CHARACTERIZATION OF THE GENOME IN TIMOTHY (*PHLEUM PRATENSE* L.): POLYPLOID NATURE, PHYLOGENETIC RELATIONSHIPS, AND PROTEIN VARIATION

by

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Genome Characterization in Timothy

ABSTRACT

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CHARACTERIZATION OF THE GENOME IN TIMOTHY (*PHLEUM PRATENSE* L.): POLYPLOID NATURE, PHYLOGENETIC RELATIONSHIPS AND PROTEIN VARIATION

The genome constitution and phylogeny of hexaploid timothy (*Phleum pratense* L., $2n = 42$) have been studied with C-banding and genome-specific DNA techniques. The cultivated species and two diploid wild relatives *P. alpinum* ($2n = 14$) and *P. bertolonii* ($2n = 14$) were karyotyped. In *P. pratense*, two sets of seven chromosomes could not be distinguished from each other either in gross morphology or in C-banding patterns and the third set was found to be different' 'ed from them. Two genomes, A and B, were established in this species presumably with the genome formula of AAAABB. The banded karyotype in *P. alpinum* was close to the A genome and that of *P. bertolonii* was analogous to the B genome, which suggests these wild species were the genome donors of *P. pratense*. To reinforce this, a molecular assay was performed with genome-specific probes. Eight clones specific to the genome of *P. alpinum* and thirteen specific to the genome of *P. bertolonii* were respectively isolated from the genomic DNA libraries of *P. alpinum* and *P. bertolonii*. Three *P. alpinum*-specific sequences and three *P. bertolonii*-specific sequences were used as probes to hybridize the DNA of *P. pratense* on slot blot and Southern blot. All the three *P. bertolonii*-specific probes and two of the three *P. alpinum* specific probes exhibited cross-hybridization to *P. pratense* DNA. This has confirmed the allopolyploid origin of hexaploid *P. pratense*. In addition, phenotypic variations of seed storage proteins were investigated within the cultivated *P. pratense* with SDS-PAGE analysis. A total of 44 protein monomers were detected in mature seed extracts from 19 cultivars of timothy. The protein banding patterns were differentiated among all the examined cultivars except for two pairs. Such differentiation makes it possible to identify the timothy cultivars.

Résumé

(Cai Qinyin)

LA CONSTITUTION GÉNOMIQUE DE LA FLÉOLE DES PRÉS, (*PHLEUM PRATENSE*): LA POLYPLOïDIE, LA PHYLOGÉNÈSE ET LA VARIATION DES PATRONS D'ÉLECTROPHORÈSE DE PROTÉINE

La constitution génomique et la phylogénèse de la fléole des prés (*Phleum pratense* L., 2*n* = 42) ont été étudiées par l'analyse des profils de bandes C et le séquençage de l'ADN spécifique aux différents génomes. Les caryotypes de l'espèce cultivée, *P. pratense* et de deux espèces sauvages apparentées, *P. alpinum* (2*n* = 14) et *P. bertolonii* (2*n* = 14) ont été examinés. En ce qui concerne l'espèce *F. pratense*, on a trouvé que deux séries de sept chromosomes étaient identiques tandis qu'une troisième série était différente des deux premières, sur la base de la morphologie et de la distribution des bandes C. Ces séries réfèrent à deux génomes, A et B et l'espèce hexaploïde se voit ainsi conférer la formule génomique AAAABB. La morphologie des chromosomes ainsi que les profils de bandes C permettent d'associer les chromosomes de *P. alpinum* au génome A et les chromosomes de *P. bertolonii* au génome B. Ces données suggèrent que *P. alpinum* serait l'espèce donnatrice du génome A de *P. pratense*, tandis que *P. bertolonii* serait l'espèce donnatrice du génome B de *P. pratense*. Afin d'appuyer ces données, des sondes spécifiques aux génomes A et B furent développées. Huit clones spécifiques de *P. alpinum* et treize clones spécifiques de *P. bertolonii* furent isolés à partir des banques génomiques de *P. alpinum* et de *P. bertolonii*. Trois séquences spécifiques de *P. alpinum* et trois séquences spécifiques de *P. bertolonii* furent utilisées comme sondes lors d'hybridation de *P. pratense* selon les techniques de slot blot et de Southern. Les trois sondes spécifiques de *P. bertolonii* et deux des trois sondes spécifiques de *P. alpinum* ont montré une hybridation croisée de *P. pratense*. Ces données confirment l'origine allopolyploïde de *P. pratense*. De plus, les variations des protéines de réserve de graines de *P. pratense* ont été examinées par une analyse de SDS-PAGE. 44 protéines monomériques ont été détectées dans les extraits de graines matures des dix-neuf cultivars. Les profils de bandes des protéines etaient différentiables à l'exception de deux paires de cultivars. Cette différentiation rend possible l'identification des différents cultivars de *P. pratense* utilisés dans cette etude.

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I thank M. P. St-Marseille, a graduate student in genetics, for his special help in preparation of the French abstract of my thesis. I also thank Mr. L. Du, a graduate student in Department of Microbiology, for his kindly help in my DNA cloning work.

My special appreciation must go to Dr. M.R. Bullen, my thesis supervisor. Dr. Bullen has offered his continuous guidance, financial and moral support, and constructive suggestions in the preparation of this thesis.

Contribution to original knowledge

To the best of the author's knowledge, all three techniques : Giemsa C-banding of chromosomes, genome-specific sequence analysis and SDS-PAGE analysis of seed storage proteins used in this study have not previously been applied on studies of timothy (*Phleum pratense* L.). The study presented in this thesis has resulted in the following original findings:

1. The chromosomes in cultivated timothy (*P. pratense*) and two wild relatives *P. alpinum* and *P. bertolonii* were successfully banded with the C-banding procedure. The distinguishable banding patterns permit identification of individual chromosome pairs and particular genomes in these species.

2. Two genomes, A and B, comprising the karyotype of *P. pratense* were established based on the C-banding patterns.

3. The cytological evidence was presented to show that *P. pratense* is a allohexaploid with the genome formula of AAAABB.

4. Two partial genomic libraries which represented the genome of *P. alpinum* and the genome of *P. bertolonii* respectively were constructed and were screened by colony hybridization using total genomic probes. Eight *P. alpinum* genome-specific and 13 *P. bertolonii* genome-specific clones were isolated from these two libraries.

5. All the genome-specific clones were characterized including stringency of

the hybridization specificity, copy-number range and insert size.

6. Three *P. alpinum*-specific clones and three *P. bertolonii*-specific clones were used as probes to hybridize the DNA samples from four *Phleum* species involving *P. pratense* in slot blot and Southern blot. It was discovered that two *P. alpinum*-specific and three *P. bertolonii*-specific probes could hybridize to *P. pratense* DNA.

7. The evidence from chromosome analysis and molecular analysis were presented to confirm that one genome in *F. pratense* may come from *P. alpinum* and the other may be donated by *P. bertolonii*.

8. The seed storage proteins from 19 timothy cultivars were SDS-PAGE-analyzed. A total of 44 protein bands were detected and phenotypic variation of the protein monomers were found between the cultivars.

9. A parameter, proportion of differentiated proteins (P_p) was presented to quantitatively evaluate the differentiation between cultivars.

10. The information from protein analysis was presented to support the identification of the cultivars in timothy.

Table of contents

Forward

This thesis is submitted under the form of three manuscripts according to the conditions outlined in the **Guidelines Concerning Thesis Preparation** which are as follows:

"The candidate has the option, **subject to the approval of their Department**, of including as part of the thesis the text, or duplicated published text, of an original paper or papers.

Manuscript-style theses must still conform to all other requirements explained in the **Guidelines Concerning Thesis Preparation.** Additional material (procedural) and design data as well as description of equipment) must be provided in sufficient detail (eg. in appendices) to allow clear and precise judgement to be made of the importance and originality of the research reported. The thesis should be more than a mere collection of manuscripts published or to be published. It must include a general abstract, a full introduction and literature review and a final overall conclusion. Connecting texts which provide logical bridges between different manuscripts are usually desirable in the interest of cohesion.

It is acceptable for theses to include, as chapter, authentic copies of papers already published, provided these are duplicated clearly and bound as an integral part of the thesis, In such instances, connecting texts are mandatory, and supplementary explanatory material is always necessary. Photographs or other materials which do not duplicate well must be included in their original form.

While the inclusion of manuscripts co-authored by the candidate and others is acceptable, the candidate is required to make an explicit statement in the thesis of who contributed to such work and to what extent, and supervisors must attest to the accuracy of the claims at the Ph.D. Oral

Defense. Since the task of the examiners is made more difficult in these cases, it is in the candidate's interest to make the responsibilities of authors perfectly clear."

All three manuscripts submitted within this thesis were coauthored by Dr. M.R. Bullen, the candidate's research supervisor.

Dr. Bullen was responsible for the administrative aspect of the research, obtaining funding and laboratory space and equipment, as well as giving suggestion for preparation of the manuscripts.

The candidate was responsible for all design and operation of experimental procedure, as well as for analysis and presentation of results. The manuscripts were written entirely by the candidate.

The first manuscript was published in **Genome** (Appendix II). The second manuscript will be submitted for publication to **Theoretical and Applied Genetics**, and the third one has been accepted by the **Canadian Journal of Plant Science**.

General introduction

Timothy (*P. pratense* L.) is an important forage crop grown either in monoculture or in mixture with legumes, particularly alfalfa (*Medicago sativa* L.) (Berg and Hill 1983). This species was brought under cultivation in North America and it has been widely grown throughout the cool, temperate regions of the world. Timothy is grown primarily for hay. It is generally found, however, in mixture for pasture (Childers and Hanson 1985). The data from feeding trials (Grant and Burgess 1978) indicated that its silage was equal to corn silage for milk production when fed as the only forage to dairying cows. Therefore, in some areas like Hokkaido Island in Japan, timothy is used as the principal forage in the dairying industry. In spite of the increase in the importance of other forage grasses and legumes, timothy remains the most important cultivated grass of eastern Canada (Lemieux 1986).

There are at least 10 species which are formally recognized in the genus *Phleum*. Only *P. pratense* is of major economic importance. Investigations aimed at the improvement of this cultivated species were begun during the early years of the present century (Nath 1967). The progress in the commercial development of this cultivated species has been rather disappointing. Only a limited number of new, improved cultivars of timothy are available. It should

be recognized that although the genus *Phleum* as a whole has considerable potential, programs designed to increase the agronomic usefulness of timothy have generally been limited to conventional breeding and selections within the species. It has been found that some wild species bear desirable characteristics such as higher seed set, higher content of storage proteins, rust resistance and frost resistance (Nath 1967). If some of these characteristics could be combined with the cultivated form of timothy, the quality of timothy cultivars might be greatly improved. The recent successful development of a new timothy cultivar by interspecific hybridization between cultivated timothy and the wild species *P. alpinum* reported in Soviet Union (Turkina 1985) has opened up broad prospects for utilization of wild resources in *Phleum* to improve the cultivated timothy. However, we are far away from our goal in this aspect. More studies of timothy, especially on its genetic basis, are required before efficient exploitation of the genetic resources in cultivated timothy as well as in the wild species of *Phleum*.

The knowledge of the genomes in timothy (*P. pratense*) including its constitution and phylogenetic relationships will be useful not only in theoretical studies but also in the practical breeding program of timothy. In addition, information about genetic variations in this species would be also useful. However, the genome structure of *P. pratense* is still poorly understood and most of the genetic variability in this cultivated species is still unknown. The main task of my study was to characterize the genome of *P. pratense*

including its constitution and phylogenetic relationships. In addition, it was to examine the genetic variation in order to understand the origin and evolution of this forage species and also to provide useful information for timothy breeders. The content of the study under the title of this thesis includes the following two aspects:

I. Cytogenetic and molecular study to characterize the genomes in *Phleum pratense* and its wild relatives

P. pratense is recognized as a hexaploid ($2n = 6x = 42$) (Gregor and Sansome 1930, Nordenskiöld 1945). In the earlier studies on timothy, the nature of this polyploid was extensively studied through interspecific hybridization and observation of chromosomal behaviour in meiosis. Two hypotheses about the nature and origin of the genome in *P. pratense* were proposed. One considered it originated as an allohexaploid from the diploid species *P. bertolonii* ($2n = 14$) (It was named *P. nodosom* or diploid *P. pratense* in the earlier literature) and the tetraploid form of *P. alpinum* ($2n = 4x = 28$); The other hypothesis proposed was an autohexaploid developed from *P. bertolonii*.

The evidence supporting the first assumption mainly came from the studies reported by Gregor and Sansome (1930), Gregor (1931), Müntzing (1935) and Müntzing and Prakken (1940). Gregor and Sansome (1930) successfully obtained triploid hybrids ($2n = 21$) from diploid *P. bertolonii* and

tetraploid *P. alpinum* (2*n* = 28). Through chromosome doubling, some hexaploid plants were obtained from these hybrids. One of them proved cross-fertile with hexaploid *P. pratense*. Müntzing (1935) and Müntzing and Prakken (1940) analyzed the meiotic chromosomal behaviour in the pentaploid hybrids between hexaploid *P. pratense* and diploid *P. bertolonii*. They found that the typical MI configuration in these hybrids was 14 II + 7 I and 15 II + 6 I. They suggested the genome formula in *P. pratense* as NNA1A1A2A2, in which A1 and A2 were very likely identical and the pairing between them was as frequent as that between A1 and A1 and between A2 and A2. Levan (1941) reported that the cytology of haploid plants of *P. pratense* was characterized by a MI configuration of 7 II + 7 I. In comments on Levan's study on the "haploid" plants of *P. pratense*, Stebbins (1950) suggested that the genome formula in hexaploid *P. pratense* should be AAAABB in which the A genome and B genome might come from *P. bertolonii* and *P. alpinum* respectively.

The second assumption, an autopolyploid origin, was proposed by Nordenskiöld (1941, 1945). The evidence which was considered to support this assumption was mainly derived from the cytological, morphological and compatibility investigation of *Phleum* species by Nordenskiöld as well as by Myers(1941, 1944). In comparison of the meiotic chromosomal behaviour between triploid *P. bertolonii* (2*n* = 21) and "haploid" *P. pratense* (2*n* = 21) and triploid hybrids (2*n* = 21) of diploid *P. bertolonii* x tetraploid *P. alpinum*,

Nordenskiöld (1941) noted that meiosis in the triploid *P. bertolonii* and that of "haploid" *P. pratense* seemed to mutually correspond more closely than with the triploid hybrid *P. bertolonii* X tetraploid *P. alpinum*. The typical MI configuration of "haploid" *P. pratense* was 7 II + 7 I and very few trivalents were observed. Meiosis of the triploid *P. bertolonii* showed a high frequency of bivalents and univalents with a rather low frequency of trivalents, although the latter was more frequent than in haploid *P. pratense*. However, chromosome pairing in the triploid hybrids between *P. bertolonii* and tetraploid *P. alpinum* appeared to be more irregular. In addition to trivalents, bivalents and univalents, associations of four chromosomes were observed. Also, *P. bertolonii* was morphologically similar to the cultivated *P. pratense*. Moreover, the data from interspecific hybridization experiments indicated that *P. bertolonii* seemed to be more compatible with *P. pratense* than *P. alpinum* and other species such as *P. commutatum*. The hybrids between *P. bertolonii* and *P. pratense* frequently behaved normally, including high viability, high fertility and normal chromosome pairing in meiosis. However, the behaviour of the other hybrids including those between *P. pratense* and *P. alpinum* were frequently abnormal including low viability, high sterility and irregular chromosome pairing in meiosis. These facts made Nordenskiöld believe that all three chromosome sets in haploid *P. pratense* were homologous and the species must have developed from *P. bertolonii*.

Very few studies related to the genome of *P. pratense* and interspecific

relationships among *Phleum* species have been reported since the 1960s. Although most people now tend to agree with the explanation of *P. pratense* as an autohexaploid, no definite conclusion can be made. It is very difficult to prove the polyploid nature of *P. pratense* and the genome relationships in *Phleum* using the conventional methods including meiotic cytological, morphological and compatibility analysis. The chromosomal behaviour in meiosis is usually considered as an important clue for identification of the polyploid nature in plants. In the case of *Phleum* species, however, it has provided no solid proof of genome constitution since chromosome pairing in meiosis of *Phleum* species failed to positively respond to the ploidy levels even in the synthesized autopolyploid (Nordenskiöld 1949). Validity of the evidence based on the compatibility analysis among *Phleum* species can be questioned, since the abnormality of the hybrids between *P. alpinum* and *P. pratense* may be attributed to the unbalanced genome structure of the asymmetric hybrids rather than to lack of genome homology between the two parents.

Because of the uncertainty in the literature, the author decided to embark on a program that would cytogenetically and molecularly characterize the polyploid genome of *P. pratense* and thus aid in verifying the hypotheses about the nature and phylogeny of this polyploid. The strategy of this study involved application of two new advanced techniques: Giemsa C-banding analysis and genome-specific sequence analysis.

The Giemsa C-banding technique is a particular chromosome staining procedure to visualize the distribution of constitutive heterochromatin in the chromosomes. Plant chromosomes are rich in constitutive heterochromatin which represents the permanent condensed regions of chromosomes and consists mainly of arrays of highly repeated late-replicating DNA sequences (Applels et al. 1978; Bedbrook et al. 1980; Jones and Flavell 1982). The polymorphic distribution patterns of heterochromatin in chromosomes often makes it possible to recognize individual chromosomes and thus to identify a particular genome as well as to cytologically characterize the genome similarities. Consequently, this is an extremely useful tool for testing models of genome evolution in plant species. Various C-banding procedures have been applied to study genome evolutions in crop plants such as wheat (Gill and Kimber 1974; Endo and Gill 1984), barley (Fernandez and Jouve 1984; Linde-Laursen et al. 1989), rye (Lelley et al. 1978; Figueiras et al. 1990) and peanut (Cai et al. 1987). This has resulted in a dramatic increase in data which has contributed to the understanding of the origin of the genome and the evolution of various species. Nevertheless, validity of this technique depends on a high content of polymorphism of heterochromatin in chromosomes. This raised the question whether this technique was feasible in *Phleum* species. The definite answer was given by a cytological experiment where the chromosomes from three *Phleum* species involving *P. pratense* and four other forage species: *Lotus corniculatus*, *Dactylis glomerata*, *Medicago sativa* and

Poa pratensis were stained with the C-banding method (Cai and Bullen, unpublished). The result obtained from this experiment suggested that the C-banding technique is well suited for genome study in *Phleum*, as the C-banding patterns in its species were much more polymorphic when compared to other forage species.

Genome-specific sequence analysis is a new molecular approach which has been actively used in genome studies in recent years. Genome-specific sequences are defined as the DNA sequences that are exclusively homologous to a particular genome and its derivatives. The specificity of DNA sequences to genomes is an evolutionary product. It is well known that genome differentiation mainly results from DNA sequence variation and structural rearrangement of chromosomes. Higher differentiation between genomes implies higher heterogeneity between their DNA sequences. However, some sequences which have been highly differentiated from distant related genomes still remain homologous between close relatives. These sequences can thus be used as genome markers or genome-specific probes to indicate genome relationships between species. Genome-specific DNA sequences have been successfully isolated in 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-Jonsson et al 1990; Fabijanski et al. 1990), as well as for identifying chromosome translocation (Lapitan et al. 1986 and Le et al. 1989). Genome-specific probes are also effective when they are used to identify genomes in polyploids and to test the models of progenitor-derivative relationships.

The objectives of the present study are: (i) to characterize the karyotype of hexaploid *P. pratense* including chromosome morphology and C-banding pattern and thus to determine whether there are distinctive genomes in this polyploid species; (ii) to evaluate the genome relationships between cultivated *P. pratense* and two wild species *P. alpinum* and *P. bertolonii* which were thought to be possible ancestors of timothy on the basis of karyotyping data; (iii) to identify and isolate the genome-specific sequences from two possible progenitor species *P. alpinum* and *P. bertolonii*; and (iv) to further test the model of progenitor-derivative relationship between *P. pratense* and the wild species using genome-specific probes.

II. Investigation of genetic variations within the cultivated species *P. pratense* with SDS PAGE electrophoresis

Understanding the genetic variability in *P. pratense* has practical

significance on timothy breeding. Phenotypic differences not only allow for selection of hybrids but also permit identification of genotypes or cultivars. Phenotypic variations have been observed in timothy for plant morphology including plant height, leaf and stem peculiarities, as well as head type and glume size (Powell and Hanson 1973). Although variations of plant morphology may provide some useful markers for genetic selection and for discrimination of cultivars in timothy, only a limited number of variations can be morphologically expressed and they are easily influenced by the environment. Thus it is very difficult to identify the genotypes or cultivars in this species depending on morphological observation. Recently, phenotypic variations have also been observed in this species for some chemical characters and physical property such as crude protein (CP) and *in vitro* dry matter digestibility (IVDMD) (Berg and Hill 1983, Surprenant et al. 1990), IVDM decline (McElroy and Christie 1986), packed volume (PV), water retention (WR) and water solubility (WS) (Surprenant and Michard 1988), and yield (Faris 1970). Unfortunately, most of these characters are quantitive traits and thus can not serve as effective markers for genetic selection and identification of genotypes.

Genetic diversity in seed storage proteins are considered to be an important source of variation. About 80% of seed proteins serve as storage function. These include globulins of dicotyledons and prolamines of monocotyledons (Konarev et al. 1987). The storage proteins in seeds usually

represent a large number of gene products which are considered to be stable quality markers. SDS polyacrylamide gel electrophoretic (PAGE) analysis of seed storage proteins has been demonstrated to be very useful for visualizing phenotypic variations in natural and cultivated populations. It has the advantage over morphological observation and other conventional methods in that it can visualize the phenotypic variations before the full development of plants. Thus, it is a powerful tool for identification of cultivars in crop plants. In view of these considerations, it was decided to undertake a survey of seed storage protein variations in cultivated timothy. The main objective of this phase of research was to evaluate the phenotypic variability in cultivated populations of timothy (*P. pratense*), which might make it possible to identify the commercial cultivars in this species.

The results of the whole study including the above two aspects are presented as the main body of the present thesis and in the form of three manuscripts for publication. The first manuscript presents the karyotypic and C-banding data for three *Phleum* species, *P. pratense*, *P. alpinum* and *P. bertolonii*. The focus is on the polyploid nature of *P. pratense* and the genetic relationship with the two diploid species based on the C-banded karyotypic data. The second manuscript presents the findings of molecular experiments associated with genome-specific sequence analysis in the *Phleum* species. The experiments include construction of the genomic DNA libraries, identification and isolation of genome-specific clones from the genomic DNA libraries and

slot and Southern blot analysis of genome homology using genome-specific probes. This work has initiated DNA manipulation in *Phleum* species with the attempt to verify the primary conclusion drawn in the cytogenetic study presented in the first manuscript. The third manuscript presents the electrophoretic data of seed storage proteins among the cultivars of timothy. The emphasis in this manuscript is on the evaluation of phenotypic differences between cultivars to identify the genotypes in this species.

References

Anamthawat-Jónsson, K., T. Schwarzacher, A.R. Leitch, M.D. Bennett, and J.S. Heslop-Harrison. 1990. Discrimination between closely related *Triticeae* species using genomic DNA as a probe. Theor. Appl. Genet., 79: 721-728.

Appels, R., W. Driscoll, and J. Peacock. 1978. Heterochromatin and highly repeated DNA sequences in rye *Secale cereale*. Chromosoma, 70: 67-89.

Bedbrook, J.R., J. Jones, M. O'Dell, R.D. Thompson, and R.B. Flavell. 1980. A molecular description of telomeric heterochromatin in *Secale* species. Cell, 19: 545-560.

Berg, C.C., and R.R. Hill, Jr. 1983. Quantitative inheritance and correlations among forage yield and quality component in timothy. Crop Sci., 23:

380-384.

Cai, Q., S. Lu, and C.C. Chinnappa. 1987. Analysis of karyotypes and Giemsa C-banding patterns in eight species of *Arachis*. Genome, 29: 187-194.

Childers, W.R., and A.A. Hanson. 1985. Timothy *in* Forage-the Science of Grassland Agriculture, 4th Edition, edited by M. E. Heath, R.F. Barnes, and D.S. Metcalfe. Iowa State University Press, pp. 217-223.

Crowhurst, R.N., and R.C. Gardner. 1991. A genome-specific repeat sequence from kiwifruit (*Actinidia deliciosa* var. *deliciosa*). Theor. Appl. Genet., 81: 71-78.

Endo, T.R., and B.S. Gill. 1984. Somatic karyotype, heterochromatin distribution, and nature of chromosome differentiation in common wheat, *Triticum aestivum* L. em. Thell. Chromosoma, 89: 361-369.

Fabijanski , S., G. Fedak, K. Armstrong, and I. Altosaar. 1990. A repeated sequence probe for the C genome in *Avena* (oats). Theor. Appl. Genet., 79: 1-7.

Faris, A.E. 1970. A comparative study of different procedures for heritability estimates of timothy (*P. pratense* L.). Ph.D. Thesis, McGill University, Montreal, Que.

Fernandez, J.A., and N. Jouve. 1984. Giemsa C-banding of the chromosomes of *Hordeum chilense* and its amphidiploid x *Triticum turgidum* conv. *durum*. Z. Pflanzenzuecht, 93: 212-221.

Figueiras, A M., M.T. González, and M. Candela. 1990. Chromosomal

identification and meiotic behavior of reciprocal translocations in a rye polymorphic population. Evolutionary implications. Theor. Appl. Genet., 79: 686-692.

Gill, B.S., and G. Kimber. 1974. Giemsa C-banding and the evolution of wheat. Proc. Nat. Acad. Sci. USA, 71: 4086-4090.

Grant, E.A., and P.L. Burgess. 1978. Can. Agr. Timothy Publ. 1640.

Gregor, J.W. 1931. Experimental delimitation of species. New Phytol., 30: 204-217.

Gregor, J.W., and F.W. Sansome. 1930. Experiments on the genetics of wild population. J.Genet., 22: 373-387.

Guidet, F., P. Rogowsky, C. Taylor, W. Song, and P. Langridge. 1991. Cloning and characterisation of a new rye-specific repeated sequence. Genome, 34: 81-87.

Hosaka, K., S.F. Kianian, J.M. McGrath, and C.F. Quiros. 1990. Development and chromosomal localization of genome-specific DNA markers of *Brassica* and the evolution of amphidiploid an $n = 9$ diploid species. Genome, 33: 131-142.

Jones, J.D.G, and R.D. Flavell. 1982. The mapping of highly repeated DNA families and their relationship to C-bands in chromosomes of *Secale cereale*. Chromosoma, 86: 595-612.

Junghans, H., and M. Metzlaff. 1988. Genome specific, highly repeated sequences of *Hordeum vulgare*: cloning, sequencing and squash dot test.

Theor. Appl. Genet., 76: 728-732.

Konarev, V.G., I.P. Gavriljuk, and N.K. Gubareva. 1987. Storage proteins in the identification of species, cultivars and lines. Seed Sci. and Technol., 15: 675-678.

Lapitan, N.L.V., R.G. Sears, A.L. Rayburn and B.S. Gill. 1986. Wheat-rye translocations. Detection of chromosome breakpoints by *in-situ* hybridization with a biotin-labeled DNA probe. J. Hered., 77: 415-419.

Lu, L.H., K.C. Armstrong, and B. Miki. 1989. Detection of rye DNA in wheat-rye hybrids and wheat translocation stocks using total genomic DNA as a probe. Plant Mol. Biol. Rep., 7: 150-158.

Lelley, T., K. Josifek, and P.J. Kaltsikes. 1978. Polymorphism in the Giemsa C-banding pattern of rye chromosomes. Can. J. Genet. Cytol., 20: 307-312.

Lemieux, C. 1986. Influence of barly, red clover and weed on timothy stands. Ph.D. Thesis, McGill University, Montreal, Que.

Levan, A. 1941. Syncyte formation in the pollen-mother cells of haploid *P. pratense*. Hereditas, 27: 243-252.

Linde-Laursen, I., R. von Bothmer, and N. Jacobsen. 1989. Giemsa C-banded karyotypes of *Hordeum marinum* and *H. murinum*. Genome, 32: 629-639.

McElroy, A.R. and B.R. Christie. 1986. Variation in digestibility decline with advance in maturity among timothy (*P. pratense* L.) genotypes. Can. J.

Plant Sci., 66: 322-328.

Metzlaff, M., W. Troebner, R. Schlegel, and J. Cullum. 1986. Wheat-specific repetitive DNA sequences-construction and characterization of four different genomic clones. Theor. Appl. Genet., 72: 207-210.

Müntzing, A. 1935. Cyto-genetic studies on hybrids between two *Phleum* species. Hereditas, 20: 103-136.

Müntzing, A., and R. Prakken. 1940. the mode of chromosome pairing in *Phleum* twins with 63 chromosomes and its cytogenetic consequences. Hereditas 26: 463-501.

Myers, W.M. 1941. Meiotic behavior of *P. pratense*, *Phleum subulatum* and their F1 hybrids. J. Agr. Res., 63: 649-655.

Myers, W.M. 1944. Cytological and genetic analysis of chromosomal association and behavior during meiosis in hexaploid timothy. J. Agr. Res., 68: 21-33.

Nath, J. 1967 Cytogenetical and related studies in the genus *Phleum* L.. Euphytica, 16: 267-282.

Nordenskiöld, H. 1941. Cytological studies in triploid *Phleum*. Bot. Not. 1941. pp. 12-32.

Nordenskiöld, H. 1945. Cytogenetic studies in the genus *Phleum*. Acta Agr. Suecana, 1: 1-137.

Nordenskiöld, H. 1949. Synthesis of *P. pratense* L. from *P. nodosum* L.. Hereditas, 35: 190-202.

Pehu, E., M. Thomas, T. Poutala, A. Karp, and M.C.K. Jones. 1990. Species-specific sequences in the genus *Solanum*: identification, characterization, and application to study somatic hybrids of *S. brevidens* and *S. tuberosum*. Theor. Appl. Genet., 80: 693-698.

Powell, J.B., and A.A. Hanson. 1973. Timothy. *In* Forage-the Science of Glassland Agriculture, 3rd Edition, edited by M.E. Heath, D.S. Metcalfe, and R.F. Bardes, Iowa State University Press, pp. 277–284.

Rayburn, A.L., and B.S. Gill. 1985. Use of biotin-labeled probe to map specific DNA sequences on wheat chromosomes. J. Hered., 76: 78-81.

Rayburn, A.L., and B.S. Gill. 1986. Molecular identification of the D genome chromosomes of wheat. J. Hered., 77: 253-255.

Rayburn, A.L., and B.S. Gill. 1987. Use of repeated DNA sequences as cytological markers. Amer. J. Bot. 74: 574-580.

Saul, M., and I. Potrykus 1984. Species-specific repetitive DNA used to identify interspecific somatic hybrids. Plant Cell Rep., 3: 65-67.

Schmidt, T., H. Junghans, and M. Metzlaff. 1990. Construction of *Beta procumbens*-specific DNA probes and their application for the screening of *B. vulgaris* x *B. procumbens* (2n =19) addition lines. Theor. Appl. Genet., 79: 177-181.

Stebbins, G.L., Jr. 1950. Variation and evolution in plants. New York, Columbia University Press, pp. 643.

Surprenant, J., and R. Michaud. 1988. Genetic variability in the physical

properties and other quality traits in timothy. Can. J. Plant Sci., 68: 713-720.

Surprenant, J., R. Michaud, and G. Allard. 1990. Effect of one cycle of divergent phenotypic selection for crude protein, digestibility and digestible yield in timothy. Can. J. Plant Sci., 70: 757-765.

Turkina, G.M. 1985. the new timothy variety **Yunona**. *In* Sozdanie novykh sortov mnogletnikh trav i razrabotka tekhnologii ikh vozdelyvaniya v usloviyakh severo-zapada RSFSR, Leningrad, USSR (1985). Plant Breeding Abst.(1988), 58: 235.

Zhao, X., T. Wu, Y. Xie, and R. Wu. 1989. Genome-specific repetitive sequences in the genus *Oryza*. Theor. Appl. Genet., 78: 201-209.

Characterization of genomes of timothy (Phleum pratense L.): I. Karyotypes and C-banding patterns in cultivated timothy and two wild relatives

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Abstract

In an attempt to know the phylogeny of timothy (*P. pratense*), the cultivated species and two wild relatives, *P. alpinum* and *P. bertolonii*, were karyotyped with conventional and Giemsa C-banding methods. In the hexaploid *P. pratense* ($2n = 6x = 42$), two sets of seven chromosomes were indistinguishable from each other both in morphology and in banding patterns and the third set of seven was found to be differentiated from them. Two genomes, A and B were tentatively established. The banded karyotype in diploid *P. alpinum* ($2n = 2x = 14$) was close to the A genome which was tetraploid in *P. pratense* and the karyotype in *P. bertolonii* ($2n = 2x = 14$) was analogous to the B genome in *P. pratense*, which suggests these species were the genome donors of *P. pratense*.

Key words: chromosome, genome, allopolyploid, Giemsa C-banding.

Résumé

Dans le but de connaitre la phlogénèse du *P. pratense*, la Fléole des prés, les caryotypes Giemsa de l'espèce cultivée et deux espèces sauvages, *P. alpinum* et *P. bertolonii* ont été examinés. Les bandes C obtenues ainsi que la longueur et les rapports des bras des chromosomes ont conduit à l'identification des génomes de ces espèces. En ce qui concerne l'espèce hexaploïde *P. pratense* ($2n = 6x = 42$), on a trouvé que deux groupes de sept chromosomes étaient identiques tandis qu'un troisième groupe de sept chromosomes était différent des deux premiers, sur la base de la morphologie et de la distribution des bandes C. Le caryotype de l'espèce diploïde *P. alpinum* ($2n = 2x = 14$) est proche de génome A (tétraploïde chez *P. pratense*) tandis que le caryotype de *P. bertolonii* ($2n = 2x = 14$) ressemble au génome B de *P. pratense*.

Mots clés: chromosome, génome, allolyploïde, Bandes C, coloration de Giemsa.

Introduction

Timothy (*Phleum pratense* L.) is an important forage crop, widely grown in cool, temperate regions of the world including North America and Europe. In the genus *Phleum*, of which at least 10 species have been positively identified while another 10 may have specific rank, only the species, *P. pratense* is of major economic importance (Nath 1967).

The cultivated species, *P. pratense* almost always appears in the literature as a hexaploid with a basic chromosome number of seven. The phylogenetic relationship between this species and the wild species of *Phleum* was a frequent subject of study during the period 1930-1954. Few investigations have been published in the last two decades. Most of the studies on phylogenetic relationships in *Phleum* species were concentrated on interspecific hybridization. A series of investigations on the fertility of interspecific hybrids and chromosomal behavior in meiosis of pollen mother cells in *P. pratense* and in interspecific hybrids have been reported (Müntzing 1935, Müntzing and Prakken 1940, Nordenskiöld 1941, 1945). From these studies came hypotheses about the nature and origin of the polyploid genome in *P. pratense*. One hypothesis saw the cultivated *P. pratense* as an allohexaploid containing two distinct genomes which were proposed to come from *P. alpinum* and *P. nodosom*, renamed *P. bertolonii*, (Müntzing and

Prakken 1940, Levan 1941 and Stebbins 1950). A second hypothesis considered the species to be an autopolyploid of *P. nodosom* (Myers 1944, Nordenskiöld 1945, Wilton and Klebesadel 1973). The latter point of view seems to be generally accepted. The authors believe, however, that a definite conclusion can not be drawn because of a lack of conclusive evidence from cytology and genetics.

The detailed karyotypes of *P. pratense* and its relatives have been poorly understood. Wilton and Klebesadel (1973) reported the chromosome morphology (length, arm ratios) of three *Phleum* species including *P. pratense* and showed that the three genomes in *P. pratense* had similar chromosomal morphology thus supporting the autopolyploid nature. Only in two out of the three species could they make statistical comparisons as cytological preparations were difficult to obtain in the third taxon. The information was limited in this study as banding was not yet available in the genus. Joachimiak (1981) showed the first banding with a *Phleum* using the Hy (hydrochloric acid) technique on the diploid *P. boehmeri*. While of interest, this technique did not prove to be a practical way to prepare banded karyotypes that could be readily repeated (Bullen, unpublished). The objective of this study was to obtain the C-banded karyotypes of *P. pratense* and two wild species which appear to be genome donors. Confirming the genome donors would contribute to the understanding of the origin and evolution of the polyploid genome of timothy.

Materials and methods

Three species of *Phleum*: *P. pratense*, *P. alpinum* ssp. *rhaeticum*, and *P. bertolonii*, were used for this study. *P. pratense* is a cultivated species and the other two are essentially wild. These species and accession numbers are listed in Table 1. Specimens will be deposited in the Herbarium of McGill University.

The plants were grown in a growth chamber with a photoperiod of 16 hours, light intensity of 94 $\mu EM^{-2}s^{-1}$ and a temperature 18°C (day) and 12°C (night). To carry out the pretreatment of the root tips, the roots still attached to the rest of the plant, were immersed in 0.002 M 8-hydroxyquinoline solution for three hours. One cm root tips were cut and fixed in 2:1 methanol:glacial acetic acid for 12 hours. For the conventional chromosome observation, the root tips were hydrolyzed in 0.2 N HCl at 60°C for 6 min. The slides were made and stained following the method described by Cai and Chinnappa (1987). For the Giemsa C-banding of the chromosomes, the fixed root tips were hydrolyzed in 0.1 N HCl at 60°C for 9 min and then squashed in 45% acetic acid. The slides were dried at room temperature for four days. Subsequently, the dried slides were denatured in 5% barium hydroxide at 20° C for three min and then incubated in 2XSSC solution at 60°C for one hour, stained with 4% Giemsa in 1/15 M phosphate buffer (pH 6.8). The

chromosomes were observed and photographed using a Leitz Ortholux II microscope. Ten cells with well spread metaphase chromosomes for each accession were used for analysis and construction of idiograms.

Results

The karyotype and C-banding pattern in P. pratense

The three accessions from *P. pratense* listed in Table 1 were karyotyped. The somatic cells in all three accessions, M44, M35 and M46, were shown to have 42 chromosomes with the exception of two plants of M46 which were found to have more than 42 chromosomes. One plant was $2n = 44$ and the other was $2n = 46$. The number of 42 chromosomes was considered to be the hexaploid number in *Phleum*, logically consisting of three sets of seven. In all the accessions, hexaploid and diploid, B-chromosomes were often observed in low numbers, although some cells showed up to five per cell. The morphology of individual chromosomes in this species can be characterized by the data presented in Table 2 and by the photos and idiograms in Fig. 3a. As shown in Table 2, the chromosome size ranges from 3.80 to 7.72 μm. Within the chromosome complement, 14 pairs of chromosomes are metacentric and the other 7 pairs are submetacentric. Two pairs of chromosomes (A6) were shown to have distinct satellites with long secondary constrictions. There were no

apparent differences in karyotype found between different accessions apart from the two plants mentioned above. In addition to the chromosome morphology, the C-banding patterns of *P. pratense* chromosomes were examined. As shown in Fig. 1 and Fig. 3a, this karyotype contained distinct telomeric bands and centromeric bands as well as some intercalary bands. All the 42 chromosomes showed telomeric bands and centromeric bands. Among them 10 chromosomes contained intercalary bands and the satellited chromosomes showed the secondary constriction band. The telomeric bands seemed to be stable, but the centromeric bands were slightly variable depending on the division stage of the cell. It was noted that some chromosomes showing centromeric bands in metaphase were not banded in the centric region in prometaphase.

In order to clarify the polyploid nature of *P. pratense*, the morphology and C-banding patterns of individual chromosome pairs were compared. Morphological similarity was found among some chromosomes. The similar chromosome pairs can be put into seven groups: A1, A2, A3, A4, A5, A6 and A7, as shown in Table 2. Each group contains two similar chromosome pairs except two groups A1 and A3 which also share a similar morphology with B2 and B3 respectively. Not only the morphology but the C-banding patterns showed the similarity between corresponding chromosome pairs in these seven groups as well. As shown in Fig. 3a, except A4 (of *P. pratense*) where the telomeric bands on the long arms are different in band intensity, the

chromosome pairs in the other A groups show apparently similar banding patterns. However, the third set of seven chromosomes are mostly distinguishable from them. These data imply that there are two homologous sets rather than three homologous sets consisting of seven chromosomes in *P. pratense*. The karyotype of this species may well be an allopolyploid of two different genomes, with one being tetraploid and the other diploid. Based on the karyotype data and C-banding patterns, two different genomes are tentatively named for *P. pratense*, the tetraploid as genome A and the diploid as genome B. In two cases, three chromosome pairs presumably from the three genomes share a similar morphology. However, they can be sorted out into genome A or B with the aid of distinct banding patterns. The C-banding patterns of individual chromosome pairs are described shortly using the following nomenclature: the lower case letter "p" is used to designate the chromosomes from *P. pratense* and the "a" is used for the chromosomes from *P. alpinum* and "b" to designate those from *P. bertolonii*. The details of the pairs of chromosomes are:

pA1 - The chromosomes were banded on both ends as well as at the centromere. In addition, an intercalary band can be seen in the long arm.

pA2 and *pA3* - There were telomeric bands on both ends and a centromeric band.

pA4, pA5 and *pA7* - The chromosomes were banded at the centromeric region and on the end of the long arm. In terms of the band intensity, the telomeric

bands in pA5 seemed to be different between homologous chromosome pairs. The band in one pair was much thicker than the other one.

pA6 - These chromosomes were found to be distinguishable from others by having distinct satellites. In addition to the telomeric and centromeric bands which were similar to pA4, pA5 and pA7, a secondary constriction band was revealed.

The members of genome B showed the following C-banding patterns:

pB1 - This is the biggest chromosome pair in *P. pratense* karyotype. Telomeric bands on both ends, a centromeric band as well as an intercalary band in the long arm were demonstrated.

pB2 - This pair of chromosomes had the richest bands in the whole chromosome complement, including two telomeric bands, two intercalary bands, one on the short arm and the other on the long arm, as well as a centromeric band.

pA3 - Two telomeric bands and one centromeric band were stably visualized. In addition, one intercalary band could, in most cases, be seen in the short arm.

pB4 and *pB6* - The chromosomes were banded at the centromere and on both distant ends.

pB5 - The chromosome pair contained a unique banding pattern, having an intercalary band in the short arm, a telomeric band on the long arm and a centromeric band.

pB7 - This chromosome pair had a similar morphology and banding pattern to pA7, containing a centromeric band and a telomeric band on the end of the long arm.

The banded karyotypes in the wild species

P. alpinum ssp. *rhaeticum*

Both of the accessions from this species were found to be diploid, with 14 chromosomes and in most cases one or two B chromosomes. In its chromosome complement, there were two submetacentric and five metacentric chromosome pairs with the lengths ranging from 4.28 to 7.27 μm. In this investigation, one satellited chromosome pair (the sixth) was identified for this species. The detailed karyotype data can be seen in Table 2.

The karyotype of this species was further assayed by the C-banding technique. The banded chromosomes and the idiograms of the C-banding pattern are shown in Fig. 2a, 3b. It can be seen from this figure that all the chromosomes in this species are banded at the centromere and on either or both ends of the chromosomes. Two chromosome pairs were found to have intercalary bands which were shown in the long arm in the first chromosome and in the short arm in the second. The telomeric bands were distributed on two distant ends of the chromosomes in the following chromosome pairs: aA1, aA2, aA3, and aA5 and only on the long arm end in aA4, and aA7. A secondary constriction band was revealed in the satellited chromosome pair,

aA6. It should be pointed out that polymorphism of C-banding patterns between the two homologous chromosomes was found in some chromosome pairs. In the aA1, one chromosome had telomeric bands on both ends, while its partner showed only a thin band on the short arm end. In the aA6, one chromosome had a telomeric band on the end of the satellite but its partner did not. This phenomenon was also reported in *Allium* (Loidl 1983, Cai and Chinnappa 1987).

Comparison of the banded karyotypes between *P. alpinum* and *P. pratense* showed that the chromsome morphology and banding pattern of *P. alpinum* was quite similar to the chromosomes in A genome of *P. pratense*. Among the chromosome complement in *P. alpinum*, the following chromosomes: aA1, aA3, aA4, aA6 and aA7 were respectively analogous to the pA1, pA3, pA4, pA6 and pA7 in the A genome of *P. pratense*. Slight differences in banding were found in another two chromosome pairs. Between the aA2 chromosome in *P. alpinum* and the pA2 in *P. pratense*, a short arm intercalary band was found in the former but not in the latter. Between the aA5 in *P. alpinum* and the pA5 in *P. pratense*, the former had a telomeric band on the short arm while the latter did not. These data suggest a close relationship between the genome of *P. alpinum* and the A genome in *P. pratense*.

P. bertolonii

Like *P. alpinum*, *P. bertolonii* was found to be diploid with 14

chromosomes in all examined accessions. The karyotype data is presented in Table 2. There were two chromosome pairs that were submetacentric and five metacentric. The sixth chromosome had a satellite. The C-banding treatment of the chromosomes revealed abundant heterochromatin in the *P. bertolonii* complement (Fig. 2b, 3c). As shown in Fig. 2b, 3c, the heterochromatin was mainly distributed in telomeric and centric regions, similar to the cases in *P. alpinum* and *P. pratense*. All the chromosomes observed in this species were found to carry telomeric bands. Among them, five chromosome pairs (bB1, bB2, bB3, bB4, and bB5) had two telomeric bands and the others had one telomeric band which was located in the long arm. In comparison with the telomeric bands, the centromeric band seemed to be thin and variable. Although in some cells which were in metaphase, all chromosomes were shown to have the centromeric band, in many cells, especially those in prometaphase, only two or three pairs of chromosomes were banded at the centromeres. Intercalary bands were found in four chromosome pairs. In the bB1 and bB4, an intercalary band was present in the long arm. The bB2 chromosome contained two intercalary bands located in the long and short arms respectively. The bB3 contained one intercalary band in the short arm. A secondary constriction band was found in the bB6 chromosome. Polymorphism of C-banding patterns between homologous chromosomes was found in the bB2 chromosome pair where one chromosome had an intercalary band in the short arm but the other did not.

The karyotypes and C-banding patterns were compared between *P. bertolonii* and *P. pratense*. The genome in *P. bertolonii* seemed to match the genome B in *P. pratense*. In terms of chromosome morphology, including the lengths and arm ratio, all the chromosomes except one in *P. bertolonii* were similar to the six chromosomes in the genome B. The exceptional chromosome is the bB6 in *P. bertolonii*, which is distinguished from pB6 in *P. pratense* in that the former had a satellite while the latter did not. The C-banding pattern revealed in *P. bertolonii* genome was to some extent similar to that shown for the B genome in *P. pratense*. Four chromosomes (bB1, bB2, bB3 and bB7) in the former appeared to be the same banding patterns as the four members respectively, (pB1, pB2, pB3 and pB7) in the latter. When the bB4 and pB4 were compared, both of them had one centromeric band and two telomeric bands and were only different in that the long arm of the former had an intercalary band while the other species did not. Relatively greater variation in banding patterns between these two genomes occurred in the remaining two chromosomes. The bB5 in the *P. bertolonii* genome, was divergent from the pB5 of timothy as it had a telomeric band on its short arm while the latter had an intercalary band in the same arm. The bB6 was different from pB6 in that the former had a secondary constriction band while the latter had a telomeric band in the short arm.

Discussion

The results with respect to the chromosome numbers in these three species mentioned above are in agreement with other reports (Wilton and Klebesadel 1973; Teppner 1980). However, the karyotypes observed in *P. pratense* seem to disagree with those reported by Wilton and Klebesadel (1973). The main difference is that they found three chromosome pairs with distinct satellites while only two pairs of satellited chromosomes were identified in this study. Differences in the appearance and number of satellites have been noted previously between biotypes in the grass family. In *Triticum monococcum* L. where six biotypes were analysed only two had satellites of the same appearance while the others were all different even varying in the number of pairs (Waines and Kimber, 1973). While there is competent sampling of the plant material in both the work of Wilton and Klebsadel and the present studies the number of biotypes studied is too small to draw any conclusions as what is a standard karyotype in respect to satellites for timothy. In Figure 3a the shift in the configuration of the karyotype in respect to a satellite is illustrated. It is the bB6 from the diploid which does not show a satellite when incorporated into the hexaploid. The similar phenomenon has also been reported in wheat. The diploid *Triticum monococcum* L., which has one to two pairs of satellites depending on the

biotype (Waines and Kimber, 1973), contributes its A genome to the hexaploid wheat *T. aestivum* (Riley, 1965). However, the karyotype of the polyploid wheat, AABBDD, exhibts only two pairs of satellited chromosomes 1B and 6B (Gill, 1987), with those of the A genome not being detected. While the mechanism is unknown it would be reasonable to suppose that part of the adaptation of the new polyploid cell would be to suppress some of the rDNA activity to the point that the end of the chromosome could no longer be recognised as a satellite.

In addition, we found it difficult to assign the *P. pratense* chromosomes into three similar groups consisting of seven chromosomes in the way that Wilton and Klebesadel did. It is clear to us that two sets of seven chromosomes are very similar to the corresponding chromosomes while the third set of seven is distinguishable from them.

One of the two earlier hypotheses considered that *P. pratense*'s origin was by way of allopolyploidy. In support of this concept, Gregor and Sansome (1930) obtained triploid hybrids ($2n = 21$) from crossing diploid *P. pratense* ($2n = 14$) with tetraploid *P. alpinum* ($2n = 28$). From this highly sterile triploid, four hexaploid plants were obtained, one of which proved cross-fertile with the natural hexaploid *P. pratense*. This implied that *P. pratense* might be an allopolyploid of a diploid *P. pratense* ($2n = 14$) and tetraploid *P. alpinum* ($2n = 28$). Müntzing (1935) further concluded that the hexaploid *P. pratense* ($2n = 42$) may be an allohexaploid at least involving the genome (N)

from *P. nodosom*. The genomic constitution in *P. pratense* was considered to be NNAABB (Müntzing 1935) or NNA1A1A2A2 where A1 and A2 are sufficiently homologous to permit formation of bivalents between chromosomes of the two genomes (Müntzing and Prakken 1940). Levan (1941) studied haploid plants of *P. pratense* and found seven bivalents and seven univalents. Stebbins (1950) commented that since they typically form seven bivalents and seven univalents their genomic formula must be AAB and that of the diploid AAAABB. However, Nordenskiöld (1941, 1945 and 1949), based on her studies on hybrids between *P. pratense* and *P. nodosom* and *P. alpinum*, proposed her different point of view that hexaploid *P. pratense* was an autoploid developed from *P. nodosom*. The evidence supporting her hypothesis is that the artificially synthesized hexaploid plants of *P. nodosom* were found to have similar chromosomal behavior in meiosis to the hexaploid *P. pratense* and also they were cross-fertile with each other. It should be pointed out that diploid *P. pratense* ($2n = 14$) and *P. nodosom* used in earlier work might have been *P. bertolonii*. According to Humphries (1980), diploid *P. pratense* could be assigned to *P. pratense* ssp. *bertolonii*. Besides diploid *P. bertolonii* ($2n = 14$), an autotetraploid of this species has been produced for breeding purposes (Borg and Ellerstrom 1986). According to Wilton and Klebesadel (1973) and Borrill (1976), *P. bertolonii* was previously known as *P. nodosom*. The chromosome association and behavior in meiosis of pollen mother cells in hexaploid timothy were expected to be an important guide to

the polyploid nature of this species. The higher frequency of quadrivalents in the first division of meiosis in *P. pratense* reported by Myers (1944) seemed to support the allopolyploid genome structure of AAB in *P. pratense*. However, Nordenskiöld (1945) reported very low frequency of quadrivalents and other multivalents formed in the meiosis of *P. pratenese*. This brought into question the contribution of the information from meiosis of hexaploid *P. pratense* to the understanding of polyploid nature of the *P. pratense* genome.

In our study, the evidence from the analysis of C-banded karyotypes seems to support the earlier hypotheses proposed by Gregor and Sansome (1930), Müntzing and Prakken (1940) and Stebbins (1950). Our results show that two sets of seven chromosomes are very similar to each other for the corresponding individuals in karyotype morphology and C-banding pattern while another set of seven seems distinguishable. The two similar chromosome sets may belong to the same genome or highly homologous genomes. They have been designated as genome A. The other one may belong to a different genome, designated as genome B. Therefore, the genomic formula of *P. pratense* should be AAB. This result coincides with the earlier observation of meiosis in haploid *P. pratense* where seven bivalents and seven univalents were found (Levan 1941).

The comparison of C-banded karyotypes between *P. pratense* and *P. alpinum* and between *P. pratense* and *P. bertolonii* in this study, implies that the genome A of *P. pratense* was donated by *P. alpinum* and the genome

B was donated by *P. bertolonii*. Almost all evidence from different investigations favors *P. bertolonii* as a progenitor of *P. pratense*, irrespective of whether *P. pratense* is considered an autohexaploid or an allohexaploid. Nevertheless, some evidence from earlier reports (Nordenskiöld 1941 and 1945) seems unfavorable for the candidate of *P. alpinum* as a progenitor. In these reports, the hybrids between *P. pratense* ($2n = 42$) and *P. alpinum* (unreduced gametes) were compared with the hybrids between *P. pratense* ($2n = 42$) and *P. nodosom* (*P. bertolonii*)($2n = 14$ and $2n = 28$). The result showed that the hybrids from *P. pratense-P. alpinum* appeared abnormal, including low viability, sterility and irregular behavior of chromosomes in meiosis. In contrast, the hybrids from *P. pratense-P. nodosom* (*P. bertolonii*) behaved normally. Therefore, Nordenskiöld concluded that *P. alpinum* is unlikely to participate in the formation of the genome in *P. pratense*.

From our point of view, the evidence from Nordenskiöld's studies does not repudiate the hypothesis that *P. alpinum* is one of the progenitors of *P. pratense*. The reason is that if the genome constitution in *P. pratense* is indeed AAAABB (in which A from *P. alpinum* and B from *P. bertolonii*), the hybrid between *P. pratense* (hexaploid) and *P. bertolonii* (diploid) will have the genome pattern of AABB or AABBB (unreduced gamete in *P. bertolonii*), while the hybrid from *P. pratense* (hexaploid)-*P. alpinum* (diploid) will have AAAB or AAAAB (unreduced gamete in *P. alpinum*). In these terms, the results reported by Nordenskiöld can readily be explained. The aberrant

results reported by Nordenskiöld can readily be explained. The aberrant behavior of the hybrid from *P. pratense-P. alpinum* might be attributed to its unbalanced genome constitution, in which only one copy of the B genome existed. On the other hand, the hybrid from *P. pratense-P. bertolonii* possessed a balanced genome structure, which contained at least two copies of either A or B, and this might account for the its normal behavior in biology and cytology.

Apart from the chromosome observation and interspecific hybrid investigation in the early studies of *Phleum* species, some studies were also carrried out to test inheritance models of individual gene loci in the hexaploid *P. pratense* by using chlorophyll markers (Wexelsen 1941, Myers 1944, Nordenskiöld 1953, 1954, Nielsen and Smith 1959). These studies were considered promising in their ability to confirm the polyploid nature of *P. pratense*. An autohexaploid would exhibit hexasomic segregation while an allohexaploid would exhibit disomic or disomic plus tetrasomic segregation. Unfortunately, too few loci were dealt with in these studies, so no conclusive evidence was obtained.

In addition to the three species reported in this paper, *P. hirsutum* and *P. montanum* were also karyotyped. Although the banding was not optimised for these two species the preliminary evidence showed that their banding pattern seemed to be quite different from either the A or B genomes. At least one can conclude that there are more than two genomes in the genus.

While the study in this report has provided some important information on the genome constitution of *P. pratense* and its phylogenetic relationship with some wild relatives, further studies on the molecular aspect are required to reinforce the initial conclusions drawn in this study. In our next research, the technique of hybridization of genome-specific probes to the genomic DNA of the *Phleum* species will be used to clarify the phylogeny.

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References

Borg, G. and S. Ellerström. 1986. Some preliminary attempts to produce species hybrids in the genus *Phleum* L.. *In* Svalöf. 1886-1986, edited by G. Olsson. LTs förlag, Stockholm. pp. 120-121.

Borrill, M. 1976. Temperate grasses. *In* Evolution of Crop Plants, edited by N. W. Simmonds. Longman, London. pp. 137-142.

Cai, Q. and C.C. Chinnappa. 1987. Giemsa C-banded karyotypes of seven North American species of *Allium*. Amer. J. Bot. 74: 1087-1092.

Gill, B. S. 1987. Chromosome banding methods, standard chromosome band nomenclature, and applications in cytogenetic analysis. *In* Wheat and Wheat Improvement. Agronomy Monograph no.13. (2nd Edition). ASA-CSSA-SSSA, Madison. pp. 243-322.

Gregor, J.W. and F.W. Sansome. 1930. Experiments on the genetics of wild populations. J. Genet. 22: 373-387.

Humphries, C.J. 1980. *Phleum* L. *In* Flora Europaea. Vol. 5, edited by T. G. Tutin, V.H. Heywood, N.A. Burges, D.M. Moore, D.H. Valentine, S.M. Walters, and Webb. Cambridge University Press, Cambridge. pp. 239-241.

Joachimiak, A. 1981. *Phleum boehmeri* Wib. Hy-banding karyotype analysis. Acta Biologica Cracoviensia, series: Botanica. XXIII: 147-154.

Levan, A. 1941. Syncyte formation in the pollen-mother cells of haploid *Phleum pratense*. Hereditas. 27: 243-252.

Loidl, J. 1983. Some features of heterochromatin in wild *Allium* species. Syst. Evol. 143: 117-131.

Müntzing, A. 1935. Cyto-genetic studies on hybrids between two *Phleum* species. Hereditas. 20: 103-136.

Müntzing, A., and R. Prakken. 1940. The mode of chromosome pairing in *Phleum* twins with 63 chromosomes and its cytogenetic consequences.

Hereditas. 26: 463-501.

Myers, W.M. 1944. Cytological and genetic analysis of chromosomal association and behavior during meiosis in hexaploid timothy.(*Phleum pratense*) J. Agr. Res. 68: 21-33.

Nath, J. 1967. Cytogenetical and related studies in the genus *Phleum* L. Euphytica 16: 267-282.

Nielsen, E.L. and D.C. Smith. 1959. Chlorophyll inheritance patterns and extent of natural self-pollination in timothy. Euphytica. 8: 169-179.

Nordenskiöld, H. 1941. Cytological studies in triploid *Phleum*. Bot. Notiser. 1941. 12-32.

Nordenskiöld, H. 1945. Cytogenetic studies in the genus *Phleum*. Acta Agr. Suecana. 1: 1-137.

Nordenskiöld, H. 1949. Synthesis of *Phleum pratense* L. from *P. nodosom* L. Hereditas. 35: 190-202.

Nordenskiöld, H. 1953. A genetical study in the mode of segregation in hexaploid *Phleum pratense*. Hereditas. 39: 469-488.

Nordenskiöld, H. 1954. A genetical study in the mode of segregation in hexaploid *Phleum pratense*. Caryologia suppl. 6: 704-707.

Riley, R. 1965. Cytogenetics and the evolution of wheat. *In* Essays on Crop Plant Evolution, edited by J. Hutchinson. Cambridge University Press, Cambridge. pp. 103-122.

Stebbins, G.L. Jr. 1950. Variation and evolution in plants. Columbia

University Press, New York.

Teppner, H. 1980. Karyologie und systematik einiger gefaBpfanzen der ostalpen : Botanische studien im Gebiet der Planneralm (Niedere Tauern, Steiermark) VII. Phyton (Austria). 20: 73-94.

Waines, J. G. and G. Kimber. 1973. Satellite number and size in *Triticum* monococcum L. and the evolution of the polyploid wheats. Can. J. Genet. Cytol. 15: 117-122.

Wexelsen, H. 1941. Chlorophyll deficient seedlings in timothy (*Phleum pratense* L.). J. Hered. 32: 227-231.

Wilton, A.C. and L.J. Klebesadel. 1973. Karyology and phylogenetic relationships of *Phleum pratense*, *P. commutatum*, and *P. bertolonii*. Crop Sci. 13: 663-665.

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

Species	Accession No.	2*n*	Source
P. pratense	M 46	42	Agriculture Canada Ste-Foy Research Station, Quebec.
	M 44	42	Agriculture Canada Plant Research Center, Ottawa.
	M 35	42	Jardin Botanique National de Belgique, Meise.
P. alpinum ssp.*rhaeticum*	M 27	14	Botanischer Garten und Botansches museum, Berlin-Dahlem.
	M 39	14	Muséum National d' Histoire Naturelle, Paris
P. bertolonii	M 40	14	Muséum National d' Histoire Naturelle, Paris.
	M 33	14	Botanischer Garten und Botanisches Museum, Berlin-Dahlem
	M 16	14	Plant Gene Resources of Canada, Ottawa.

Table 2. The lengths (μ) and arm ratios (L/S) of the chromosomes from three *Phleum* species

	Genome designation						
	A1	A2	A3	A4	A5	A6	A7
P. pratense							
Length	7.09±.51	6.49±.30	6.10±.37	5.67±.41	4.97±.35	4.54±.27*	4.28±.43
L/S	1.18±.05	1.22±.09	1.38±.08	1.64±.20	1.52±.14	2.32±.43	2.81±.37
Length	6.72±.29	6.28±.43	5.87±.53	5.34±.48	4.82±.35	4.24±.22*	3.80±.25
L/S	1.23±.07	1.14±.07	1.44±.19	1.80±.18	1.82±.22	2.34±.28	2.72±.43
P. alpinum							
Length	7.26±.79	6.47±.70	5.85±.59	5.70±.54	5.26±.43	4.46±.56*	4.28±.43
L/S	1.28±.11	1.41±.11	1.34±.08	1.56±.16	1.49±.24	2.05±.19	2.72±.27
	B1	B2	B3	B4	B5	B6	B7
P. pratense							
Length	7.72±.47	6.52±.40	6.12±.38	5.60±.38	5.33±.39	5.05±.28	4.52±.30
L/S	1.53±.13	1.20±.07	1.55±.14	1.19±.20	1.13±.08	1.12±.08	2.85±.26
P. bertolonii							
Length	7.62±.80	6.98±.80	6.40±.80	6.23±.78	5.79±.67	5.25±.62*	4.78±.34
L/S	1.53±.11	1.17±.08	1.51±.15	1.17±.10	1.17±.06	1.84±.26	2.77±.34

* Not including the satellites L/S

Fig. 1, The Giemsa C-banded mitotic chromosomes of *P. pratense* ($2n$=42), 2300x.

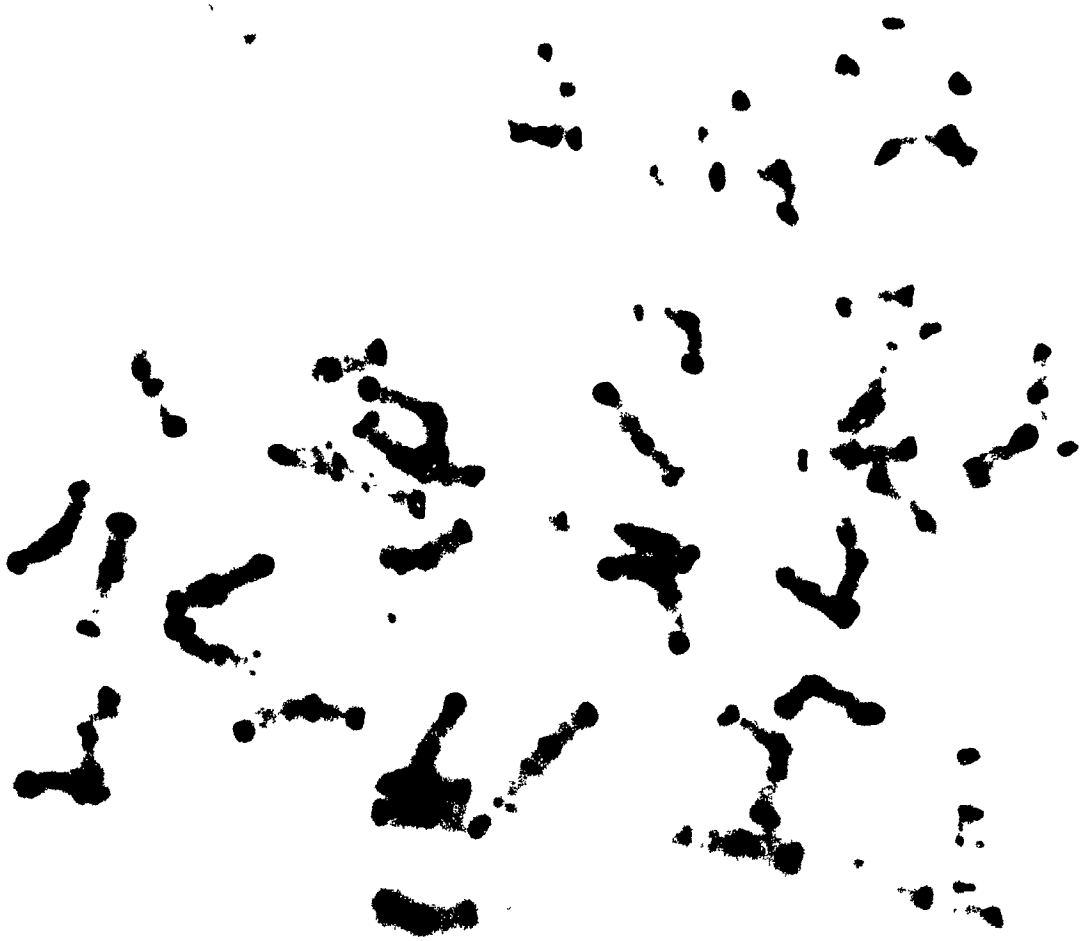

Fig. 2. The Giemsa C-banded mitotic chromosomes of two diploid *Phleum* species, *P. alpinum* (a) and *P. bertolonii* (b), 2300 x.

a b

Fig. 3, The banded karyotypes and idiograms of C-banding patterns for three *Phleum* species: (a) *P. pratense*, where two of the A genomes and the one of the B genomes of this hexaploid are shown; (b) *P. alpinum*, where the A genome is shown; (c) *P. bertolonii*, where the B genome is shown. For the nomenclature of the individual chromosomes, the lower case letters "p", "a" and "b" are used to designate the corresponding species (*P. pratense*, *P. alpinum* and *P. bertolonii*).


A
pA1
pA2
pA3
pA4
pA5
pA6
pA7
B
pB1
pB2
pB3
pB4
pB5
pB6
pB7
a
aA1
aA2
aA3
aA4
aA5
aA6
aA7
b
bB1
bB2
bB3
bB4
bB5
bB6
bB7
c

Analysis of genome-specific sequences in Phleum species: Identification and use for study of genome relationships

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Abstract

Sau3AI "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 Sau3AI and cloned using the plasmid pUC19 as a vector to an *E. coli* strain DH5αmcr. A partial genomic DNA library consisting of 3030 colonies for *P. alpinum* and one consisting of 3240 colonies for *P. bertolonii* were constructed. 1230 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. 24 colonies exhibiting signals specific to the genome of *P. alpinum* and 34 specific to the genome of *P. bertolonii* were isolated. Further dot blot analysis in four different stringencies confirmed 8 clones specific to the genome of *P. alpinum* and 13 specific to the genome of *P. bertolonii*. Most of these clones may carry highly or moderately repetitive sequences. Three clones from the genome of *P. alpinum* and seven from *P. bertolonii* exhibited their genome specificity at the lowest stringency (hybridization in 50% formamide and washing at 55°C). Three highly repetitive sequences specific to *P. bertolonii* genome, and three high-copy-number sequences specific to *P. alpinum* genome were used as probes to hybridize the EcoRI-digested DNA samples from four species, *P. alpinum, P. bertolonii, P. pratense* and *P. montanum*, on slot blot and Southern blot. The results from these hybridization experiments showed that all three *P. bertolonii*-specific probes

and two of three *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 which was drawn in our previous study using the C-banding technique.

Key words: genome-specific sequences, DNA hybridization, repetitive sequences, phylogeny, *Phleum*.

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 genome markers or genome-specific probes which can be used to identify a particular genome type (Zhao et al. 1989), or to trace genome phylogenetic relationships (Hosaka et al. 1990). Hybridization of genome-specific probes to isolated DNA or to chromosomes *in situ* is believed to be reliable for genome characterization and the estimation of phylogenetic relationships of plant species (Junghans and Metzlaff 1988; Anamthawat-Jónsson 1990). It is also effective when used to discriminate the polyploid nature in plants or to test the models of progenitor-derivator relationships (Crowhurst and Gardner 1991). As a consequence, it has become an extremely useful tool for the studies on the 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 to identify somatic cell hybrids (Saul and Potrykus 1984; Pehu et al. 1990), to detect hybrid addition lines (Schmidt et al. 1990) and to study genome evolution (Rayburn and Gill 1985, 1987; Hosaka et al. 1990; Anamthawat-Jónsson et al. 1990; Fabijanski et al. 1990). The application of genome-specific sequences has contributed to genome studies in higher plants 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 10 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 ($2n = 6x = 42$)(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 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 lack of conclusive evidence both from cytology and from genetics. Our previous study using Giemsa C-banding technique has suggested that the hexaploid karyotype of *P. pratense* may consist of two genomes, A and B, with a genome formula of AAAABB; the A genome from *P. alpinum* and the B genome donated by *P. bertolonii* (Cai and Bullen 1991). To verify this, 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 used in this study

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 Macdonald Campus of McGill University for two weeks. Before isolation, the young plants were placed in the dark for 24 hours. Isolation of total DNA from these plants followed the procedure described by Ausubel et al. (1989) with some modifications. All solutions, plastic and glassware were autoclaved under about 7 Kg pressure for 18 min and the whole process of DNA isolation was performed at 4°C. The fresh tissue was harvested, frozen with liquid nitrogen and ground to a fine powder in a mortar and pestle. A volume of extraction buffer containing 10 M Tris-Cl (pH 8.0), 100 mM EDTA, 250 mM NaCl and 100 µg/ml proteinase K was added to the frozen powder (4 ml/g). Subsequently, 10% (wt/vol) sarkosyl was added to achieve a final concentration of 1%. The solution was incubated at 55°C for one hour. The lysate was centrifuged in a SS-34 rotor at 7000 x g for 15 min, the supernatant was moved to 3 volumes of 95% chilled ethanol, and nucleic acid precipitate was spooled out with a glass rod and resuspended in 10 ml TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 8.0). 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 volumes of ethanol. The

DNA resuspended in TE buffer was purified by CsCl gradient (5.15 g/8 ml)-ethidium bromide ultracentrifugation in Beckman 41 STi rotor at 38000 x g for 48 hours. The DNA was collected and repeatedly extracted with isopropanol. The purified DNA was quantified in a Beckman DU-40 UV Spectrophotometer.

Construction of partial genomic DNA libraries

Total DNA from *P. alpinum* (M39) and *P. bertolonii* (M33) was used for this experiment. The plant DNA was digested with Sau3AI at 37°C for 40 min and then purified with the Gene Clean II Kit (Bio 101 Inc.) as suggested by the manufacturer. Plasmid pUC19 was used as a vector for DNA cloning. The plasmid was digested with BamHI at the same temperature, purified with the Gene Clean II Kit and dephosphorylated by treating the DNA with calf intestinal alkaline phosphatase (CIP). The Sau3AI-digested fragments of plant DNA were ligated to BamHI-digested, dephosphorylated PUC19 with a insert-vector ratio of 2:1 (Sambrook et al. 1989). The ligated DNA mixture was used to transform an *E. coli* strain DH5αmcr (Jessee and Bloom 1988). The recombinant colonies were identified on the agar plates with Ampicillin and X-gal.

Colony hybridization

The colonies bearing *Phleum* genomic DNA libraries were blotted to Hybon-N membranes (Amersham) on which the DNA was denatured in alkaline solution and fixed by UV irradiation as suggested by the manufacturer. The total DNA from *P. alpinum* (M39) and *P. bertolonii* (M33) was used as total genomic probes representing two different genome. The total genomic probes were prepared by completely digesting the DNA with

Sau3AI at 37°C for one hour and purified with the Gene Clean II. The probes were labelled with [α-^{32}P]-dCTP by nick translation using the Nick Translation Labelling System (BRL). The specific activity of the labelled DNA was calculated based on the data from counting of 3 µl labelled DNA mixture in LS7500 scintillation counter (Beckman). The labelled probes were hybridized to the blotted colonies in the following procedure: The fixed colony DNA was prehybridized at 42°C in a solution containing 50% formamide, 0.25 M $NaHPO_4$ (pH7.2), 0.25 M NaCl, 7% (w/v) SDS, 1 mM EDTA and 10% (w/v) PEG-3000 for one hour, followed by hybridization overnight in the same but fresh new solution to which the labelled probes were added (5 ng/ml, about 1 x 10^6 cpm). Subsequently the membranes were washed for 15 min in each of the following steps: 2 X SSC + 0.1% SDS at room temperature, 0.5 X SSC + 0.1% SDS at the same temperature and finally 0.1 X SSC +0.1% SDS at 60°C. Immediately the filters were exposed to Hyperfilm™-ECL (Amersham).

Dot-blot analysis

All the clones isolated from colony hybridization were amplified and subject 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. 0.2 µg DNA was loaded for each sample. 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 in the same procedure as in colony hybridization but changing the temperature in the last step. To determine whether sequence overlapping is involved between the clones, the dot-blotted plasmids were hybridized to various labelled probes from the isolated clones. The conditions of hybridization and washing were the same as in colony hybridization except the temperature in the last step of washing was changed to 65°C.

Slot-blot hybridization

DNA from all the plants listed in Table 1 was used for the slot-blot analysis. The plant DNA (50 ng for each) was slot-blotted to a Zeta-Probe membrane with the same procedure used in dot blotting. Three clones specific to *P. alpinum*, pPa07, pPa08, pPa11 and three *P. bertolonii* genome-specific clones, pPb27, pPb43 and pPb68 were used as probes for slot-blot hybridization. The inserts were excised from these plasmids by digesting them with EcoRI and HindIII and labelled with [α-^{32}P]-dCTP by nick translation. They were used to hybridize against slot blot. Slot-blot hybridization was performed with the same procedure as in colony hybridization.

Southern blot analysis

Plant DNA was digested with EcoRI (5 units/µg) at 37°C for one hour 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. All the probes and hybridization procedures used in this experiment were the same as used in slot-blot hybridization.

Results

Identification and analysis of genome-specific sequences

Isolation and cloning of Phleum DNA DNA was isolated from the *Phleum* plants (Table 1) through the procedure described above. The typical size of the DNAs obtained was estimated to be about 50 kbp. They were digested by different restriction endonucleases (data not shown). This indicates that these DNAs can be used for molecular cloning to construct a partial genomic DNA library. The DNA from diploid *P. alpinum* and *P. bertolonii* were partially digested with Sau3AI (1 unit/μg DNA) at 37°C for 40 min. About 80% of the fragments generated ranged in size from 200 bp to 3 kbp (Fig. 1). These DNA fragments were randomly inserted into the plasmid pUC19 at the BamHI site. By transforming the *E. coli* strain DH5αmcr, a high frequency of recombinant colonies was obtained for the genomic library of *P. alpinum* (91%) and for the genomic library of *P. bertolonii* (93%) (Table 2).

Screening the genomic libraries by colony hybridization In order to isolate the clones specific respectively to the genome of *P. alpinum* and the genome of *P. bertolonii* from the two genomic libraries, total DNA from *P. alpinum* and from *P. bertolonii* was used as total genomic probes to self- and cross-hybridize the colonies from these two genomic libraries. 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 genome-specific clones. A total of 1230 recombinant colonies from the genomic library of *P. alpinum* were hybridized with the total genomic probe from *P. alpinum* and that from *P. bertolonii* respectively. 920 colonies gave detectable signals upon self-hybridization with labelled total probe from *P. alpinum*. The rest of the colonies showed no detectable signals either in self-hybridization or in cross-hybridization. The signal intensity was very high in 20% of those colonies, moderate in 35% of them and low in 45% of them (Table 3). The colonies showing high-intensity signals may bear highly repetitive sequences and those giving middle-intensity or low-intensity signals may contain sequences with moderate copy number or low copy number. In a comparison of hybridization signals between self-hybridization and cross-hybridization, signal specificity in some colonies was found (Fig. 2). As indicated by the arrows in Fig. 2, two colonies show intense signals using total genomic probe of *P. alpinum* (Fig. 2a) but show no signals using the genomic probe of *P. bertolonii* (Fig. 2b). A total of 24 colonies exhibited hybridization specificity and they were isolated for further analysis.

In screening 1320 colonies in the genomic library of *P. bertolonii*, 1035 colonies gave hybridization signals upon self-hybridization. 25% of the hybridized colonies showed signals with high intensity, 36% with moderate intensity and 39% with low intensity (Table 3). Hybridization specificity could be readily identified in some colonies when their signals were compared between self-hybridization and cross-hybridization experiments. Fig. 3 shows the hybridization results of some colonies from the B-genomic library probed by labelled total DNA of *P. alpinum* (cross-hybridization) and that of *P. bertolonii* (self-hybridization). As pointed by the arrows, four

colonies show intense hybridization to the genomic probe from *P. bertolonii* (Fig. 3a) but very weak hybridization to the genomic probe from *P. alpinum* (Fig. 3b), suggesting that these sequences bind preferentially to the genome of *P. bertolonii*. Through colony hybridization, a total of 34 colonies recognized to be specific to *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 are really specific to a genome and also to determine in which level of stringency their genome specificity can be identified. Recombinant plasmids from selected colonies were isolated by the alkaline method of Birnboin and Doly (1979) and dot-blotted to duplicate Zeta-Probe membranes.

The dot blot was hybridized with the total genomic probe from *P. alpinum* and that from *P. bertolonii*. The following four stringencies were used to characterize the genome specificity of all isolated clones: hybridizing in 50% formamide and washing at 55°C, hybridizing in 50% formamide and washing at 65°C, hybridizing in 60% formamide and washing at 55°C and hybridizing in 60% formamide and washing at 65°C. The results of dot-blot analysis of some clones from the two genomic libraries are shown in Fig. 4 and Fig. 5 respectively. A total of eight clones isolated were confirmed to be specific to *P. alpinum* (Table 4). Among them, three clones: pPa08, pPa24 and pPa42 can be identified as genome-specific at lower stringencies of hybridization (in 50% formamide) and of washing (at 55°C). Upon hybridization under such stringency, pPa08 showed no signal to the genomic probe from *P. bertolonii* and the other two pPa24 and pPa42 gave extremely weak signals to this probe, in contrast to intense signals with the genomic

probe from *P. alpinum*. When washing stringency was raised by increasing the temperature to 65°C, all signals with pPa24 and pPa42 were lost in cross-hybridization to the genomic probe from *P. bertolonii* (Fig. 4). Another three clones: pPa16, pPa33 and pPa47 showed their genome specificity only at higher stringencies of hybridization (in 60% formamide) and of washing (at 65°C), implying that their genome specificity was relatively low. The genome specificity of the rest of the clones: pPa07 and pPa11 can be characterized as follows: pPa07 showed the signal specific to the genomic probe from *P. alpinum* in the lower hybridization stringency (in 50% formamide) but higher washing stringency (65°C) and pPa11 showed the same feature at higher hybridization stringency but lower washing stringency.

Among the clones isolated from the genomic library of *P. bertolonii*, 13 were confirmed to be specific to *P. bertolonii* by dot blot analysis (Table 5). Out of these clones seven showed their signal specificity identifiable at the lower stringency of hybridization and washing. Upon hybridization in 50% formamide and washing at 55°C, five clones: pPb03, pPb68, pPb69, pPb81 and pPb82 gave signals unique to the total genomic probe from *P. bertolonii*. Another two pPb27 and pPb43 showed highly intensity signals to the same genomic probe but very weak signals to the genomic probe from *P. alpinum* (Fig. 5). Under this stringency, no other clones showed their genome specificity. Upon increasing the washing stringency to the higher level (65°C), however, another three clones: pPb08, pPb75 and pPb88 immediately responded to this by showing hybridization exclusively to the genomic probe from *P. bertolonii*. For the rest of the three clones, genome specificity could be identified only at higher hybridization stringency. Among these, one clone pPb05 showed its hybridization specificity to the genomic probe of *P.*

bertolonii at the lower washing stringency and the other two: pPb70 and pPb78 showed their specificity only at the higher washing stringency.

It was found that 16 clones isolated from *P. alpinum* genome and 21 from the *P. bertolonii* genome were not really specific to either of the genomes because they showed no hybridization specificity even in the higher hybridization stringency and the higher washing stringency. Their signal specificity revealed in colony hybridization may be attributed to copy-number differences between species or to technical problems rather than to genome specificity.

Analysis of genome-specific clones The sizes of plant DNA inserts in the genome-specific clones (Table 4 and 5) were determined by comparing EcoRI and HindIII-digested plasmids after electrophoresis on 1.0% agarose gels (Fig. 6, 7). Three classes of relative sequence abundance in these clones were deduced from the signal intensities in dot blot hybridization.

To determine whether the same or overlapping sequences were carried among some of these selected clones, an experiment was performed by cross-hybridizing the dot-blotted plasmid DNA to various labelled insert probes from some of these clones. Within the group of *P. alpinum*-specific clones, all eight target DNAs were hybridized to the labelled insert probes from five clones. The results are presented in Table 6. 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. In the cross-hybridization within the group of *P. bertolonii*-specific clones, all 13 isolated plasmids were dot-blotted as targets and hybridized to six labelled insert probes (Table 7). As shown in Table 7, sequence overlapping was confirmed

between pPb03 and pPb68 and between pPb27 and pPb69.

Analysis of genome relationships using genome-specific probes

In order to examine genetic relationships between hexaploid *P. pratense* and the two diploid species *P. alpinum* and *P. bertolonii*. a slot blot and a Southern blot analysis were carried out using genome-specific probes isolated from *P. alpinum* and *P. bertolonii*. Three *P. alpinum* genome-specific clones and three *P. bertolonii* genome-specific clones (Fig. 8) were used as probes to screen the DNA samples from four *Phleum* species, *P. pratense*, *P. alpinum*, *P. bertolonii* and *P. montanum* on slot blot and Southern blot. Among these species, tetraploid *P. montanum* ($2n = 4x = 28$) seemed to have a karyotype differentiated from the genomes of *P. alpinum* and *P. bertolonii* (unpublished) and it was used for these analyses as a control to highlight the genome specificity of the selected probes. The results from slot blot and Southern blot hybridizations are presented below..

Slot-blot hybridization Fig. 9 summarizes the results of slot-blot hybridizations. When all three *P. bertolonii*-specific clones: pPb27, pPb43 and pPb68 were used as probes, all DNA samples from *P. bertolonii* and *P. pratense* showed hybridization signals. One sample (M39) from *P. alpinum* showed very weak signal with pPb43. The other sample (M27) from this species and the sample from *P. montanum* showed no detectable signals. This indicates that at least one part of the genome in *P. pratense* may be homologous to the genome in *P. bertolonii*. In the case of hybridizations with the *P. alpinum*-specific probes, apart from the DNA samples from *P. alpinum* which showed intense signals to all these probes, the DNA samples from *P. pratense* showed hybridization to two probes, pPa08 and pPa11 but

gave no signal to pPa07. *P. bertolonii* showed very weak signals to pPa11 and no signals to the other two probes. *P. montanum* gave a weak signal to pPa08 and no signals to the other two probes. These results indicate that the genome in *P. alpinum* is closely related to the genome in *P. pratense*. A possible explanation for lack of hybridization between *P. pratense* DNA and pPa07 is that the related sequence may have been highly differentiated between these two species or may have been completely lost in *P. pratense*.

Southern blot hybridization In Southern blot analysis, DNA samples from the four species, *P. pratense*, *P. alpinum*, *P. bertolonii* and *P. montanum* were digested by EcoRI, separated on agarose gel by electrophoresis, Southern blotted to the membrane filter Zeta Probe, and finally hybridized to the same probes as used in slot blot hybridization. The results of hybridization with the three *P. bertolonii*-specific probes are shown in Fig. 10. In these hybridizations, besides *P. bertolonii*, the DNA samples from *P. pratense* exhibited strong hybridization signals to the probes, but no DNA from another two species, *P. alpinum* and *P. montanum* gave signals. Two probes, pPb27 and pPb43 seemed to result in hybridization with a ladder pattern in the EcoRI-digested DNA samples (Fig. 10a, b). The similar ladder pattern, was observed for the tandemly repeated, interspersed sequences (Zhao et al. 1989; Pehu et al. 1990). The ladder pattern was obvious in the *P. pratense* samples but not very clear in the *P. bertolonii* samples. To confirm this, the DNA of *P. bertolonii* (M33) was digested by Sau3AI and then probed by the same two sequences (pPb27 and pPb43). As a result, very clear ladder pattern was observed with the Sau3AI-digested sample (Fig. 11). This ladder pattern may be attributed to these probes which may be interspersed sequences. It is also possible that this ladder

pattern is caused by imcomplete diggestion.

Southern blot hybridization with the probe pPa07 (specific to *P. alpinum*) confirmed the result in slot blot hybridization. Only *P. alpinum* exhibited hybridization and *P. pratense* did not hybridize to this species. The hybridization with the other two *P. alpinum* genome-specific probes, pPa08 and pPa11 revealed signals both in *P. alpinum* and in *P. pratense* but no signals were apparent in other DNA samples (Fig. 12), suggesting that the DNA from *P. pratense* share DNA sequences with the *P. alpinum* DNA. According to the banding pattern revealed in Southern blot hybridization, these two clones were unlikely to be an interspersed sequence.

Both slot blot and Southern blot hybridizations confirmed that the *P. pratense* DNA was closely related to the genome of *P. alpinum* and the genome of *P. bertolonii* in the diploid species. In contrast, the *P. montanum* DNA did not hybridize to the probes from these two genome, suggesting that its genome was highly differentiated from these two.

Discussion

Successful construction of species- or genome-specific clones by "shot gun" cloning of Sau3AI-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). In our paper, we describe the cloning, isolation and characterization of genome-specific sequences in *Phleum* species. Sau3AI "shot gun" cloning of *Phleum* DNA resulted in a genomic library consisting of 3030 recombinant colonies for *P. alpinum* and a library consisting of 3240 recombinant colonies for *P. bertolonii*. By screening 1230 recombinant

colonies of *P. alpinum* genome and 1320 of *P. bertolonii* genome through colony hybridization and further dot blot analysis using total DNA from these two species as probes, eight clones specific to the genome of *P. alpinum* and 13 specific to the genome of *P. bertolonii* were identified. 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). These differences may be attributed to differences of genetic distances between species used in different studies rather than to technical problems. Distantly related species contain a larger proportion of sequences differentiated from one another than closely related species. Thus more genome-specific sequences are expected to be obtained in the distantly related species. In the case of *Secale* and *Triticum* (Metzlaff et al. 1986), a very high frequency of clones specific to wheat (10%) was detected. However, only about one-third of that was detected between two potato species, *Solanum brevidens* and *S. tubersum* (Pehu et al. 1990). The species used in the latter case are taxonomically more closely related than those in the former. In the case of *P. alpinum* and *P. bertolonii*, the low frequency of genome-specific clones identified in this study may imply that there is to some extent close relationship between these two species. 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 related to *p. alpinum*.

Total genomic DNA has been used as probes for identification of genome-specific sequences. The rationale of detection of genome-specific sequences using total genomic probes is that highly differentiated sequences between genomes is expected to result in a marked contrast of hybridization signals

between self-hybridization and cross-hybridization. In self-hybridization the signals are very strong while in cross-hybridization they are very weak or missed. Therefore, when comparing the signals shown between self- and cross-hybridization, any cloned DNA exhibiting the signals specific to self-hybridization can be identified to be genome-specific. A remarkable advantage of this strategy is that it permits the screening of many recombinant clones in one pair of hybridizations, thus making it possible to select genome-specific clones more efficiently. Alternatively, hybridization of total DNA samples to random cloned probes may be used for isolation of genome-specific sequences. But it is too time consuming when compared with the former.

One problem may occur with colony hybridization in that it is sometimes difficult to localize the colonies with hybridization specificity on master plates by comparing the signals in X-ray films. To overcome this problem, it is necessary to reduce the cell concentration added to an agar plate or a membrane filter. According to our study the suitable number of colonies for each filter is 100-150. Another problem may occur with colony hybridization in that it is difficult to determine whether the hybridization specificity exhibited by the colonies is due to genome specificity of related sequences or to differences of copy numbers of the sequences. It is also difficult to characterize the stringencies of the hybridization specificity. To solve these problems, further analysis of the isolated clones with dot-blot hybridization in different stringencies is required. In this study, plasmids from all the isolated clones were dot-blotted and hybridized to the total genomic probes in two stringencies, low in 50% formamide and high in 60% formamide, and washed in two stringencies, low at 55°C and high at 65°C. A combination of the hybridization and washing stringencies represented four levels of

stringencies, from the lowest level: hybridization in 50% formamide and washing at 55°C to the highest: hybridization in 60% formamide and washing at 65°C. One-third of the clones were confirmed in this analysis to be genome-specific and the others were eliminated for not exhibiting hybridization specificity even at the highest level of stringency. Ten clones, 3 from the A genome and 7 from the B genome, exhibited hybridization specificity at the lowest level of stringency. Five clones, 3 from the A genome and 2 from the B genome, showed this feature only at the highest stringency. The others were identified at the intermediated stringencies. It can be argued that the levels of stringencies of exhibition of hybridization specificity may inversely reflect the degrees of genome specificity of a particular sequence. The lower the stringency under which the hybridization specificity of a sequence can be identified, the higher degree of genome specificity the sequence should be. This argument is based on the fact that at lower stringency, hybridization is allowed between the single-strands with a relatively wide range of sequence differentiation whereas in high stringency, hybridization is only allowed between the single-strands with a narrow range of differentiation. Those clones exhibiting hybridization specificity in the lowest stringency are considered to be highly genome-specific.

Based on the hybridization intensity on dot blot (data not shown), most of the genome-specific clones seleted in this study may carry highly or intermidiately repetitive sequences. Almost all the species- or genome-specific sequences reported for the other plants were repetitive sequences. Genome specificity seems easier to be identified in repetitive sequences compared to low- or single-copy sequences because the former gives higher contrast of signals between self-hybridization and cross-hybridization. In

addition, a larger proportion of intergenomic divergent sequences may be involved in repetitive sequences so that there are more opportunities to obtain genome-specific sequences from them. 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 appears to evolve rapidly between genomes presumably through amplification, divergence and transposition (Hake and Walbot 1980; Singer 1982; Zhao et al. 1989). Therefore it may be considered to be an important source of probes that are species-specific or genome-specific. Repetitive DNA in plants is found in three possible arrangements: interspersed with single copy DNA, interspersed with repetitive DNA of different reiteration frequency, or in long uninterrupted similar repeats. Each of these pattern of repeats has been well characterized in some plants, especially in wheat and rye. Six repetitive sequence families have been characterized in rye, including the 630-, 610-, 480- and 120-bp families (Bedbrook et al. 1980), the 350-480 bp family (Appels and McIntyre 1985), the 5.3 H_3 family (Appels et al. 1986) and the R173 family (Guidet et al. 1991). In the case of *Phleum*, no repetitive DNA has been well characterized. Although most of the genome-specific sequences isolated in this study are proposed to be repeated sequences, no information regarding their lengths of units and definite copy numbers per genome is available. The Southern analysis with pPb27 and pPb43 suggests that the arrangement pattern of these two highly repetitive sequences may be interspersed. More studies are required to confirm their arrangement pattern and further to characterize these sequences.

This study has provided the first molecular evidence for genome

relationships in *Phleum*. Three clones specific to *P. alpinum* and three specific to *P. bertolonii* were used as probes to hybridize to the DNA from four species, *P. alpinum, P. bertolonii, P. pratense* and *P. montanum* on slot blot and Southern blot. All three *P. bertolonii*-specific probes and two of the three *P. alpinum*-specific probes were shown to hybridize to the DNA of *P. pratense*, thus confirming that the polyploid genome in *P. pratense* was closely related to the genome of *P. alpinum* and the genome 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, involving two genomes. One genome may have come from *P. alpinum* and the other is most likely donated by *P. bertolonii*. In contrast to the case of *P. pratense*, none of the probes from *P. alpinum* and *P. bertolonii* were shown to hybridize to the DNA from *P. montanum*, except pPa08 which showed a weak hybridization signal. This suggests the genome distinctiveness between this species and the two diploid species, *P. alpinum* and *P. bertolonii*. It should be pointed out that *P. alpinum* may share a common genome with *P. pratense*, there appears to be some sequence differentiations between these species since one clone pPa07 from *P. alpinum* can not hybridize to the *P. pratense* DNA. The data of the C-banded karyotype of *P. pratense* (Cai and Bullen 1991) seemed 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 the genome-specific probes to its chromosomes. This work has been attempted in our laboratory but has not succeeded as yet. So no information has been obtained on this aspect.

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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References

Anamthawat-Jónsson, K., T. Schwarzacher, A.R. Leitch, M.D. Bennett, and J.S. Heslop-Harrison. 1990. Discrimination between closely related *Triticeae* species using genomic DNA as a probe. Theor. Appl. Genet., 79: 721-728.

Appels, R., and C.L. McIntype. 1985. Cereal genome organisation as revealed by molecular probes. Oxf. Surv. Plant Mol. Cell Biol., 2: 235-252.

Appels, R., L.B. Moran, and J.P. Gustafson. 1986. Rye heterochromatin. I.

Studies on clusters of the major repeating sequences and the identification of a new dispersed repetitive sequence element. Can. J. Genet. Cytol., 28: 645-657.

Ausubel, F.M., R. Brent, R.E. Kingston, D.D. Moore, J.G. Scidman, J.A. Smith, and K. Struhl. 1989. Current Protocols in Molecular Biology. Greene Publishing Associates and Wiley-Interscience: J. Wiley. New York.

Bedbrook, J.R., J. Jones, M. O'Dell, R.D. Thompson, and R.B. Flavell. 1980. A molecular description of telomeric heterochromatin in *Secale* species. Cell, 19: 545-560.

Birnboin, H.C., and J. Doly. 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acid Res., 7: 1513-1525.

Cai, Q., and M.R. Bullen. 1991. Characterization of genomes of timothy (*Phleum pratense* L.). I. Karyotypes and C-banding patterns in cultivated timothy and two wild relatives. Genome, 34: 52-58.

Crowhurst, R.N., and R.C. Gardner. 1991. A genome-specific repeat sequence from kiwifruit (*Actinidia deliciosa* var. *deliciosa*). Theor. Appl. Genet., 81: 71-78.

Fabijanski , S., G. Fedak, K. Armstrong, and I. Altosaar. 1990. A repeated sequence probe for the C genome in *Avena* (oats). Theor. Appl. Genet., 79: 1-7.

Flavell, R.B., M.D. Bernett, J.B. Smith, and D.B. Smith. 1974. Genome size and the proportion of repeated nucleotide sequence DNA in plants. Biochem. Genet., 12: 257-269.

Gregor, J.W. 1931. Experimental delimitation of species. New Phytol., 30: 204-217.

Gregor, J.W., and F.W. Sansome. 1930. Experiments on the genetics of wild

populations. J. genet., 22: 373-387.

Guidet, F., P. Rogowsky, C. Taylor, W. Song, and P. Langridge. 1991. Cloning and characterisation of a new rye-specific repeated sequence. Genome, 34: 81-87.

Hake, S., and V. Walbot. 1980. The genome of *Zea mays*, its organization and homology to related grasses. Chromosoma, 79: 251-270.

Hosaka, K., S.F. Kianian, J.M. McGrath, and C.F. Quiros. 1990. Development and chromosomal localization of genome-specific DNA markers of *Brassica* and the evolution of amphidiploid an $n = 9$ diploid species. Genome, 33. 131-142.

Jessee, J., and F. Bloom. 1988. DH5αMCR™: New competent cells for cloning methylated DNA. Focus 10· 69.

Jones, J.D., and R.B. Flavell. 1982. The mapping of highly-repeated DNA families and their relationship to C-bands in chromosomes of *Secale cereale*. Chromosoma, 86. 595-612.

Junghans, H., and M. Metzlaff. 1988. Genome specific, highly repeated sequences of *Hordeum vulgare*: cloning, sequencing and squash dot test. Theor. Appl. Genet., 76: 728-732.

Metzlaff, M., W. Troebner, R Schlegel, and J. Cullum. 1986. Wheat-specific repetitive DNA sequences-construction and characterization of four different genomic clones. Theor Appl Genet , 72. 207-210.

Müntzing, A. 1935. Cyto-genetic studies on hybrids between two *Phleum* species. Hereditas, 20 103-136.

Müntzing, A., and R. Prakken. 1940. The mode of chromosome pairing in *Phleum* twins with 63 chromosomes and its cytogenetic consequences. Hereditas 26· 463-501.

Nordenskiold, H. 1941. Cytological studies in triploid *Phleum*. Bot. Notiser.

1941. pp. 12-32.

Nordenskiöld, H. 1945. Cytogenetic studies in the genus *Phleum*. Acta Agr. Suecana, 1: 1-137.

Pehu, E., M. Thomas, T. Poutala, A. Karp, and M.C.K. Jones. 1990. Species-specific sequences in the genus *Solanum*: identification, characterization, and application to study somatic hybrids of *S. brevidens* and *S. tuberosum*. Theor. Appl. Genet., 80: 693-698.

Rayburn, A.L., and B.S. Gill. 1985. Use of biotin-labeled probe to map specific DNA sequences on wheat chromosomes. J. Hered., 76: 78-81.

Rayburn, A.L. and B.S. Gill. 1986. Isolation of a D-genome specific repeated DNA sequences from *Aegilops squarosa*. Plant Mol. Biol. Rep., 4: 102-109.

Rayburn, A.L., and B.S. Gill. 1987. Use of repeated DNA sequences as cytological markers. Amer. J. Bot. 74: 574-580.

Sambrook, J., E.F Fritsch, and T. Maniatis. 1989. Molecular Cloning: a laboratory manual Cold Spring Harbor Laboratory, Cold Spring, New York.

Saul, M., and I. Potrykus. 1984. Species-specific repetitive DNA used to identify interspecific somatic hybrids Plant Cell Rep., 3: 65-67.

Schmidt, T., H. Junghans, and M. Metzlaff. 1990. Construction of *Beta procumbens*-specific DNA probes and their application for the screening of *B. vulgaris* x *B. procumbens* ($2n$ = 19) addition lines. Theor. Appl Genet., 79. 177-181.

Singer, M.F. 1982. Highly repeated sequences in mammalian genomes. Int. Rev. Cytol., 76. 67-112.

Stebbins, G.L., Jr. 1950. Variation and evolution in plants. Columbia University Press, New York. pp. 643.

Walbot, V., and C.A. Cullis. 1985. Rapid genomic change in higher plants. Annu. Rev. Physio. 36: 367-396.

Zhao, X., T. Wu, Y. Xie, and R. Wu. 1989. Genome-specific repetitive sequences in the genus *Oryza*. Theor. Appl. Genet., 78: 201-209.

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

Species	Accession No.	2*n*	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 Botanischer Museum, Berlin-Dahlem

Table 2. Cloning test of genomic DNA fragments from *P. alpinum* and *P. bertolonii* using pUC19 as a vector and transforming the *E. coli* strain DH5αmcr

DNA source	Observed colonies			Transformation frequency	
	Total No.	Recombinant No	%	Ligated plasmid with inserts	Control*
P. alpinum	3030	2760	91	$3.5X10^6$/μg	$2.1X10^7$/μg
P. bertolonii	3240	3010	93	$4.1X10^6$/μg	$2.5X10^7$/μg

* Ligated plasmid only.

Table 3. The signal intensity* and specificity of the screened colonies bearing genomic libraries of *P. alpinum* and of *P. bertolonii*

Genomic library	Screened colonies		Signal intensity						Specific
	Total No.	Signalled No.	High		Middle		Low		No.
			No.	%	No.	%	No	%	
P. alpinum	1230	920	185	20	325	35	410	45	24
P. bertolonii	1320	1035	255	25	370	36	410	39	34

* Three ranges of signal intensity were sorted based on sizes and intensity of spots in X-ray film. The intensity was finally tested by measurement of radioactivity of the probed DNA (50 ng each) from 30 colonies randomly sampled from each range and dot-blotted to a membrane. High intensity: > 80 cpm/mm^2; middle: 20-80 cpm/mm^2; low: 5-20 cpm/mm^2.

Table 4. The insert size, copy-number range, and stringency of genome specificity of the isolated clones specific to *P. alpinum*

Clone	Size of insert(Kbp)	Range of copy number	Stringency of genome specificity
pPa07	0.85	High	***
pPa08	0.15	Middle	****
pPa11	2.50	High	**
pPa16	4.55	Middle	*
pPa24	0.75	Low	****
pPa33	0.70	High	*
pPa42	2.05	Low	****
pPa47	0.90	High	*

DNA hybridization was carried at 42°C and 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 5. The insert size, copy-number range, and stringency of genome specificity of the isolated clones specific to *P. bertolonii*

Clone	Size of insert(Kbp)	Range of copy number	Stringency of genome specificity
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	***

DNA hybridization was carried at 42°C and 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 6. Cross-hybridization of *P. alpinum*-specific clones

Probes	Target							
	pPa07	pPa08	pPa11	pPa16	pPa24	pPa33	pPa42	pPa47
pPa07	+++	-	-	-	++	-	-	-
pPa08	-	+++	-	-	-	-	-	++
pPa11	-	-	+++	-	-	-	-	-
pPa24	-	-	-	-	++	-	-	-
pPa42	-	-	++	-	-	++	-	-

Table 7. Cross-hybridization of *P. bertolonii* -specific clones

Probes	Target												
	pPb03	pPb05	pPb08	pPb27	pPb43	pPb68	pPb69	pPb70	pPb75	pPb78	pPb81	pPb82	pPb88
pPb03	+++	-	-	-	-	+++	-	-	-	-	-	-	-
pPb08	-	-	+++	-	-	-	-	-	-	-	-	-	-
pPb27	-	-	-	+++	-	-	++	-	-	-	-	-	-
pPb43	-	-	-	-	+++	-	-	-	-	-	-	-	-
pPb68	+++	-	-	-	-	+++	-	-	-	-	-	-	-
pPb82	-	-	-	-	-	-	-	-	-	-	-	+++	-

Fig. 1. The sau3AI-digested total DNA from two diploid species of *Phleum*. The partial digestion of DNA was performed in Sau3AI (1 unit/μg DNA) at 37°C for 40 min. 5 μg DNA was loaded in each well and fractionated electrophoretically on 0.8% agarose gel. Lane 1: undigested DNA of *P. bertolonii* (M33), lane 2: its digested DNA, lane 3: undigested DNA of *P. alpinum*, lane 4: its digested DNA, and lane 5: 1 Kbp ladder fragment markers.

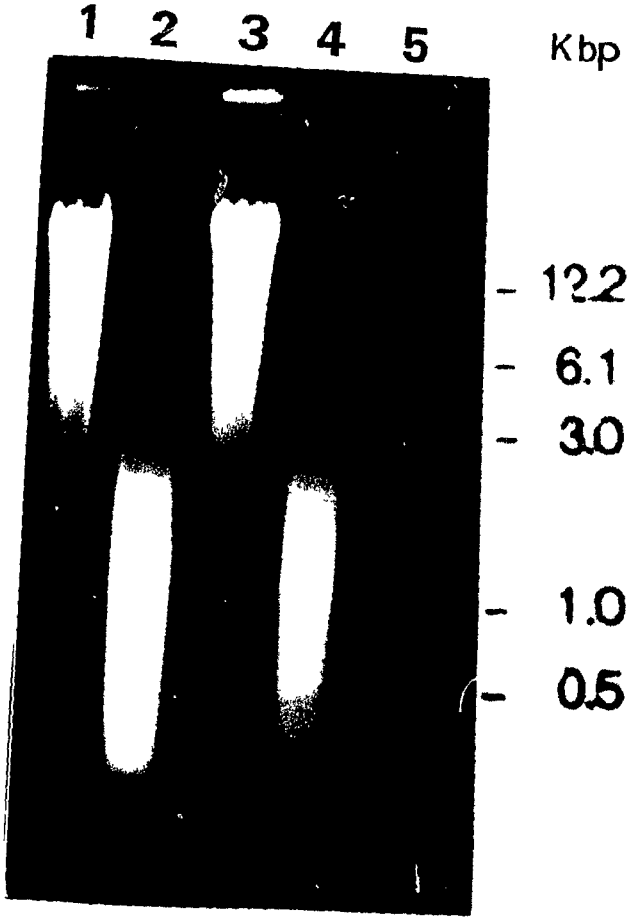
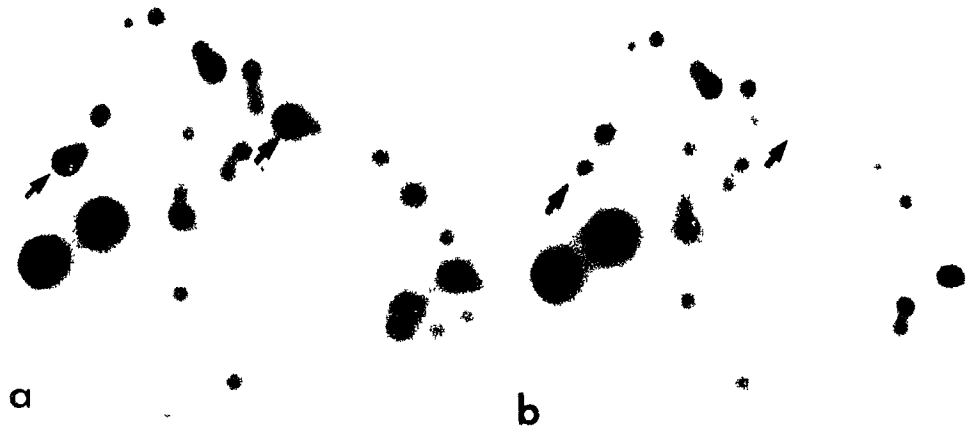

1
2
3
4
5
Kbp
12.2
6.1
3.0
1.0
0.5

Fig. 2. Comparison of the hybridizations of the 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 total genomic probe from *P. alpinum*.



a b

Fig. 3. Comparison of the hybridizations of the 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*.


b
a

Fig. 4. Dot-blot analysis of the colonies exhibiting hybridization signals specific to the genomic probe from *P. alpinum* in colony hybridization. The dot-blotted plasmid DNA from these colonies were hybridized to the total DNA probes from *P. alpinum* (a) and from *P. bertolonii*(b) under the following stringencies: hybridization in 50% formamide and washing at 55°C in the final step (1); hybridization in 50% formamide and washing at 65°C (2); hybridization in 60% formamide and washing at 55°C (3); and hybridization in 60% formamide and washing at 65°C (4). The washing in the first two steps was performed at room temperature for all stringencies. The exposure time of the hybridized membranes was 24 h.

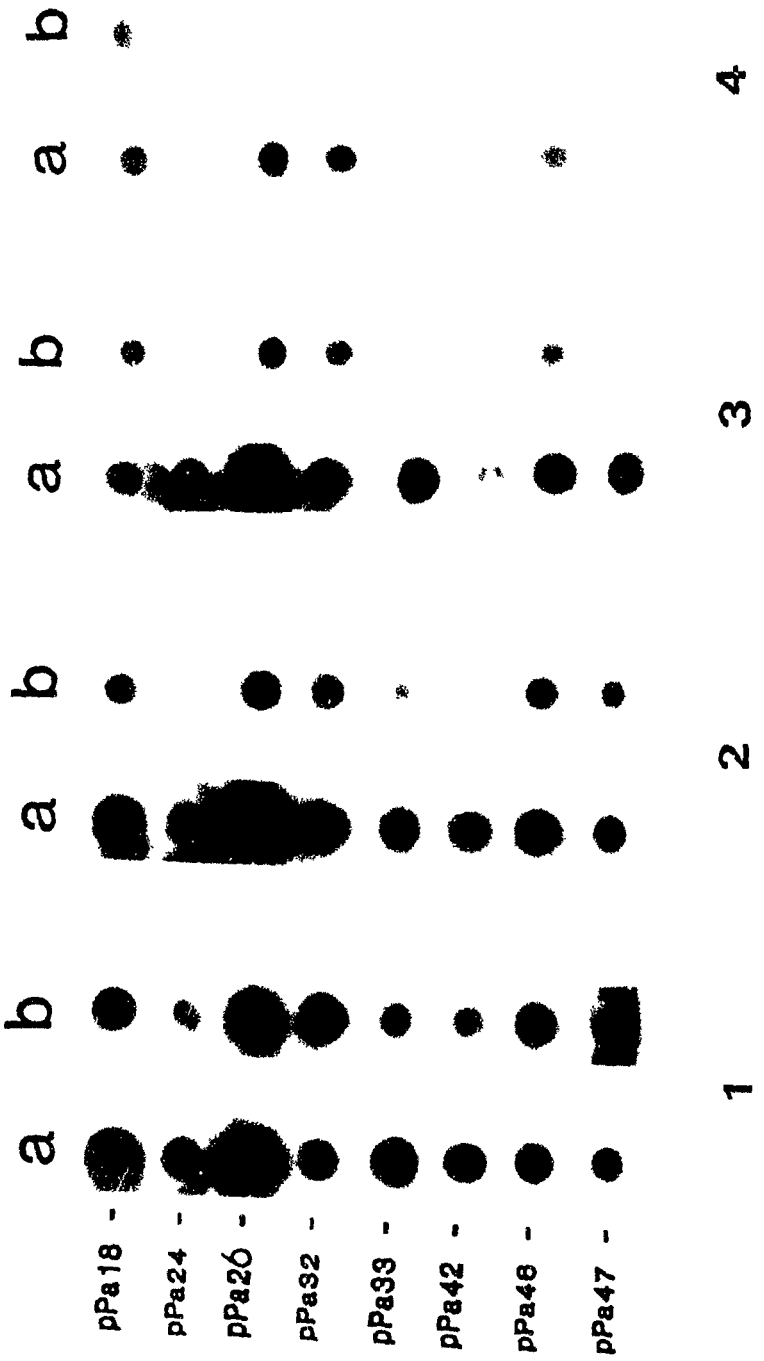

a
b
1
2
3
4
pPa18 -
pPa24 -
pPa26 -
pPa32 -
pPa33 -
pPa42 -
pPa48 -
pPa47 -

Fig. 5. Dot-blot analysis of the colonies exhibiting hybridization signals specific to the genomic probe from *P. bertolonii* in colony hybridization. The dot-blotted plasmid DNA from these colonies were hybridized to the total DNA from *P. bertolonii* (a) and from *P. alpinum* (b) under the following stringencies: hybridization in 50% formamide and washing at 55°C in final step (1); hybridization in 50% formamide and washing at 65°C (2); hybridization in 60% formamide and washing at 55°C (3); and hybridization in 60% formamide and washing at 65°C (4).

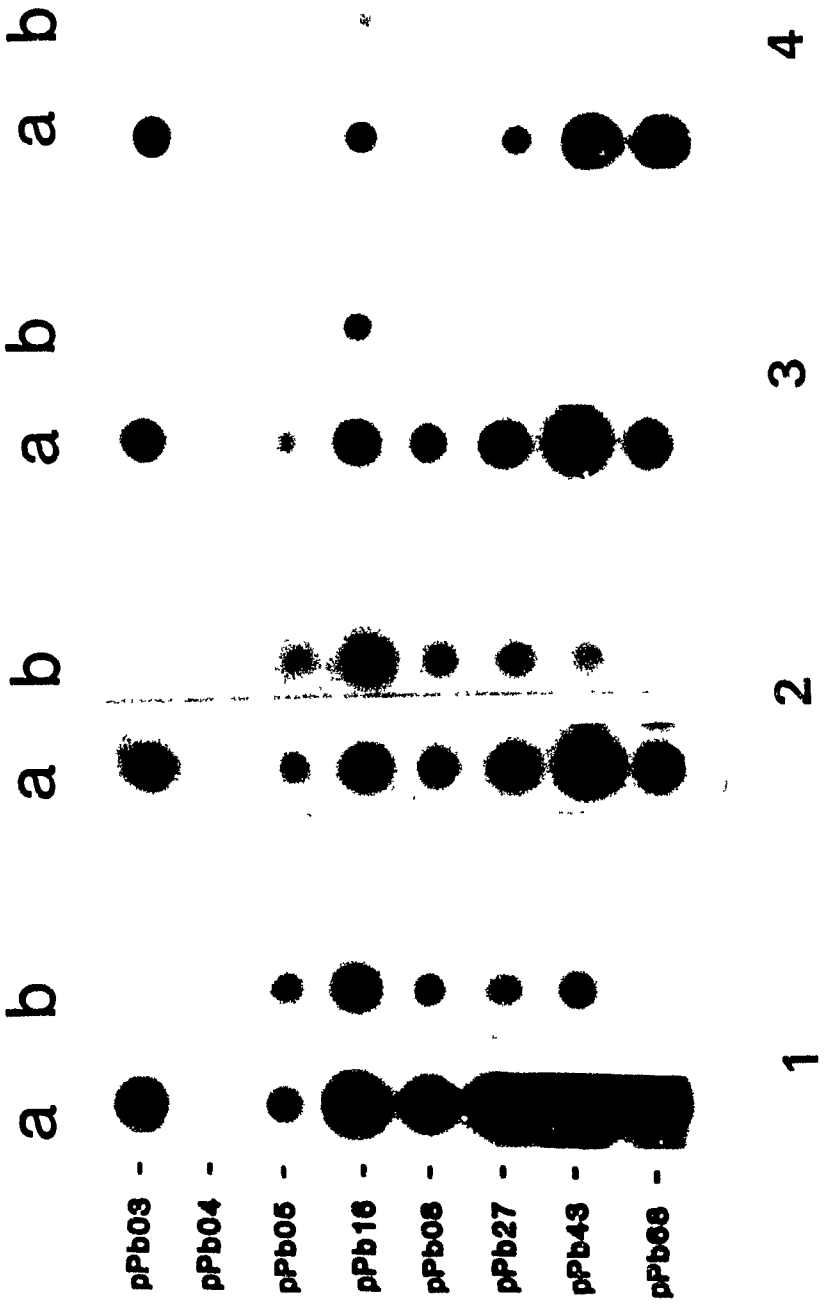

a b
a b
a b
a b
4
3
2
1
pPb03 -
pPb04 -
pPb05 -
pPb16 -
pPb08 -
pPb27 -
pPb43 -
pPb68 -

Fig. 6. The inserted fragments of the *P. alpinum* genome-specific clones and the vector plasmid pUC19. The inserts were excised by EcoRI and HindIII. Lane 1-8: pPa07, pPa08, pPa11, pPa16, pPa33, pPa24, pPa42 and pPa47; lane 9: 1 Kbp ladder fragment markers.

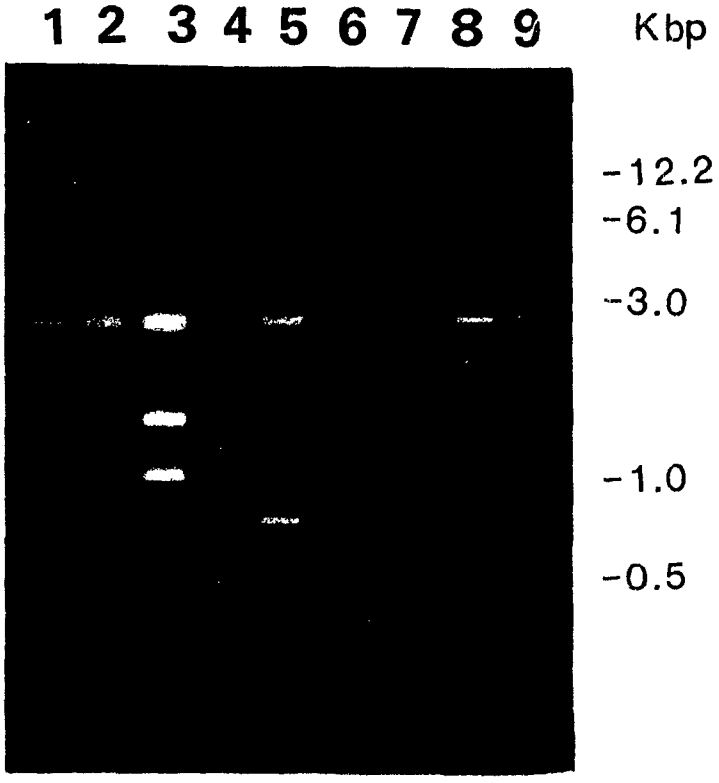

1 2 3 4 5 6 7 8 9
Kbp
-12.2
-6.1
-3.0
-1.0
-0.5

Fig. 7. The inserted fragments of the *P. bertolonii* genome-specific clones and the vector plasmid pUC19. Lane 1-13: pPb03, pPb05, pPb08, pPb27, pPb43, pPb68, pPb69, pPb70, pPb75, pPb78, pPb81, pPb82 and pPb88; lane 14: The fragment markers.

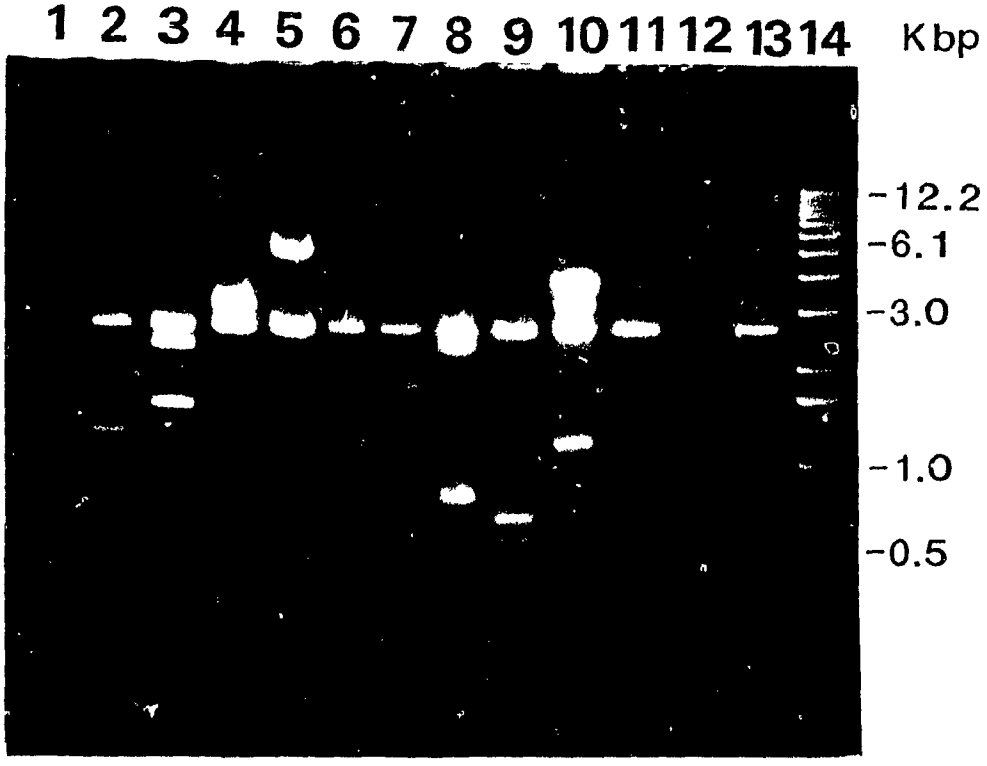

1 2 3 4 5 6 7 8 9 10 11 12 13 14
Kbp
-12.2
-6.1
-3.0
-1.0
-0.5

Fig. 8. The schematic diagram of the inserts of 6 genome-specific clones used for slot-blot and Southern blot analyses.

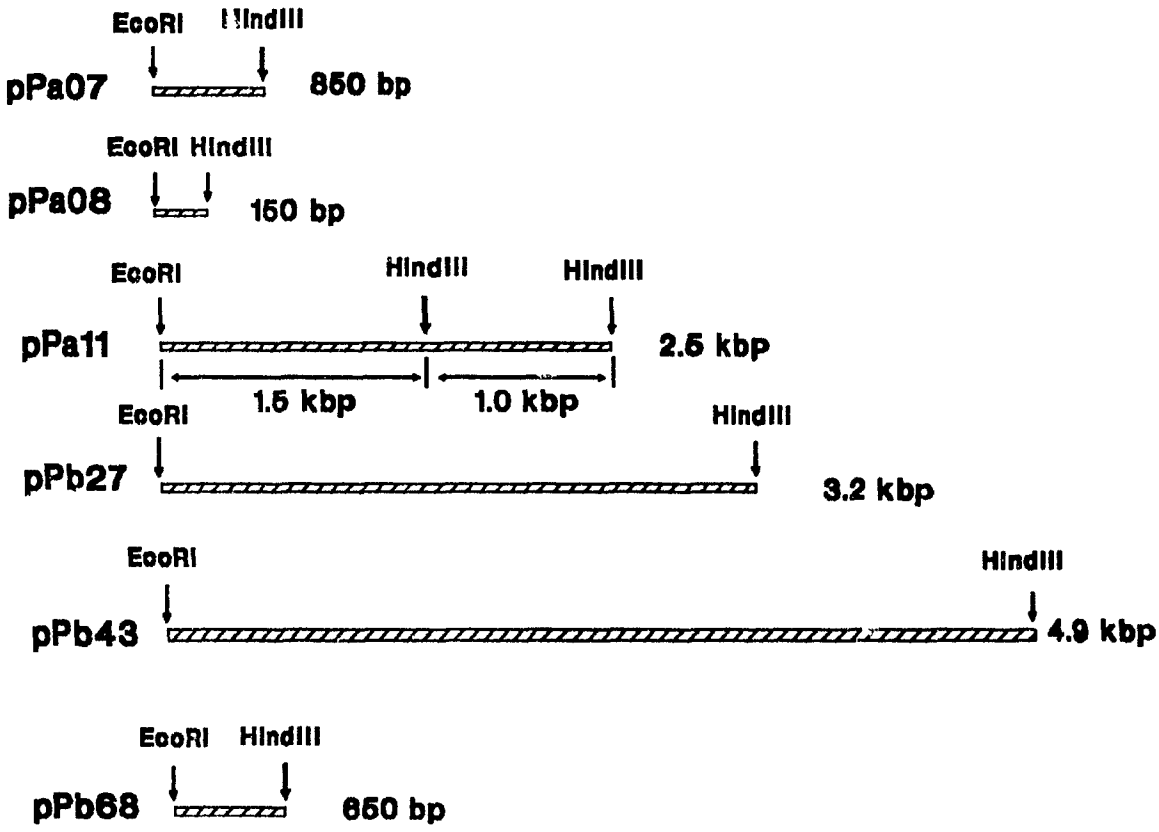

EcoRI
HindIII
pPa07
850 bp
EcoRI HindIII
pPa08
150 bp
EcoRI
HindIII
HindIII
pPa11
2.5 kbp
1.5 kbp
1.0 kbp
EcoRI
HindIII
pPb27
3.2 kbp
EcoRI
HindIII
pPb43
4.9 kbp
EcoRI
HindIII
pPb68
650 bp

Fig. 9. Slot-blot hybridization of the total DNA from four *Phleum* species using genome-specific clones as probes. 50 ng DNA of each sample was loaded onto a membrane using a slot-blot template and probed with pPb68 (column I), pPb43 (column II), pPb27 (column III), pPa08 (column IV), pPa07 (colum V), and pPa11 (column VI). The hybridized membranes were exposed for 24 h (column I) and 15 h (all the other columns).


P. pratense
M46
M103
M35
M104
P. bertolonii
M33
M40
p. alpinum
M39
M27
P. montanum
M29
I
II
III
IV
V
VI

Fig. 10. Southern-blot hybridization of the total DNA from four *Phleum* species: *P. pratense* (lane 1-2: M46, M103), *P. bertolonii* (lane 3-4: M33, M40), *P. alpinum* (lane 5-6: M39, M27) , and *P. montanum* (lane 7: M29) using *P. bertolonii*-specific clones as probes. 5 μg EcoRI-digested total DNA was loaded each lane and the Southern-blotted DNA was probed by pPb27 (a), pPb43 (b) and pPb68 (c). The exposure time of the hybridized membrane was 20 h.

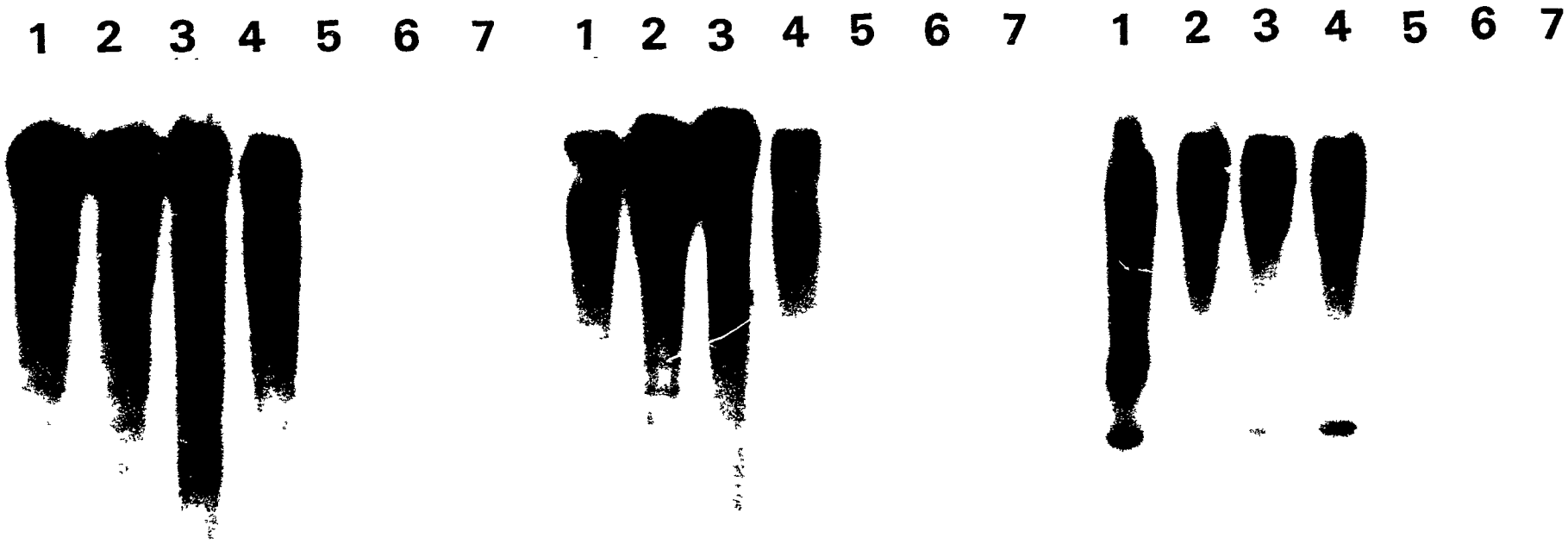
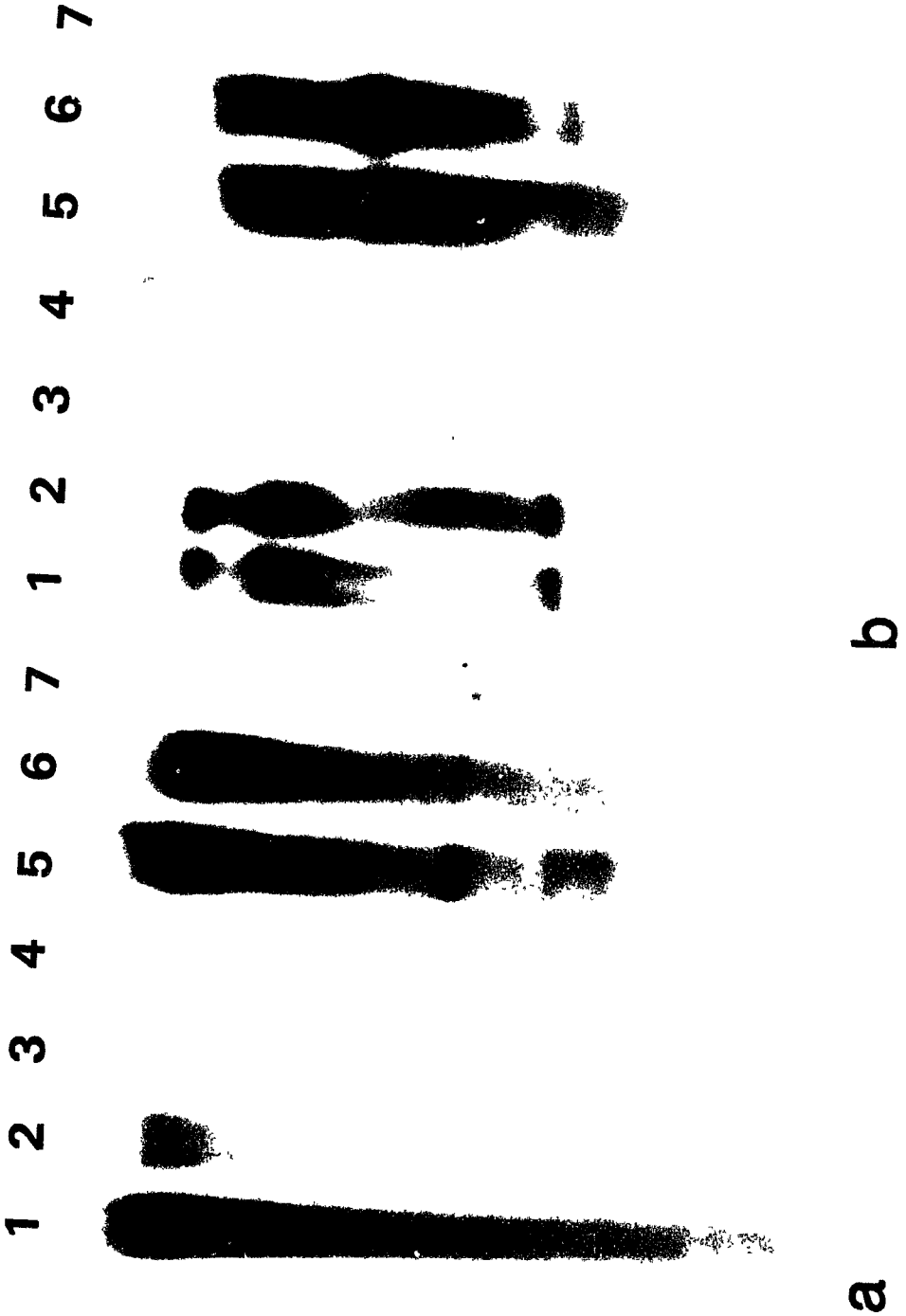

1 2 3 4 5 6 7
1 2 3 4 5 6 7
1 2 3 4 5 6 7
a
b
c

Fig. 11. Southern-blot hybridization of the Sau3AI-digested *P. bertolonii* DNA to pPb27 (a) and pPb43 (b) showing ladder patterns. The digestion was performed at 37°C for one hour in the following enzyme concentrations: 0 unit/μg DNA (lane 1), 0.5 unit/μg DNA (lane 2), 1 unit/μg DNA (lane 3) and 2 units (lane 4).


1 2 3 4
1 2 3 4
a
b

Fig. 12. Southern blot screening of the total DNA from four *Phleum* species: *P. pratense* (lane 1-2: M46, M103), *P. bertolonii* (lane 3-4: M33, M40), *P. alpinum* (lane 5-6: M39, M27), and *P. montanum* (lane 7: M29) using *P. alpinum*-specific probes: pPa08 (a) and pPa11 (b).


7
6
5
4
3
2
1
b
7
6
5
4
3
2
1
a

Protein variation in timothy and identification of its cultivars by SDS-PAGE analysis

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Abstract

SDS-PAGE analysis of seed proteins was carried out to evaluate the genetic variability and to identify the cultivars in the forage crop, timothy (*Phleum pratense* L.). Nineteen cultivars of timothy were examined. Among them five were from Europe and fourteen from North America. A total of 44 protein monomers were detected in mature seed extract by SDS-PAGE followed by Coomassie blue staining. Except for two pairs, all the cultivars were differentiated by SDS-PAGE analysis of seed storage proteins. In the electrophoretic profile, no protein bands were found to be specific either to European or to North American cultivars, which is an indication of their genetic similarity. Twelve samples of cultivar Toro harvested from Alberta and Manitoba (Canada), Idaho and Minnesota (USA), were compared and no significant differences were found in their seed protein banding patterns, which suggests environmental stability of timothy seed proteins.

Key words: SDS PAGE, variation, timothy cultivar identification, seed storage proteins

Résumé

[Variation de la protéine et identification de cultivars de fléole des prés par électrophorèse des protéines des semence sur gels dénaturants de polyacrylamide.]

Titre abrégé: Identification des cultivars de fléole des prés par électrophorèse.

L'électrophorèse sur gel de polyacrylamide au sulfate de dodécyl (SDS-PAGE) nous a permis de distinguer 19 cultivars de la fléole des prés (*Phleum pratense* L.) et démontrer la variation génétique. Cinq étaient d'origine européenne et 14 de l'Amerique du Nord. L'analyse SDS-PAGE nous a permis de séparer 44 protéines à partir d'extraits de graines mures. Tous les cultivars peuvent être identifiés d'après le profil électophorétique à l'exception de deux paires. Puisqu' aucune protéine n'était spécifique soit aux cultivars européens soit à ceux de l'Amérique du Nord on peut supposer une origine génétique commune pour tous les cultivars. Nous avons comparé les profils électrophorétiques des protéines de semence de 12 échantillons du cultivar Toro, récoltés a différents endroits (Alberta et Manitoba au Canada, et Idaho et Minnesota aux Etats Unis). Aucune différence significative n'a été détectée. Cette donnée suggère une stabilité face à l'environment au niveau des protéines de semence chez la fléole des prés.

Mots clés: Électrophorèse sur gel de polyacrylamide au SDS, identification de cultivars des fléole de prés, protéines d'emmagasinage des semences.

Introduction

Timothy (*Phleum pratense* L), a perennial grass, is grown extensively in the northeast quarter of North America, the Pacific northwest states of the U.S.A. and northern Europe. It is a major forage species in Norway, Sweden, Finland and Iceland (Klebesadel and Helm 1986), as well as in eastern Canada. In the province of Quebec, Canada, timothy accounts for about 50% of all forage seed sold each year.

The most recent studies on the genome in timothy (*P. pratense* L) with Giemsa C-banding and genome-specific sequence analyses (Cai and Bullen 1991a, b) have provided some important information which adds to our understanding of the genome constitution and evolution in timothy. However, information about genetic variation, which can aid in the identification of genotypes or cultivars is quite limited in this species. Investigation of genetic variability in *P. pratense* has practical significance for timothy breeding. Phenotypic difference not only allows selection of hybrids but also permits identification of genotypes or cultivars. Phenotypic variation in timothy has been traditionally studied by examining the morphology. Although morphological characters are considered to be an source of markers for genetic selection and for discrimination between cultivars, only a limited number of variations can be detected by morphological observation. Many differences such as disease resistance and storage products in the endosperm or other organs are not expressed morphologically. Recently, phenotypic variation has also been observed in this species for some chemical characters and physical properties such as crude protein and *in situ* dry matter digestibility (Surprenant et al. 1990), packed volume, water retention and

solubility (Surprenant and Michaud 1988), and yield (Faris 1970). Unfortunately, most of these characters are quantitative traits and thus are not effective markers for genetic selection or for identification of genotypes.

Phenotypic diversity in seed storage proteins are considered to be an important source of variation. About 80% of seed proteins serve as a storage function. These include globulins of dicotyledons and prolamines of monocotyledons (Konarev et al. 1987). The storage proteins in seeds belong to a large protein family, which covers a lot of structural gene products, i.e. polypeptides. Most variation in polypeptides can be easily detected by SDS-electrophoresis. Electrophoretic phenotypes of seed storage proteins can therefore serve as useful markers for selection and they are also considered to be potential identifiers and descriptors of cultivars. SDS-polyacrylamide gel electrophoresis (PAGE) of seed storage proteins is thus a useful technique for investigating phenotypic variation in natural and cultivated populations and is a powerful tool for cultivar identification. This technique has the advantage over morphological observation in that it offers a rapid and reliable evaluation of genotypic differences among most of the cultivars before the development of the mature plants. It has been widely used to discriminate cultivars and their hybrids in breeding programs and to a lesser extent in the seed trade. This has led to the successful identification of cultivars in a variety of cereals (Ferguson and Grabe 1986; Wrigley et al. 1987) and pasture grasses (Gardiner et al. 1986; Gardiner and Ford 1987), as well as some other crops (Barratt 1980; Kapse and Nerkar 1985; Onokpise et al. 1988).

In forage grasses, the successful identification of cultivars by SDS-PAGE has been reported in at least 10 species, including species in the

genera *Lolium* and *Festuca* which are close to the grass timothy. Perennial (*L. perenne* L.) and Italian (*L. multiflorum* Lam.) ryegrasses were readily differentiated, and distinctive banding patterns were obtained for seed proteins from a range of cultivars and varieties of each (Gardiner et al 1986). There is no report on the analysis of seed storage proteins in timothy (*P. pratense* L.) to demonstrate variation and cultivar identification.

For successful timothy production one needs good management and seed of high genetic quality. The use of a rapid and reliable technique to identify the cultivars in this species and to establish the purity of seeds should benefit both the breeders and the farmers. In the present paper we report the variation of SDS-PAGE banding patterns of seed storage proteins of timothy which can be used to distinguish the cultivars of this species.

Materials and Methods

Nineteen cultivars of timothy (*P. pratense* L.) were used in this study. Five of them are from Europe and the others are from North America. The seeds of five European cultivars: Erecta, Pecora, Alpage, Tiller and A.S.352 were supplied by the Institute National de la Recherche Agronomique, France. The North American cultivars used in this study are all from Canada: Mariposa and Richmond, from a commercial supplier, Pickseed, Ontario; Itasca, from the University of Guelph; Champ, Basho, Bounty, Salvo, Climax and Toro, from the Plant Research Centre, Ottawa; Glenmor, Timfor and Winmor, from Northrup King Seeds Ltd, Ontario; Bottina and Tiiti, from S.

S. Johnson Seeds Ltd, Manitoba. The following samples of Toro supplied by Oseco Inc. in Ontario were used to test the environmental influence on protein banding patterns: Lot 6-97, Lot 6-426, Lot 7-195, and Lot 7-217 (Alberta, Canada); Lot 7-684 and Lot 6-521 (Manitoba, Canada); Lot 6-567, Lot 6-1026, Lot 7-702 and Lot 7- 947 (Idaho, USA); and Lot 7-540 and Lot 7-651 (Minnesota, USA). The seed storage proteins were extracted using a method described by Gifford and Chinnappa (1986). 0.2 g mature seeds were ground in a mortar in 2 ml 0.05M sodium phosphate (pH 7.5). The homogenate was centrifuged in a Sorvall RC2-B centrifuge at 7000 rpm for five minutes. The supernatant containing soluble proteins was removed, the pellet was reextracted twice, and the supernatants were combined. Insoluble proteins in the pellet were extracted by boiling the suspension for five minutes in an equal volume of 65 mM Tris-HCl (pH 6.8) containing 2% (w/v) SDS, 10% (v/v) glycerol and 2.5% (v/v) 2-mercaptoethanol (ME) followed by centrifugation and collection of the supernatant as described above. Before electrophoresis, extracts of soluble proteins were boiled for five minutes in an equal volume of 65 mM Tris-HCl (pH 6.8) containing 2% SDS, 10% glycerol and 2.5% ME. Extracts of insoluble proteins were used directly.

The SDS-PAGE process was conducted in a Bio-Rad Protein II Slab Cell in 14% acrylamide gel using the technique outlined by Gifford and Bewley (1983). After electrophoresis, the gel was fixed in 45.4% (v/v) methanol and 9.2% (v/v) glacial acetic acid for one hour and then was stained in 2.5% (w/v) Coomassie Blue R250 solution (stain dissolved in fixer solution) over night. The gel was subsequently destained in an aqueous solution containing 5% ethanol and 7.5% acetic acid until the background colour was completely washed out. The electrophoretic experiment for all the cultivars was repeated

four times. The Coomassie Blue stained gel profile was scanned and quantified by a Bio-Rad Model 620 Video densitometer and 1-D Analyst computer program. Differentiation of protein banding patterns between cultivars was evaluated by the proportion of differentiated protein bands (P_p) which is calculated according to the following formula:

$$P_p = (d_1 + \bar{\gamma}d_2)/T_n \quad (d_1 \geq d_2)$$

where, d_1 and d_2 are the numbers of protein bands respectively specific to either of two cultivars in a comparison; T_n is the total number of protein bands, which includes the numbers of differentiated bands ($d_1 + \bar{\gamma}d_2$) and the bands common for two cultivars; $\bar{\gamma}$ is an average frequency of null bands.

Results

The storage seed proteins from 19 cultivars of timothy (*P. pratense* L.) were SDS-PAGE analyzed. Following extraction of the seed proteins from all 19 cultivars of timothy in this study, about 50% of the protein content was found in the insoluble fraction. As no obvious differences in the SDS-PAGE profile were shown between soluble and insoluble fractions (data not shown), subsequent gel profiles were obtained using a combined soluble and insoluble fraction. In the reduced condition under which the proteins were treated with 2-mercaptoethanol, a total of 44 protein bands were separated by SDS-PAGE among the timothy cultivars observed in this study. These protein bands were distributed along the SDS acrylamide gel in a range of Rf 0.16 to 0.97. The

molecular weight of them were determined using the curve constructed with Bio-Rad standard proteins (14.4 to 97.4 KDa). Each of these protein bands should represent one protein monomer or one polypeptide if all the protein monomers are completely separated. In fact, overlapping usually exists in some bands and thus some protein monomers can not be identified in SDS-PAGE gel profile. A computor-monitored densitometer (BioRad) was used to scan the gel and quantify the band intensities. Some thin bands which were difficult to be identified with the naked eyes could be recognized by the densitometer. Table 1 shows the relative mobility (Rf), molecular weight and average band intensities of the proteins bands detected in the examined cultivars. The densitometer-scanned data showing the band intensities in the individual cultivars are presented in Appendix (Table I, II). The Coomassie Blue gel profile showed two zones (from Rf 0.44 to 0.49 and from Rf 0.61 to 0.66) with very high intensity (Fig. 1). Among these protein bands, 14 were monomorphic, revealed in all 19 cultivars and the others were polymorphic. Among the polymorphic bands, seven were detected in 18 out of the 19 cultivars. The following protein bands: No. 11, 17, and 24 were found only in a minority of the cultivars.

SDS-PAGE banding patterns of seed proteins were compared among these 19 cultivars. The proportion of differentiated proteins (protein monomers which were electrophoretically different between cultivars in a pair-wise comparison), P_p, was used to evaluate the phenotypic divergence of seed storage proteins between cultivars. The band intensity data recorded by the densitometer was used as a reference for the identification of cultivars but not included in P_p values. The comparison of protein banding patterns among five European cultivars is summarized in Table 2. Based on the data listed in

Table 2, all these five cultivars can be distinguished from one another. Among ten pair-comparisons shown in Table 2, A.S.352 and Tiller shows the highest degree of protein divergence. In A.S.352, 30 protein bands were detected and in Tiller 33 bands were found. Among these protein bands, 26 are in common and the rest are specific to one or the other cultivars. Although most of these differentiated bands do not stain intensely, they are nonetheless reproducible when the analysis is repeated. These incompatible proteins may be due to differences in amino acid sequences in the polypeptides or to loss of the polypeptides as a result of the failure of gene expression. Regardless of the cause it is clear that cultivar A.S.352 does not have three of the differentiated protein bands present in Tiller.

The comparison of seed protein banding patterns among the North American cultivars (Table 3) shows that all the 14 North American cultivars can be distinguished from one another by the SDS-PAGE banding patterns except three pairs of cultivars, Climax:Richmond, Basho:Bounty and Timfor:Winmor, in which the P_p values are too low to allow them to be distinguished. Between Climax and Richmond, only about 6% of the proteins are different and between Basho and Bounty, only 4% of the proteins are different. These differentiated protein bands are neither major bands nor readily reproducible. The protein banding patterns for Timfor and Winmor are even closer, only about 3% of the seed proteins are different. The densitometer scanning data does not distinguish the protein banding patterns in these three pairs of cultivars. In contrast to these three pairs of cultivars, another three pairs of cultivars, Salvo-Timfor, Toro-Basho and Salvo-Glenmor, exhibited high degree of phenotypic differentiation. The P_p value calculated in these three pairs were respectively 0.31, 0.32 and 0.31 (Table 3). This may

to some extent reflect the distant genetic relationships in these cultivar pairs.

Among all the seed proteins revealed in the cultivars examined, no protein bands were found to be specific to European or to North American cultivars, and hence it is impossible to distinguish cultivars from the two continents on the basis of banding patterns. The proportion of differentiated proteins (P_p) between European and North American cultivars are listed in Table 4. In terms of the P_p values calculated within and between European and North American cultivars, the variation of seed proteins revealed between European and North American cultivars is not greatly different from those detected within each group. The P_p values calculated within European cultivars range from 0.14 to 0.24; while within North American cultivars, the P_p values range from 0.12 to 0.32 except for those obtained in three pairs (Climax:Richmond, Basho:Bounty and Timfor:Winmor); and they vary from 0.10 to 0.33 when the two geographic groups are compared. These results indicate the genotypic similarity between European and North American cultivars. This finding agrees well with the reported domestication of the species in Massachusetts in the eighteenth century (Piper,1924) and its subsequent shipment to Europe as well as the exchange of breeding material across the Atlantic in this century.

The samples from the cultivar, Toro, harvested in different sites were compared to see if there is an environmental influence on protein banding patterns. The seeds of Toro were harvested from four production sites: Alberta (four seed lots) and Manitoba (two seed lots) in Canada and Idaho (four seed lots) and Minnesota (two seed lots) in the USA. The SDS-PAGE profile shows no outstanding differences in banding patterns among these samples and the data from densitometer scanning (Fig. 2) show relative stability of individual

bands among these samples. The data indicates no significant influence from the environment on the banding patterns of seed proteins. Similar results have been reported in other grass species (Ferguson and Grabe 1986; Gardiner et al 1986). The environmental stability of seed storage proteins has been confirmed not only by PAGE electrophoresis but also in other studies by reversed phase high performance liquid chromatography (RP-HPLC). More recently in an assessment of soybean seed proteins by RP-HPLC technique, Buehler et al (1989) demonstrated that different seed production sites did not significantly affect either quantitative or qualitative aspects of the chromatogram for a given cultivar.

Discussion

In order to quantitatively evaluate the phenotypic divergence of seed storage proteins between timothy cultivars, a parameter, termed proportion of differentiated proteins (P_p), was calculated in this study. In calculation of this value, the total number of protein bands represented by T_n and the number of differentiated bands represented by D_n were involved. If the differentiated bands (D_n) is defined as those protein bands specific to either of two cultivars, respectively represented by d_1 and d_2, then $D_n = d_1 + d_2$ and thus $P_p = D_n/T_n = (d_1 + d_2)/T_n$. It should be pointed out that this formula in most cases results in a biased value of P_p because differentiation of one polypeptide without loss of it (null band) between any two cultivars results in two divergent bands which are respectively specific to one or to the other of the two cultivars, thus leading to increase the number of cultivar-specific bands. In the case of A.S.352 and Tiller, for instance, $D_n = d_1 + d_2 = 7 + 4 =$

11, $T_n = 7 + 4 + 26 = 37$ and hence $P_p = D_n/T_n = 11/37 = 0.30$. This value is higher than it should be. In order to refine the P_p value so that it can correctly reflect the genetic differentiation between cultivars, a modified formula, $P_p = (d_1 + \bar{\gamma}d_2)/T_n$ was used to calculate the P_p. The $\bar{\gamma}$ in this formula is an average frequency of null bands, which is calculated by

$$\bar{\gamma} = 1/n \sum_{i=1}^{n} [(d_1 - d_2)/(d_1 + d_2)]_i$$

where, $[(d_1 - d_2)/(d_1 + d_2)]_i$ is the frequency of null bands in the ith comparison. When calculated among all the 19 examined cultivars $\bar{\gamma} = 0.29$. T_n is calculated by $T_n = d_1 + \bar{\gamma}d_2 + C_n$, where C_n is the number of those protein bands common for two cultivars. The above modified formula for calculation of P_p has eliminated the portion of increased specific bands from polypeptide differentiation. With the modified formula, the proportion of differentiated protein between A.S.352 and Tiller is $P_p = (7 + 0.29 \times 4)/(7 + 0.29 \times 4 + 26) = 0.24$.

In SDS-PAGE of seed proteins for identification of cultivars, the situation in outbreeding species is likely to be different from that found in inbreeding species. In inbreeding species, such as most cereals, in which SDS-PAGE of seed proteins is extensively used for distinguishing the cultivars (see review by Cooke 1984), each seed in a pure line gives rise to the same protein banding pattern. In outbreeding species, however, genetic variation may well exist within cultivars to a greater or lesser degree. This was confirmed in Westwolds ryegrass (*L. multiflorum* Lam.) (Gardiner et al 1986). In timothy, no test of within-cultivar variation of seed proteins was conducted in this

study and hence no information on this aspect is available. However, the research on some other traits such as forage yield, *in vitro* dry matter disappearance (IVDMD), percentage dry matter and mineral content reported by Berg and Hill (1983) showed some variation within cultivars of timothy. A high degree of genotypic variation within a cultivar would make identification based on the frequency of particular bands obtained from individual seeds extremely time consuming. In timothy, it would be very difficult to analyze the protein pattern in individual seeds because the small seeds do not contain sufficient protein to allow SDS-PAGE analysis. Nevertheless, the pattern of bands obtained by analyzing a meal made from a bulk seed sample sufficiently large to represent the interbreeding populations provides an average picture of the banding pattern of proteins present in the individual genotypes which together comprise the cultivar. According to Gardiner et al (1986), 0.2 g seeds gave a reproducible pattern in ryegrass. In our pre-analysis of seed proteins in five cultivars of timothy (Climax, Salvo, Champ, Bounty and Alpage), the comparison of banding patterns of proteins extracted from 0.1 g, 0.2 g, 0.4 g, 0.6 g and 1 g bulk seed sample confirmed that the 0.2 g random seed sample is sufficiently large to give rise to a reproducible pattern in timothy.

Seed protein profiles have been shown in the present paper to be a discriminating tool for identification of timothy cultivars. Together with the scanning by the densitometer, SDS-PAGE banding patterns of seed proteins can serve as a 'fingerprint' for the purpose of seed identification to establish plant variety rights or for identifying unknown or wrongly labelled lines of timothy. In addition, seed protein profiles may provide evidence of origin and genetic relationships of the timothy cultivars. Most of the timothy cultivars

were developed by polycross and mass selection (Powell and Hanson 1973), hence, the precise parents of them are mostly unknown. Data from SDS-PAGE analysis of seed proteins may help to identify the ancestors of the cultivars.

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References

Barratt, D.H. 1980. Cultivar identification *Vicia faba* (L.) by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of seed globulins. J. Sci. Food Agric. 31: 813-819.

Berg, C.C., and R.R. Hill Jr. 1983. Quantitative inheritance and correlation among forage yield and quality components in timothy. Crop Sci. 23: 380-384.

Buehler, R.E., M.B. McDonald, Jr., T.T. Van Toai, and S.K.St. Martin. 1989. Soybean cultivar identification using high performance liquid chromatography of seed proteins. Crop Sci. 29: 32-37.

Cai, Q., and M.R. Bullen. 1991a. Characterization of genomes of timothy (*Phleum pratense* L.). I. Karyotypes and C-banding patterns in cultivated timothy and two wild relatives. Genome, 34: 52-58.

Cai, Q., and M.R. Bullen. 1991b. Identification and analysis of genome specific sequences in the genus *Phleum*. 36th Annual Meeting of the Genetic Society of Canada at Queen's University, Kingston, Ontario. June 11-14, 1991 (Oral presentation), Bulletin of the Genetics Society of Canada, 22: 40, PPG-1 (Abstract).

Cooke, R.J. 1984. The characterization and identification of crop cultivars by electrophoresis. Electrophoresis 5: 59-72.

Faris, A.E. 1970. A comparative study of different procedures for heritability estimates of timothy (*Phleum pratense* L.). Ph.D. Thesis, McGill University, Montreal, Que.

Ferguson, J.M., and D.F. Grabe. 1986. Identification of cultivars of perennial ryegrass by SDS-PAGE of seed proteins. Crop Sci. 26: 170-176.

Gardiner, S.E., M.B. Forde, and C.R. Slack. 1986. Grass cultivar identification by sodium dodecylsulphate polyacrylamide gel electrophoresis. New Zealand J. Agric. Res. 29: 193-206.

Gardiner, S.E., and M.B. Forde. 1987. SDS polyacrylamide gel electrophoresis of grass seed proteins: a method for cultivar identification of pasture grasses. Seed Sci. Technol. 15: 663-674.

Gifford, D.J., and J.D. Bewley. 1983. An analysis of the subunit structure of the crystalloid protein complex from castor bean endosperm. Plant Physiol. 72: 376-381.

Gifford, D.J., and C.C. Chinnappa. 1986. Studies on the *Stellaria longipes* (Caryophyllaceae). VII. The seed proteins. Can. J. Bot. 64: 1327-1330.

Kapse, S.S., and Y.S. Nerkar. 1985. Polyacrylamide gel electrophoresis of soluble seed proteins in relation to cultivar identification in cotton.

Seed Sci. Technol. 13: 847-852.

Klebesadel, L.J. and Dot Helm. 1986. Food reserve storage, low-temperature injury winter survival and forage yields of timothy in subartic Alaska as related to latitude-of-origin. Crop Sci. 26: 325-334.

Konarev, V.G., I.P. Gavriljuk, and N.K. Gubareva. 1987. Storage proteins in the identification of species, cultivars and lines. Seed Sci. & Technol. 15: 675-678.

Onokpise, O.U., B.A. McBlain, and R.L. Cooper. 1988. Electrophoretic identification of semi-dwarf soybean (*Glycine max*) Cultivars. Seed Sci. Technol. 16: 555-562.

Piper, C.V. 1924. Forage plants and their culture. New York, Macmillan.

Powell, J.B. and A.A. Hanson. 1973. Timothy, *In* Forage (Ed. by M.E. Heath, D.S. Metcalfe and R.F. Barnes), The Iowa State Univ. Press/Ames, Iowa, USA. pp. 277-284.

Surprenant, J., and R. Michaud. 1988. Genetic variability in the physical properties and other quality traits in timothy. Can. J. Plant Sci., 68: 713-720.

Surprenant, J., R. Michaud, and G. Allard. 1990. Effect of one cycle of divergent phenotypic selection for crude protein, digestibility and digestible yield in timothy. Can. J. Plant Sci., 70: 757-765.

Wrigley, C.W., I.L. Batey, W.P. Campbell, and J.H. Skerritt. 1987. Complementing traditional methods of identifying cereal varieties with novel procedures. Seed Sci. Technol. 15: 679-688.

Table 1. The migration rate (Rf), molecular weight and average band intensity of the storage seed proteins in nineteen timothy cultivars

Band number	Rf	Molecular weight(KDa)	Band intensity (O.D.)	Band number	Rf	Molecular weight(KDa)	Band intensity (O.D.)
1	0.161	?	0.056	17	0.411	37.9	0.083
2	0.170	?	0.051	18	0.437	36.4	0.218
3	0.178	?	0.060	19	0.444	35.8	0.235
4	0.191	92.1	0.066	20	0.452	35.1	0.213
5	0.226	76.4	0.062	21	0.474	34.2	0.200
6	0.237	70.5	0.084	22	0.481	33.9	0.154
7	0.256	63.3	0.072	23	0.488	33.3	0.106
8	0.274	58.3	0.144	24	0.519	31.7	0.084
9	0.283	56.2	0.139	25	0.526	31.0	0.074
10	0.297	52.2	0.134	26	0.540	30.4	0.073
11	0.305	49.7	0.082	27	0.570	29.0	0.068
12	0.319	46.4	0.114	28	0.578	28.3	0.093
13	0.333	42.7	0.092	29	0.630	25.3	0.330
14	0.340	42.1	0.064	30	0.667	23.5	0.300
15	0.353	41.2	0.062	31	0.678	22.7	0.286
16	0.370	40.4	0.065	32	0.693	22.1	0.171

Table 1. (continued)

Band number	Rf	Molecular weight(KDa)	Band intensity (O.D.)	Band number	Rf	Molecular weight(KDa)	Band intensity (O.D.)
33	0.707	20.9	0.128	39	0.809	16.6	0.126
34	0.727	20.2	0.132	40	0.815	16.0	0.123
35	0.752	19.1	0.185	41	0.863	?	0.109
36	0.763	18.7	0.171	42	0.889	?	0.121
37	0.771	18.2	0.142	43	0.926	?	0.122
38	0.796	17.1	0.138	44	0.969	?	0.257

Table 2. Proportion of differentiated seed proteins (P_p) among European cultivars of timothy (*Phleum pratense* L.)

	A.S.352	Erecta	Pecola	Tiller
Alpage	0.22	0.18	0.22	0.21
A.S.352	-	0.17	0.22	0.24
Erecta	-	-	0.18	0.14
Pecola	-	-	-	0.20

Table 3. Proportion of differentiated seed proteins (P_p) among North American cultivars of timothy (*Phleum pratense* L.)

	Bot.	Bou.	Cha.	Cli.	Gle.	Ita.	Mar.	Ric.	Sal.	Tii.	Tim.	Tor.	Win.
Basho	0.20	0.04	0.18	0.23	0.22	0.22	0.25	0.24	0.22	0.22	0.26	0.32	0.24
Bottina		0.20	0.20	0.15	0.23	0.14	0.14	0.16	0.22	0.23	0.21	0.20	0.23
Bounty			0.18	0.21	0.22	0.18	0.25	0.24	0.22	0.24	0.29	0.29	0.27
Champ				0.22	0.26	0.18	0.27	0.28	0.24	0.25	0.19	0.21	0.18
Climax					0.23	0.12	0.21	0.06	0.22	0.16	0.19	0.19	0.16
Glenmor						0.21	0.18	0.21	0.31	0.19	0.23	0.27	0.19
Itasca							0.20	0.15	0.23	0.18	0.16	0.18	0.13
Mariposa								0.15	0.22	0.17	0.21	0.16	0.22
Richmond									0.26	0.14	0.16	0.20	0.17
Salvo										0.18	0.31	0.25	0.29
Tiiti											0.23	0.25	0.21
Timfor												0.20	0.03
Toro													0.20

Table 4. Proportion of differentiated seed proteins (P_p) between European and North American cultivars of timothy

	Bas.	Bot.	Bou.	Cha.	Cli.	Gle.	Ita.
Alpage	0.21	0.22	0.21	0.16	0.27	0.18	0.20
A.S.352	0.19	0.19	0.16	0.25	0.20	0.11	0.15
Erecta	0.17	0.14	0.21	0.23	0.12	0.18	0.16
Pecola	0.26	0.17	0.22	0.29	0.17	0.23	0.12
Tiller	0.27	0.20	0.27	0.20	0.14	0.24	0.17

	Mar.	Ric.	Sal.	Tii.	Tim.	Ter.	Win.
Alpage	0.19	0.25	0.33	0.26	0.15	0.28	0.18
A.S.352	0.21	0.15	0.22	0.12	0.26	0.29	0.26
Erecta	0.11	0.12	0.17	0.10	0.21	0.19	0.19
Pecola	0.22	0.13	0.28	0.19	0.23	0.24	0.23
Tiller	0.18	0.13	0.25	0.16	0.18	0.17	0.16

Fig. 1. SDS-PAGE profiles of seed proteins from eighteen timothy cultivars: Itasca (lane 1), Mariposa (lane 2), Pecola (lane 3), Richmond (lane 4), Salvo (lane 5), Tiiti (lane 6), Tiller (lane 7), Climax (lane 8), Glenmor (lane 9), Alpage (lane 10), AS352 (lane 11), Bottina (lane 12), Basho (lane 13), Bounty (lane 14), Champ (lane 15), Timfor (lane 16), Winmor (lane 17), and Erecta (lane 18).

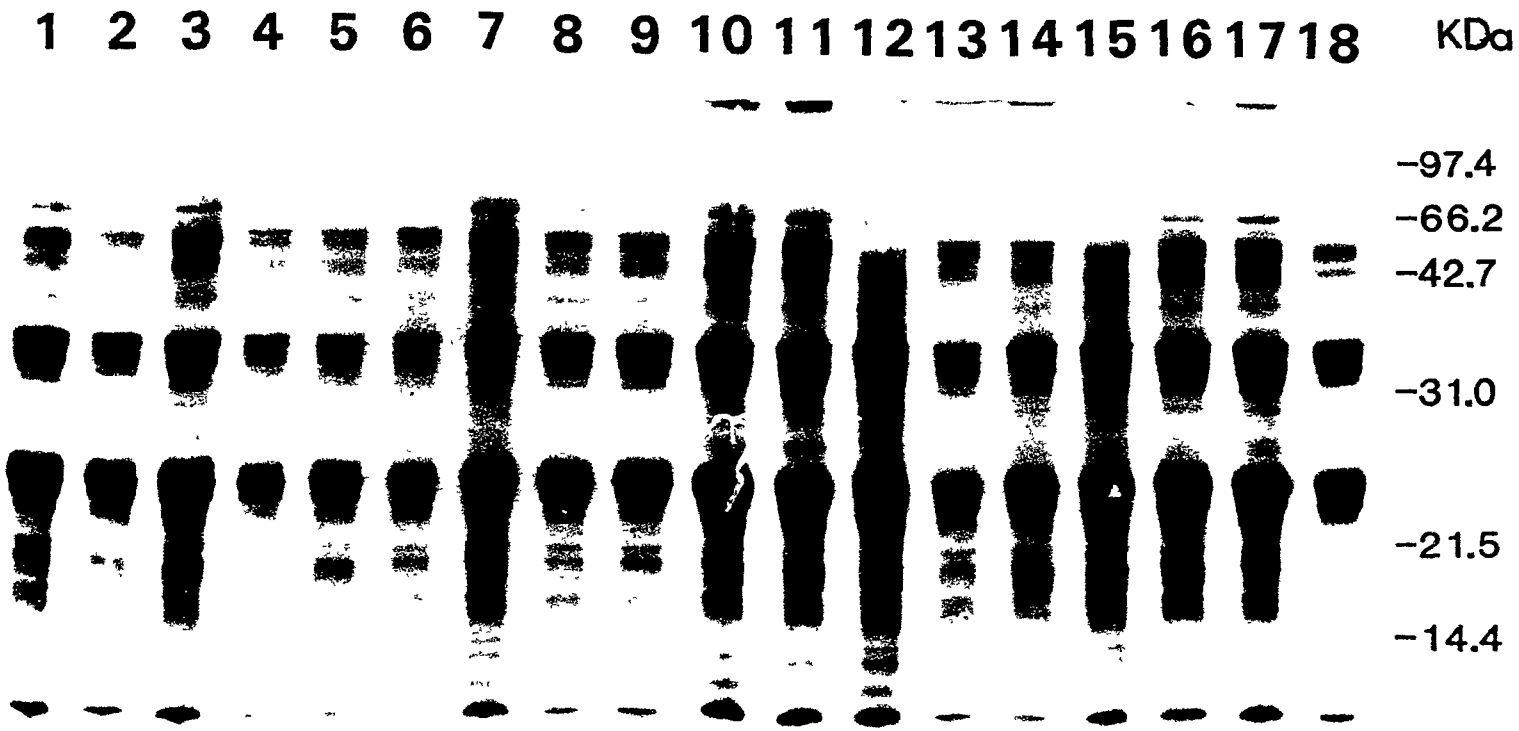
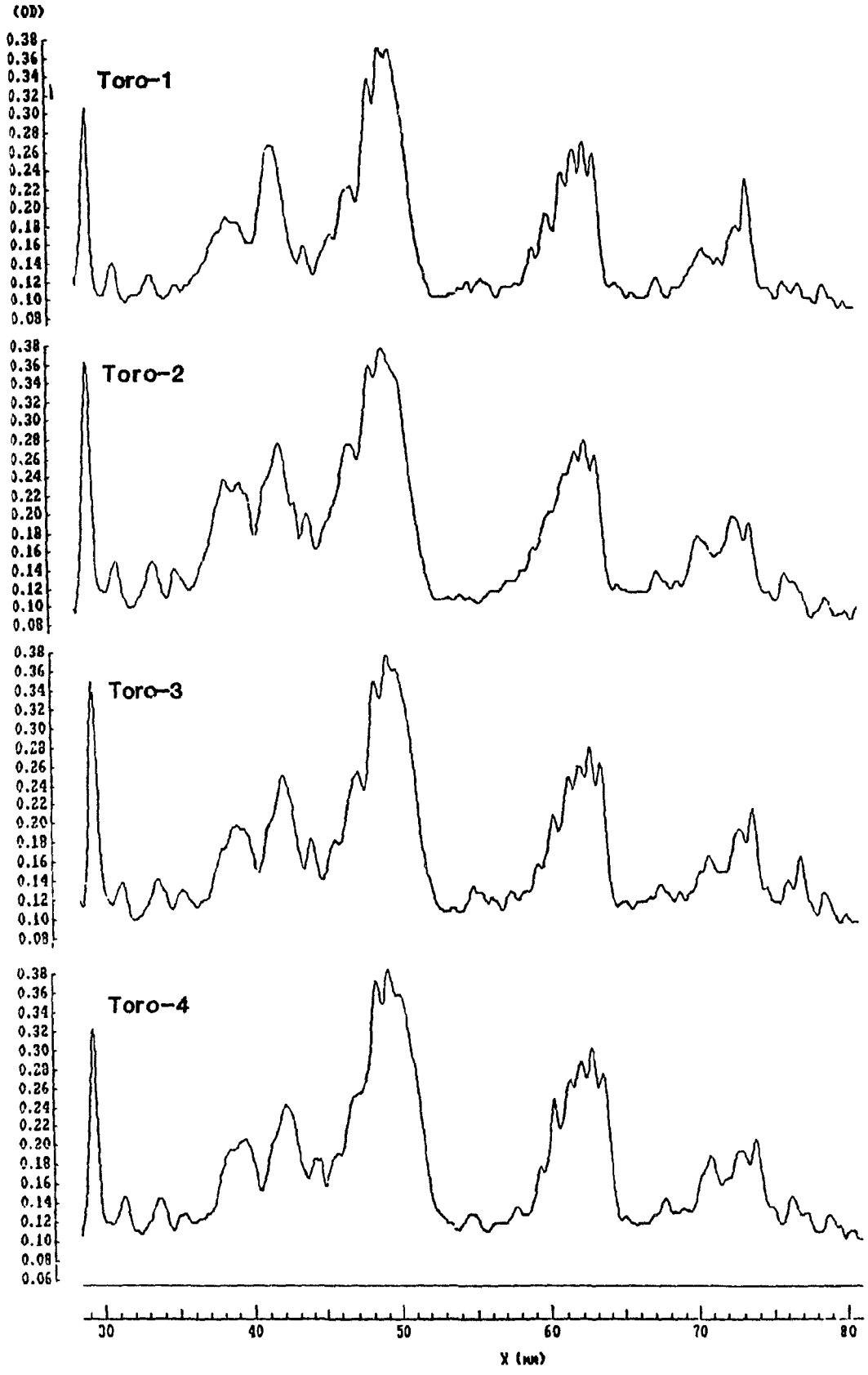

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
KDa
-97.4
-66.2
-42.7
-31.0
-21.5
-14.4

Fig. 2. Densitometer scans of protein bands of the cultivar, Toro, harvested from four sites: Alberta (Toro-1), Manitoba (Toro-2), Idaho (Toro - 3) and Minnesota (Toro-4).


(OD)
Toro-1
Toro-2
Toro-3
Toro-4
0.38
0.36
0.34
0.32
0.30
0.28
0.26
0.24
0.22
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
30
40
50
60
70
80
X (mm)

General conclusions

Genome constitution and genome phylogenetic relationships

The fundamental cytological distinction between autopolyploid and allopolyploid may include two aspects: (1) chromosomal behaviour in meiosis where an autopolyploid may exhibit a high frequency of multivalents while an allopolyploid is not expected to show multivalents but a high frequency of bivalents, and (2) chromosome morphology and heterochromatin patterns, which are potentially similar between chromosome sets in autopolyploids but differentiated in allopolyploids. However, no distinction can be made between these two forms of polyploids in the genus *Phleum*, depending on the patterns of chromosome association in meiosis, because the data from meiotic observation in earlier studies of timothy showed no response of chromosome association to the ploidy levels. A survey of somatic chromosomes was attempted in this study using C-banding to determine the genomic constitution in hexaploid *P. pratense*. This procedure made it possible to distinguish between the two forms of polyploid in this species. In terms of the characteristics of the C-banded karyotype, two chromosome sets in *P. pratense* could not be distinguished from each other but the other set was obviously differentiated from them. This finding along with the evidence from the karyotypic comparison between *P. pratense* and two diploid species *P. alpinum* and *P. bertolonii* led to two primary conclusions: (1) *P. pratense* is most likely an allohexaploid consisting of genomes, A and B, presumably with the genome of AAAABB; (2) These two genomes may have come from *P. alpinum* and *P. bertolonii* respectively. These tentative conclusions were further confirmed by a molecular study using genome-specific probes. DNA/DNA hybridization on slot blot and Southern blot demonstrated that the blotted DNA of *P. pratense* could hybridize to the probes specific to *P.*

alpinum and to the probes specific to *P. bertolonii*. This clarifies the allopolyploid nature of *P. pratense* and suggests the participation of these two diploid species in the genome construction in this allohexaploid. In contrast to *P. pratense*, another species *P. montanum* showed no hybridized signal either to *P. alpinum*-specific probes or to *P. bertolonii*-specific probes, thus implying that it was unlikely involved in the genome construction of *P. pratense*.

While this study has brought about a concrete advance in the understanding of the phylogenetic origin and evolution of *P. pratense*, it would be more fruitful if the analyses were extended to the remainder of the species in this genus. Further research is suggested to extend the conclusions drawn in this study. A fruitful area of extension is to screen the karyotypes for the remainder of the species in *Phleum* using the C-banding technique. In addition, it would be very interesting to screen the genomic DNA of all these species through Southern blot analysis using the probes specific respectively to these two genomes which are available in this laboratory. Thus further research may be expected to add more knowledge to our understanding of the gene pool in *Phleum*.

Phenotypic variation of seed storage proteins and cultivar identification

The data from SDS-PAGE analysis of seed storage proteins presents a new field of information on the genetic variation in cultivated timothy. Such data confirmed that the SDS-PAGE patterns of seed storage proteins were distinguishable among all 19 cultivars except for two pair. The following two kinds of data seem crucial for characterization of phenotypic differentiation of seed storage proteins between the cultivars:

The first category of the data was from the calculation of the proportion of differentiated proteins (p_p). The formula used to calculate P_p was first

proposed in this thesis and seems to work well in measurement of differences of the protein banding patterns in this species. The P_p data make it possible to quantitatively evaluate the protein phenotype differentiation between the cultivars in timothy, thus presenting a criteria for cultivar discrimination in this forage species.

The second category came from the computer-monitored densitometer scanning of the SDS-PAGE profile. Coomassie blue-stained protein patterns showed many thin or minor bands in timothy. Scanning of the protein bands with the densitometer seems very important in that it not only aids in the identification of these minor bands but also provides a density for each band, which makes it possible to characterize the protein banding patterns quantitatively. Therefore, densitometer scanning data played an important role in confirming phenotypic differentiation between the cultivars in timothy.

It has been shown that SDS-PAGE analysis can be used for the identification of genotypes or cultivars in timothy. It may also provide some information on the origin and genetic relationships of the cultivars in this species.

Appendix I

The densitometer-scanned data of the seed storage proteins in timothy

Table I. The band intensities (O.D.)* of the seed storage proteins in five European cultivars of timothy

Band No.	Alpage	A.S.352	Erecta	Pecola	Tiller
1	0.038	0.038	0.058	0.088	-
2	-	-	0.060	-	0.054
3	0.036	0.042	0.068	0.066	0.081
4	0.053	0.067	0.068	0.080	0.120
5	0.040	0.055	0.093	0.075	0.079
6	0.080	0.087	0.119	0.100	0.118
7	0.052	0.066	0.075	0.096	0.040
8	0.112	0.048	0.181	0.186	0.167
9	0.124	0.139	-	-	-
10	0.119	0.111	0.171	0.158	0.137
11	-	-	-	-	-
12	0.091	0.113	0.058	0.148	0.125
13	0.080	-	-	-	0.093
14	-	-	-	0.078	0.081
15	-	-	0.089	-	-
16	0.045	-	0.067	0.095	0.088
17	0.046	-	-	-	-
18	0.202	0.243	0.291	0.282	0.192
19	0.215	0.243	0.288	0.282	0.220
20	0.216	0.223	0.273	-	0.170
21	0.216	0.232	0.266	0.250	-
22	0.180	0.187	0.229	-	0.165
23	0.123	0.117	0.161	0.146	0.128
24	-	-	-	-	-
25	0.057	0.080	0.105	0.093	0.080
26	0.045	0.067	0.134	0.068	0.082

Table I, Continued

Band No.	Alpage	AS352	Erecta	Pecola	Tiller
27	0.044	0.071	0.095	0.073	0.068
28	-	-	0.096	0.109	0.088
29	0.299	0.318	0.389	0.375	0.281
30	-	0.294	0.370	-	0.279
31	0.298	-	-	0.346	0.259
32	0.206	0.220	0.237	0.213	0.160
33	0.116	-	0.166	0.130	0.121
34	-	0.119	0.182	0.166	0.140
35	0.186	0.167	0.211	0.276	0.171
36	0.132	-	-	-	-
37	-	0.135	-	0.188	-
38	0.115	0.134	0.114	0.184	0.149
39	-	0.141	-	-	0.143
40	-	-	-	0.173	-
41	0.163	0.108	0.127	0.101	0.067
42	0.151	0.091	0.138	0.127	0.071
43	0.114	0.108	0.159	0.135	0.083
44	0.332	0.249	0.300	0.308	0.262

* The O.D. value of each band is an average calculated among those recorded in four repeats.

Table II. The band intensities (O.D.)* of seed storage proteins in 14 American cultivars of timothy

Band No.	Bash.	Bott.	Boun.	Cham.	Clim.	Glen.	Itas.	Mari.	Rich.	Salv.	Tiit.	Toro.	Winm	Timf.
1	-	0.040	-	-	0.066	0.056	0.054	0.041	0.054	-	0.064	0.051	0.087	0.049
2	-	-	-	-	0.042	-	-	-	0.043	-	-	0.055	-	-
3	-	0.048	-	-	0.050	0.049	0.062	0.037	0.071	0.067	0.057	0.069	0.093	0.060
4	0.056	0.040	0.098	0.032	0 051	0.062	0.085	0.056	0.054	0.072	0.040	0.092	0.042	0.086
5	-	0.050	-	0.050	0.071	0.052	0.067	0.046	0.053	-	0.040	0.106	0.072	0.060
6	0.066	0.057	0.082	0.069	0.089	0.060	0.111	0.054	0.054	0.059	0.053	0.142	0.097	0.106
7	0.096	-	0.082	0.069	0.076	0.060	0.108	-	0.074	-	0.050	-	0.072	0.065
8	0.175	0.038	0.182	0.050	0.165	0.144	0.189	0.146	0.138	0.125	0.170	0.180	0.159	0.177
9	0.144	-	0.191	0.110	-	0.110	0.173	-	-	-	-	-	0.125	0.139
10	0.126	0.108	0.182	0.095	0.158	-	0.183	0.079	0.104	0.085	0.131	0.190	-	-
11	-	0.089	-	-	-	-	-	0.068	0.081	-	-	-	-	0.089
12	0.143	0.082	0.178	0.105	0.150	0.098	0.138	0.079	0.103	0.082	0.075	0.169	0.120	0.107
13	-	-	-	0 081	-	0.079	-	0.071	-	-	-	0.162	0.081	0.088
14	-	-	-	-	0.071	-	-	-	0.060	-	0.040	-	0.031	0.042
15	0.101	0.039	-	0.055	0.081	-	-	-	-	0.040	-	-	0.038	0.053
16	0.068	0.053	0.121	0.046	0 071	-	0.074	0.059	0.052	0.056	0.046	0.086	0.038	0.049
17	-	-	-	0.046	-	0.052	-	-	-	-	-	-	-	-
18	0.246	0.150	0.289	0.117	0.245	0.250	0.275	0.184	0.196	0.135	0.171	0.270	0.225	0.218
19	0.255	0.139	0.289	0.177	0.251	0.245	0.279	0.191	0.207	0.175	0.152	O.294	0.256	0.264
20	0.226	0.137	0.274	0.159	0.235	0.220	0.251	0.162	0.182	0.112	0.148	0.283	0.229	0.230
21	0.226	0.137	0.274	0.110	0.216	0.212	0.251	0.149	0.162	0.126	0.152	0.217	0.202	0.206
22	0.175	0.086	0.196	0.099	0.154	0.206	0.178	0.148	0.118	0.101	0.075	0.164	0.160	0.150
23	0.132	0.061	0.169	0.073	0.114	0.078	0.134	0.058	0.082	0.073	0.061	0.114	0.082	0.101
24	-	-	-	-	-	-	-	0.054	-	-	-	0.113	-	-
25	0.101	0.092	0.130	0.095	0 078	0.052	0.081	0.047	0.053	0.050	0.045	-	0.050	0.051
26	0 112	0.051	0 163	-	0.112	0.045	0.064	0.042	0.044	-	-	0.090	0.025	0.048
27	0.123	0.049	0.151	0.058	0.072	0.050	0.079	0.054	0.044	0.039	0.046	0.087	0.051	0.052

Table II, continued

Band No.	Bash.	Bott.	Boun.	Cham.	Chm.	Glen.	Itas.	Mari.	Rich	Salv	Tiit.	Toro.	Winm	Timf
28	-	-	-	-	0.072	0.087	0.112	0 083	0 096	0 100	0 085	0 060	0 120	0 096
29	0.335	0.265	0.386	0.287	0.353	0.338	0.377	0 310	0.307	0 284	0 277	0 369	0 358	0 360
30	0.327	0.350	0.361	0.254	0 329	0.309	-	0.273	0 269	0.220	0 233	0 344	-	-
31	-	0.193	-	0 177	-	-	0.350	-	-	-	-	0.250	0 319	0 316
32	0.211	0.167	0.239	0 095	0.173	0 134	0.202	0 136	0 144	0 111	0 119	-	0 173	0 152
33	0.159	0.069	0.208	0.082	0.114	-	0.116	0.077	0.089	0.068	-	0.193	0 103	0 091
34	0.141	0.080	0 179	0.101	0.142	0.101	0.153	0 077	0.085	0.079	0 071	0 169	0 111	0 105
35	0.238	0.119	0.181	0 140	0.186	0.110	0.228	0.155	0.124	0 149	0 121	0.213	0 168	0 140
36	0.220	-	0.201	0.146	-	-	-	-	-	-	-	0.190	0 139	0 133
37	0.162	0.091	0.140	-	0.140	0.083	0.168	-	0 109	-	-	-	-	-
38	0.155	0.104	0.178	0.138	0.173	0.090	0.170	0.109	0.109	-	0.081	0 183	0 130	0 116
39	-	-	-	0.117	0.140	-	0.158	-	0.111	0 083	0 106	0 195	0 140	0 052
40	-	0 091	0.140	0 101	0 104	-	0 094	-	-	0.070	-	0 191	-	
41	0.121	0.082	0.161	0.088	0.109	0.079	0.118	0.090	0 076	0 074	0 091	0.112	0 114	0 089
42	0.157	0.096	0 188	0.103	0.131	0 088	0.092	0 081	0 075	0 082	0 077	0 130	0 114	0 089
43	0.157	0.115	0.211	0.121	0 126	0.088	0.130	0 090	0.093	0 102	0 091	0 129	0 124	0 106
44	0.315	0.190	0.336	0.222	0 248	0.206	0.269	0 211	0.195	0 198	0 180	0 347	0 235	0 216

* The O.D. value of each band is an average calculated among those recorded in four repeats.

Appendix II

The reprint of paper 1 published in "Genome"

Characterization of genomes of timothy (*Phleum pratense* L.). I. Karyotypes and C-banding patterns in cultivated timothy and two wild relatives[1]

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CAI, Q., and BULLEN, M R 1991 Characterization of genomes of timothy (*Phleum pratense* L.) I Karyotypes and C-banding patterns in cultivated timothy and two wild relatives Genome, **34** 52-58

In an attempt to know the phylogeny of timothy (*Phleum pratense*), the cultivated species and two wild relatives, *Phleum alpinum* and *Phleum bertolonii*, were karyotyped with conventional and Giemsa C-banding methods In the hexaploid *P. pratense* ($2n = 6x = 42$), two sets of seven chromosomes were indistinguishable from each other both in morphology and in banding patterns and the third set of seven was found to be differentiated from them Two genomes, A and B, were tentatively established The banded karyotype in diploid *P. alpinum* ($2n = 2x = 14$) was close to the A genome, which was tetraploid in *P. pratense*, and the karyotype in *P. bertolonii* ($2n = 2x = 14$) was analogous to the B genome in *P. pratense*, which suggests these species were the genome donors of *P. pratense*

Key words chromosome, genome, allopolyploid, Giemsa C-banding

CAI, Q., et BULLEN, M R 1991 Characterization of genomes of timothy (*Phleum pratense* L.) I Karyotypes and C-banding patterns in cultivated timothy and two wild relatives Genome, **34** 52-58

Dans le but de connaître la phylogenese du *Phleum pratense*, la fleole des pres, les caryotypes Giemsa de l'espece cultivée et deux espèces sauvages, *Phleum alpinum* et *Phleum bertolonii* ont ete examines Les bandes C obtenues ainsi que la longueur et les rapports des bras des chromosomes ont conduit a l'identification des genomes de ces especes En ce qui concerne l'espèce hexaploide *P. pratense* ($2n = 6x = 42$), on a trouve que deux groupes de sept chromosomes étaient identiques tandis qu'un troisieme groupe de sept chromosomes etait different des deux premiers, sur la base de la morphologie et de la distribution des bandes C Le caryotype de l'espece diploide *P. alpinum* ($2x = 2x = 14$) est proche de génome A (tétraploide chez *P. pratense*) tandis que le caryotype de *P. bertolonii* ($2n = 2x = 14$) ressemble au génome B de *P. pratense*

Mots clés chromosome, génome, allopolyploide, bandes C, coloration de Giemsa

Introduction

Timothy (*Phleum pratense* L.) is an important forage crop, widely grown in cool, temperate regions of the world, including North America and Europe In the genus *Phleum*, of which at least 10 species have been positively identified, while another 10 may have specific rank, only the species *P. pratense* is of major economic importance (Nath 1967)

The cultivated species *P. pratense* almost always appears in the literature as a hexaploid with a basic chromosome number of seven. The phylogenetic relationship between this species and the wild species of *Phleum* was a frequent subject of study during the period 1930–1954 Few investigations have been published in the last two decades. Most of the studies on phylogenetic relationships in *Phleum* species were concentrated on interspecific hybridization. A series of investigations on the fertility of interspecific hybrids and chromosomal behavior in meiosis of pollen mother cells in *P. pratense* and in interspecific hybrids have been reported (Muntzing 1935; Muntzing and Prakken 1940; Nordenskiold 1941, 1945). From these studies came hypotheses about the nature and origin of the polyploid genome in *P. pratense* One hypothesis saw the cultivated *P. pratense* as an allohexaploid containing two distinct genomes, which were proposed to come from *P. alpinum* and *P. nodosum*, renamed *P. bertolonii* (Muntzing and Prakken 1940, Levan 1941, Stebbins 1950). A second hypothesis considered the species to be an autopolyploid of *P. nodosum* (Myers 1944, Nordenskiold 1945, Wilton and Klebesadel 1973) The latter point of view seems to be generally accepted However, the authors believe that a definite conclusion cannot be drawn because of a lack of conclusive evidence from cytology and genetics.

The detailed karyotypes of *P. pratense* and its relatives have been poorly understood Wilton and Klebesadel (1973) reported the chromosome morphology (length, arm ratios) of three *Phleum* species including *P. pratense* and showed that the three genomes in *P. pratense* had similar chromosomal morphology, thus supporting the autopolyploid nature Only in two of the three species could they make statistical comparisons, as cytological preparations were difficult to obtain in the third taxon The information was limited in this study because banding was not yet available for the genus Joachimiak (1981) showed the first banding with a *Phleum* using the Hy (hydrochloric acid) technique

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

Species	Accession No	2n	Source
P. pratense	M46	42	Agriculture Canada, Ste-Foy Research Station, Que
	M44	42	Agriculture Canada, Plant Research Centre, Ottawa
	M35	42	Jardin botanique national de Belgique, Meise
P. alpinum ssp. *rhaeticum*	M27	14	Botanischer Garten und Botanisches Museum, Berlin-Dahlem
	M39	14	Museum national d'histoire naturelle, Paris
P. bertolonii	M40	14	Museum national d'histoire naturelle, Paris
	M33	14	Botanischer Garten und Botanisches Museum, Berlin-Dahlem
	M16	14	Plant Gene Resources of Canada, Ottawa

on the diploid *P. boehmeri*. While of interest, this technique did not prove to be a practical way to prepare banded karyotypes that could be readily repeated (M. R. Bullen, unpublished results). The objective of this study was to obtain the C-banded karyotypes of *P. pratense* and two wild species which appear to be genome donors. Confirming the genome donors would contribute to the understanding of the origin and evolution of the polyploid genome of timothy.

Materials and methods

Three species of *Phleum* (*P. pratense*, *P. alpinum* ssp. *rhaeticum*, and *P. bertolonii*) were used for this study. *Phleum pratense* is a cultivated species and the other two are essentially wild. These species and accession numbers are listed in Table 1. Specimens will be deposited in the Herbarium of McGill University.

The plants were grown in a growth chamber with a photoperiod of 16 h, at a light intensity of 94 μE M^{-2} s^{-1} and a temperature of 18/12°C (light/dark). To carry out the pretreatment of the root tips, the roots, still attached to the rest of the plant, were immersed in 0.002 M 8-hydroxyquinoline solution for 3 h. One centimetre root tips were cut and fixed in methanol – glacial acetic acid (2:1) for 12 h. For the conventional chromosome observation, the root tips were hydrolyzed in 0.2 M HCl at 60°C for 6 min. The slides were made and stained following the method described by Cai and Chinnappa (1987). For the Giemsa C-banding of the chromosomes, the fixed root tips were hydrolyzed in 0.1 M HCl at 60°C for 9 min and then squashed in 45% acetic acid. The slides were dried at room temperature for 4 days. Subsequently, the dried slides were denatured in 5% barium hydroxide at 20°C for 3 min and then incubated in 2× SSC (1× = 0.15 M NaCl plus 0.015 M sodium citrate) solution at 60°C for 1 h and stained with 4% Giemsa in 1/15 M phosphate buffer (pH 6.8). The chromosomes were observed and photographed using a Leitz Orthoplan II microscope. Ten cells with well-spread metaphase chromosomes for each accession were used for analysis and construction of idiograms.

Results

The karyotype and C-banding pattern in P. pratense

The three accessions from *P. pratense* listed in Table 1 were karyotyped. The somatic cells in all three accessions, M44, M35, and M46, were shown to have 42 chromosomes with the exception of two plants of M46, which were found to have more than 42 chromosomes. One plant was $2n = 43$ and the other was $2n = 46$. The number of 42 chromosomes was considered to be the hexaploid number in *Phleum*, typically consisting of three sets of seven. In all the accessions hexaploid and diploid, B chromosomes were often observed in low numbers, although some cells showed up to three per cell. The morphology of individual chromosomes in the species can be characterized by the data presented in Table 2 and by the photos and idograms in Fig. 2*a*. As shown in Table 2, the chromosome size ranges from 3.80 to 7.12 μm. Within the chromosome complement, 14 pairs of chromosomes are metacentric and the other seven pairs are submetacentric. Two pairs of chromosomes (A6) were shown to have distinct satellites with long secondary constrictions. There were no apparent differences in karyotype found between different accessions apart from the two plants mentioned above. In addition to the chromosome morphology, the C-banding patterns of *P. pratense* chromosomes were examined. As shown in Figs. 1 and 2*a*, the karyotype contained distinct telomeric bands and centromeric bands as well as some intercalary bands. All the 42 chromosomes showed telomeric bands and centromeric bands. Among them 10 chromosomes contained intercalary bands and the satellited chromosomes showed the secondary constriction band. The telomeric bands seemed to be stable, but the centromeric bands were slightly variable, depending on the division stage of the cell. It was noted that some chromosomes showing centromeric bands in metaphase were not banded in the centric region in prometaphase.

To clarify the polyploid nature of *P. pratense*, the morphology and C-banding patterns of individual chromosome pairs were compared. Morphological similarity was found among some chromosomes. The similar chromosome pairs can be put into seven groups: A1, A2, A3, A4, A5, A6, and A7, as shown in Table 2. Each group contains two similar chromosome pairs, except two groups, A1 and A3, which also share a similar morphology with B2 and B3, respectively. Not only the morphology but the C-banding pattern showed the similarity between corresponding chromosome pairs in these seven groups as well. As shown in Fig. 2*a*, except for A4 (of *P. pratense*), where the telomeric band on the long arms are different in band intensity, the chromosome pairs in the other A groups show apparently similar banding patterns. However, the third set of seven chromosomes are mostly distinguishable from them. This would imply that there are two homologous sets rather than three homologous sets consisting of seven chromosomes in *P. pratense*. The karyotype of this species may well be that of allopolyploid of two different genomes, with one being tetraploid and the other diploid. On the basis of the karyotype data and C-banding patterns, two different genomes are tentatively named for *P. pratense*, the tetraploid as genome A and the diploid as genome B. In two cases, three chromosome pairs presumably from the three genomes share a similar morphology. However, they can be sorted into genome A or B with the aid of distinct banding patterns. The C-banding patterns of individual chromosome pairs are described shortly using the following nomenclature: the lower case letter "p" is used to designate the chromosomes from *P. pratense*, "a" is used for the chromosomes from *P. alpinum*, and "b" is used for those from

TABLE 2. The lengths (μm) and arm ratios (L/S) of the chromosomes from three *Phleum* species

	A1	A2	A3	A4	A5	A6	A7
P. pratense							
Length	7.09 ± 0.51	6.49 ± 0.30	6.10 ± 0.37	5.67 ± 0.41	4.97 ± 0.35	4.54 ± 0.27*	4.28 ± 0.43
L/S	1.18 ± 0.05	1.22 ± 0.09	1.38 ± 0.08	1.64 ± 0.20	1.52 ± 0.14	2.32 ± 0.43	2.81 ± 0.37
P. nodosum							
Length	6.72 ± 0.29	6.28 ± 0.43	5.87 ± 0.53	5.34 ± 0.48	4.82 ± 0.35	4.24 ± 0.22*	3.80 ± 0.25
L/S	1.23 ± 0.07	1.14 ± 0.07	1.44 ± 0.19	1.80 ± 0.18	1.82 ± 0.22	2.34 ± 0.28	2.72 ± 0.43
P. alpinum							
L/S	7.26 ± 0.79	6.47 ± 0.70	5.85 ± 0.59	5.70 ± 0.54	5.26 ± 0.43	4.46 ± 0.56*	4.28 ± 0.43
	1.28 ± 0.11	1.41 ± 0.11	1.34 ± 0.08	1.56 ± 0.16	1.49 ± 0.24	2.05 ± 0.19	2.72 ± 0.27
	B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**
P. pratense							
Length	7.72 ± 0.47	6.52 ± 0.40	6.12 ± 0.38	5.60 ± 0.38	5.33 ± 0.39	5.05 ± 0.28	4.52 ± 0.30
L/S	1.53 ± 0.13	1.20 ± 0.07	1.55 ± 0.14	1.19 ± 0.20	1.13 ± 0.08	1.12 ± 0.08	2.85 ± 0.26
P. bertolonii							
Length	7.62 ± 0.80	6.98 ± 0.80	6.40 ± 0.80	6.23 ± 0.78	5.79 ± 0.67	5.25 ± 0.62*	4.78 ± 0.34
L/S	1.53 ± 0.11	1.17 ± 0.08	1.51 ± 0.15	1.17 ± 0.10	1.17 ± 0.06	1.84 ± 0.26	2.77 ± 0.34

Note: *Phleum pratense* shows the two A genomes.
*Not including the satellite.

P. bertolonii The details of the pairs of chromosomes are as follows.

pA1—The chromosomes were banded on both ends as well as at the centromere. In addition, an intercalary band was seen in the long arm.

pA2 and pA3— There were telomeric bands on both ends and a centromeric band.

pA4, pA5, and pA7— The chromosomes were banded at the centromeric region and on the end of the long arm. In terms of the band intensity, the telomeric bands in pA5 seemed to be different between homologous chromosome pairs. The band in one pair was much thicker than the other one.

pA6— These chromosomes were found to be distinguishable from others by having distinct satellites. In addition to the telomeric and centromeric bands which were similar to pA4, pA5, and pA7, a secondary constriction band was revealed.

The members of genome B showed the following C-banding patterns.

pB1 - This is the biggest chromosome pair in the *P. pratense* karyotype. Telomeric bands on both ends and a centromeric band as well as an intercalary band in the long arm were demonstrated.

pB2 - This pair of chromosomes had the richest bands in the whole chromosome complement, including two telomeric bands, two intercalary bands, one on the short arm and the other on the long arm, as well as a centromeric band.

pB3 - Two telomeric bands and one centromeric band were stably visualized. In addition, one intercalary band could, in most cases, be seen in the short arm.

pB4 and pB6 - The chromosomes were banded at the centromere and on both distant ends.

pB5 - The chromosome pair contained a unique banding pattern, having an intercalary band in the short arm, a telomeric band on the long arm, and a centromeric band.

pB7— This chromosome pair had a similar morphology and banding pattern to pA7, containing a centromeric band and a telomeric band on the end of the long arm.

The banded karyotypes in the wild species

P. alpinum *ssp.* rhaeticum

Both of the accessions from this species were found to be diploid, with 14 chromosomes and in most cases one or two B-chromosomes. In its chromosome complement, there were two submetacentric and five metacentric chromosome pairs, with the lengths ranging from 4.28 to 7.27 μm. In this investigation, one satellited chromosome pair (the sixth) was identified for this species. The detailed karyotype data can be seen in Table 2.

The karyotype of this species was further assayed by the C-banding technique. The banded chromosomes and the idiograms of the C-banding pattern are shown in Fig. 2*b*. It can be seen from this figure that all chromosomes in this species are banded at the centromere and on either or both ends of the chromosomes. Two chromosome pairs were found to have intercalary bands, which were shown in the long arm in the first chromosome and in the short arm in the second. The telomeric bands were distributed on two distant ends of the chromosomes in the following chromosome pairs: aA1, aA2, aA3, and aA5, and only on the long arm end in aA4 and aA7. A secondary constriction band was revealed in the satellited chromosome pair, aA6. It should be pointed out that polymorphism of C-banding patterns between the two homologous chromosomes was found in some chromosome pairs. In the aA1, one chromosome had telomeric bands on both ends, while its partner showed only a thin band on the short arm end. In the aA6, one chromosome had a telomeric band on the end of the satellite but its partner did not. This phenomenon was also reported in *Allium* (Loidl 1983; Cai and Chinnappa 1987).

Comparison of the banded karyotypes between *P. alpinum* and *P. pratense* showed that the morphology and banding pattern of *P. alpinum* was quite similar to the chromosomes

FIG. 1. The Giemsa C-banded mitotic chromosomes of *Phleum pratense* ($2n = 42$). 2300×.

in the A genome of *P. pratense*. Among the chromosome complement in *P. alpinum*, chromosomes aA1, aA3, aA4, aA6, and aA7 were, respectively, analogous to the pA1, pA3, pA4, pA6, and pA7 in the A genome of *P. pratense*. Slight differences in banding were found in another two chromosome pairs. A short-arm intercalary band was found in the aA2 chromosome in *P. alpinum* but not in the pA2 in *P. pratense*. The aA5 in *P. alpinum* had a telomeric band on the short arm, while the pA5 in *P. pratense* did not. These data suggest a close relationship between the genome of *P. alpinum* and the A genome in *P. pratense*.

P. bertolonii

Like *P. alpinum*, *P. bertolonii* was found to be diploid, with 14 chromosomes in all examined accessions. The karyotype data are presented in Table 2. There were two chromosome pairs that were submetacentric and five metacentric. The sixth chromosome had a satellite. The C-banding treatment of the chromosomes revealed abundant heterochromatin in the *P. bertolonii* complement. As shown in Fig. 2*c*, the heterochromatin was mainly distributed in telomeric and centric regions, similar to the cases in *P. alpinum* and *P. pratense*. All the chromosomes observed in this species were found to carry telomeric bands. Among them, five chromosome pairs (bB1, bB2, bB3, bB4, and bB5) had two telomeric bands and the others had one telomeric band, which was located in the long arm. In comparison with the telomeric bands, the centromeric band seemed to be thin and variable. Although in some cells that were in metaphase, all chromosomes were shown to have the centromeric band, in many cells, especially those in prometaphase, only two or three pairs of chromosomes were banded at the centromeres. Intercalary bands were found in four chromosome pairs. In the bB1 and bB4, an intercalary band was present in the long arm. The bB2 chromosome contained two intercalary bands located in the long and short arms, respectively. The bB3 contained one intercalary band in the short arm. A secondary constriction band was found in the bB6 chromosome. Polymorphism of C-banding patterns between homologous chromosomes was found in the bB2 chromosome pair, where one chromosome had an intercalary band in the short arm but the other did not.

The karyotypes and C-banding patterns were compared between *P. bertolonii* and *P. pratense*. The genome in *P. bertolonii* seemed to match the genome B in *P. pratense*. In terms of chromosome morphology, including the lengths and arm ratio, all the chromosomes except one in *P. bertolonii* were similar to the six chromosomes in the genome B. The exceptional chromosome is the bB6 in *P. bertolonii*, which is distinguished from pB6 in *P. pratense* in that the former had a satellite while the latter did not. The C-banding pattern revealed in the *P. bertolonii* genome was to some extent similar to that shown for the B genome in *P. pratense*. Four chromosomes (bB1, bB2, bB3, and bB7) in the former had exactly the same banding pattern as the four members (pB1, pB2, pB3, and pB7, respectively) in the latter. When the bB4 and pB4 were compared, both had one centromeric band and two telomeric bands and were only different in that the long arm of the former had an intercalary band while the other species did not. Relatively greater variation in banding patterns between these two genomes occurred in the remaining two chromosomes. The bB5 in the *P. bertolonii* genome was divergent from the pB5

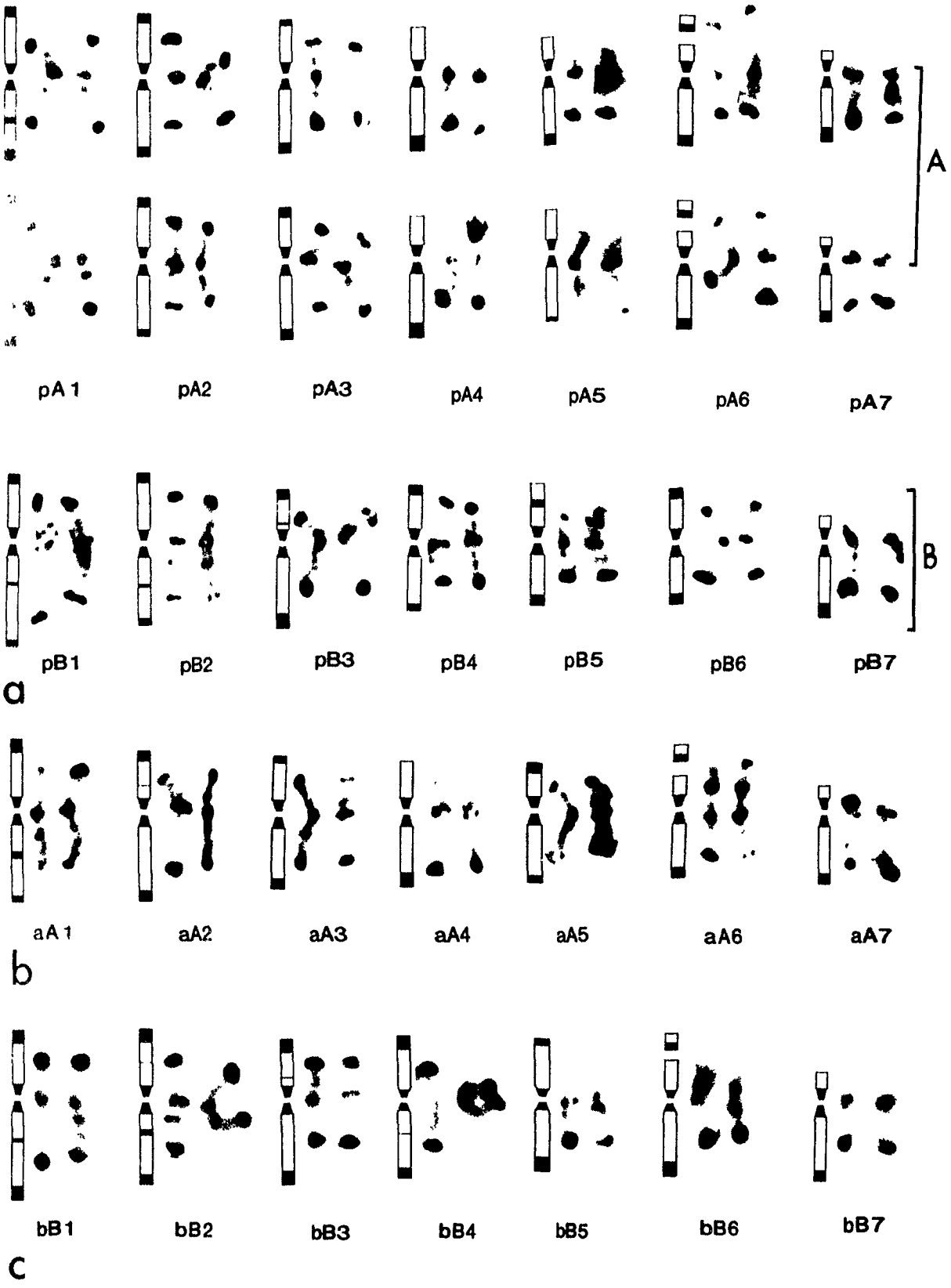

FIG. 2. The banded karyotypes and idiograms of C-banding patterns for three *Phleum* species. (*a*) *P. pratense*, where two of the A genomes and the one of the B genomes of this hexaploid are shown; (*b*) *P. alpinum*, where the A genome is shown; (*c*) *P. bertolonii*, where the B genome is shown. For the nomenclature of the individual chromosomes, the lower case letters "p," "a," and "b" are used to designate the corresponding species *P. pratense*, *P. alpinum*, and *P. bertolonii*, respectively.

of timothy as it had a telomeric band on its short arm whereas the latter had an intercalary band in the same arm. The bB6 was different from pB6 in that the former had a secondary constriction band whereas the latter had a telomeric band in the short arm.

Discussion

The results with respect to the chromosome numbers in these three species mentioned above are in agreement with other reports (Wilton and Klebesadel 1973, Teppner 1980). However, the karyotypes observed in *P. pratense* seem to disagree with those reported by Wilton and Klebesadel (1973). The main difference is that they found three chromosome pairs with distinct satellites whereas only two pairs of satellited chromosomes were identified in this study. Differences in the appearance and number of satellites have been noted previously between biotypes in the grass family. In *Triticum monococcum* L., where six biotypes were analysed, only two had satellites of the same appearance while the others were all different, even varying in the number of pairs (Waines and Kimber 1973). While there is competent sampling of the plant material in both the work of Wilton and Klebesadel and the present studies, the number of biotypes studied is too small to draw any conclusions about what is a standard karyotype in respect to satellites for timothy. In Fig. 2*a* the shift in the configuration of the karyotype in respect to a satellite is illustrated. It is the bB6 from the diploid that does not show a satellite when incorporated into the hexaploid. A similar phenomenon has also been reported in wheat. The diploid *Triticum monococcum* L., which has one or two pairs of satellites, depending on the biotype (Waines and Kimber 1973), contributes its A genome to the hexaploid wheat *T. aestivum* (Riley 1965). However, the karyotype of the polyploid wheat, AABBDD, exhibits only two pairs of satellited chromosomes, 1B and 6B (Gill 1987), which those of the A genome not being detected. Although the mechanism is unknown, it would be reasonable to suppose that part of the adaptation of the new polyploid cell would be to suppress some of the rDNA activity to the point that the end of the chromosome could no longer be recognized as a satellite.

In addition, we found it difficult to assign the *P. pratense* chromosomes into three similar groups consisting of seven chromosomes in the way that Wilton and Klebesadel did. It is clear to us that two sets of seven chromosomes are very similar to the corresponding chromosomes, while the third set of seven is distinguishable from them.

One of the two earlier hypotheses considered that *P. pratense*'s origin was by way of allopolyploidy. In support of this concept, Gregor and Sansome (1930) obtained triploid hybrids ($2n = 21$) from crossing diploid *P. pratense* ($2n = 14$) with tetraploid *P. alpinum* ($2n = 28$). From this highly sterile triploid, four hexaploid plants were obtained, one of which proved cross-fertile with the natural hexaploid *P. pratense*. This implied that *P. pratense* might be an allopolyploid of a diploid *P. pratense* ($2n = 14$) and tetraploid *P. alpinum* ($2n = 28$). Muntzing (1935) further concluded that the hexaploid *P. pratense* ($2n = 42$) may be an allohexaploid at least involving the genome (N) from *P. nodosum*. The genomic constitution in *P. pratense* was considered to be NNAABB (Muntzing 1935) or $NNA_1A_1A_2A_2$, where A_1 and A_2 are sufficiently homologous to permit formation of bivalents between chromosomes of the two genomes (Muntzing and Prakken 1940). Levan (1941) studied haploid plants of *P. pratense* and found seven bivalents and seven univalents. Stebbins (1950) commented that since they typically form seven bivalents and seven univalents, their genomic formula must be NAB and that of the diploid NNAABB. However, Nordenskiold (1941, 1945, 1949), based on her studies of hybrids between *P. pratense* and *P. nodosum* and *P. alpinum*, proposed the different point of view that hexaploid *P. pratense* was an autoploid developed from *P. nodosum*. The evidence supporting her hypothesis is that the artificially synthesized hexaploid plants of *P. nodosum* were found to have similar chromosomal behavior in meiosis to the hexaploid *P. pratense* and also they were cross-fertile with each other. It should be pointed out that diploid *P. pratense* ($2n = 14$) and *P. nodosum* used in earlier work might have been *P. bertolonii*. According to Humphries (1980), diploid *P. pratense* could be assigned to *P. pratense* ssp. *bertolonii*. In addition to diploid *P. bertolonii* ($2n = 14$), an autotetraploid of this species has been produced for breeding purposes (Borg and Ellerstrom 1986). According to Wilton and Klebesadel (1973) and Borrill (1976), *P. bertolonii* was previously known as *P. nodosum*. The chromosome association and behavior in meiosis of pollen mother cells in hexaploid timothy were expected to be an important guide to the polyploid nature of this species. The higher frequency of quadrivalents in the first division of meiosis in *P. pratense* reported by Myers (1944) seemed to support the allopolyploid genome structure of NAB in *P. pratense*. However, Nordenskiold (1945) reported a very low frequency of quadrivalent and other multivalent formation in the meiosis of *P. pratense*. This brought into question the contribution of the information from the meiosis of hexaploid *P. pratense* to the understanding of polyploid nature of the *P. pratense* genome.

In our study, the evidence from the analysis of C-banded karyotypes seems to support the earlier hypotheses proposed by Gregor and Sansome (1930), Muntzing and Prakken (1940), and Stebbins (1950). Our results show that two sets of seven chromosomes are very similar to each other for the corresponding individuals in karyotype morphology and C-banding pattern, while another set of seven seem distinguishable. The two similar chromosome sets may belong to the same genome or highly homologous genomes. They have been designated as genome A. The other one may belong to a different genome, designated as genome B. Therefore, the genomic formula of *P. pratense* should be AAB. This result coincides with the earlier observation of meiosis in haploid *P. pratense* where bivalents and seven univalents were found (Levan 1941).

The comparison of C-banded karyotypes between *P. pratense* and *P. alpinum* and between *P. pratense* and *P. bertolonii* in this study implies that the genome A of *P. pratense* was likely donated by *P. alpinum* and the genome B was likely donated by *P. bertolonii*. Also, all evidence from different investigations favor *P. bertolonii* as a progenitor of *P. pratense*, irrespective of whether *P. pratense* is considered an autohexaploid or an allohexaploid. Nevertheless, some evidence from earlier reports (Nordenskiold 1941, 1945) seems unfavorable for *P. alpinum* as a progenitor candidate. In these reports, the hybrid between *P. pratense* ($2n = 42$) and *P. alpinum* (unreduced

gametes) were compared with the hybrids between *P. pratense* (2*n* = 42) and *P. nodosum* (*P. bertolonii*) (2*n* = 14 and 2*n* = 28). The result showed that the hybrids from *P. pratense* × *P. alpinum* appeared abnormal, including low viability, sterility, and irregular behavior of chromosomes at meiosis. In contrast, the hybrids from *P. pratense* × *P. nodosum* (*P. bertolonii*) behaved normally. Therefore, Nordenskiold concluded that *P. alpinum* is unlikely to participate in the formation of the genome in *P. pratense*.

From our point of view, the evidence from Nordenskiold's data does not repudiate the hypothesis that *P. alpinum* is one of the progenitors of *P. pratense*. The reason is that if the genome constitution in *P. pratense* is indeed AAAABB (A from A from *P. alpinum* and B from *P. bertolonii*), the hybrid between *P. pratense* (hexaploid) and *P. bertolonii* (diploid) will have the genome pattern of AABB or AABBB (B reduced gamete in *P. bertolonii*), while the hybrid from *P. pratense* (hexaploid) × *P. alpinum* (diploid) will have AAB or AAAAB (unreduced gamete in *P. alpinum*). In these terms, the results reported by Nordenskiold can readily be explained. The aberrant behavior of the hybrid from *P. pratense* × *P. alpinum* might be attributed to its unbalanced genome constitution, in which only one copy of the B genome existed. On the other hand, the hybrid from *P. pratense* × *P. bertolonii* possessed a balanced genome structure, which contained at least two copies of either A or B, and this might account for its normal behavior in biology and cytology.

In addition to chromosome observation and interspecific hybrid investigation in the early studies of *Phleum* species, some studies were also carried out to test inheritance models of individual gene loci in the hexaploid *P. pratense* by using chlorophyll markers (Wexelsen 1941, Myers 1944; Nordenskiold 1953, 1954, Nielsen and Smith 1959). These studies were considered promising in their ability to confirm the polyploid nature of *P. pratense*. An autohexaploid would exhibit hexasomic segregation, while an allohexaploid would exhibit disomic or disomic plus tetrasomic segregation. Unfortunately, too few loci were dealt with in these studies, so no conclusive evidence was obtained.

In addition to the three species reported in this paper, *P. hirsutum* and *P. montanum* were also karyotyped. Although the banding was not optimized for these two species, the preliminary evidence showed that their banding pattern seemed to be quite different from either the A or B genomes. At least one can conclude that there are more than two genomes in the genus.

While the study in this report has provided some important information on the genome constitution of *P. pratense* and its phylogenetic relationship with some wild relatives, further work on the molecular aspect is required to reinforce the initial conclusions drawn in this study. In our next study, the technique of hybridization of genome-specific probes to the chromosomes of the *Phleum* species will be used to study the phylogeny.

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Bothmer, R. von, and Ellerström, S. 1986. Some preliminary attempts to produce species hybrids in the genus *Phleum* L. *In* Svalöf 1886–1986. *Edited by* G. Olsson. LTs förlag, Stockholm. pp. 120–121.

Borrill, M. 1976. Temperate grasses. *In* Evolution of crop plants. *Edited by* N. W. Simmonds. Longman, London. pp. 137–142.

Cai, Q., and Chinnappa, C. C. 1987. Giemsa C-banded karyotypes of seven North American species of *Allium*. Am. J. Bot. **74**: 1087–1092.

Gill, B. S. 1987. Chromosome banding methods, standard chromosome band nomenclature, and applications in cytogenetic analysis. *In* Wheat and wheat improvement. Agronomy Monograph 13. 2nd ed. ASA-CSSA-SSSA, Madison. pp. 243–322.

Gregor, J. W., and Sansome, F. W. 1930. Experiments on the genetics of wild populations. I. Genet. **22**: 373–387.

Humphries, C. J. 1980. *Phleum* L. *In* Flora Europaea. Vol. 5. *Edited by* T. G. Tutin, V. H. Heywood, N. A. Burges, D. M. Moore, D. H. Valentine, S. M. Walters, and D. A. Webb. Cambridge University Press, Cambridge. pp. 239–241.

Joachimiak, A. 1981. *Phleum boehmeri* Wib. Hy-banding karyotype analysis. Acta Biol. Cracov. Ser. Bot. **13**: 147–154.

Levan, A. 1941. Syncyte formation in the pollen-mother cells of haploid *Phleum pratense*. Hereditas, **27**: 243–252.

Löidl, J. 1983. Some features of heterochromatin in wild *Allium* species. Syst. Evol. **143**: 117–131.

Müntzing, A. 1935. Cyto-genetic studies on hybrids between two *Phleum* species. Hereditas, **20**: 103–136.

Müntzing, A., and Prakken, R. 1940. The mode of chromosome pairing in *Phleum* twins with 63 chromosomes and its cytogenetic consequences. Hereditas, **26**: 463–501.

Myers, W. M. 1944. Cytological and genetic analysis of chromosomal association and behavior during meiosis in hexaploid timothy (*Phleum pratense*). J. Agric. Res. **68**: 21–33.

Nath, J. 1967. Cytogenetical and related studies in the genus *Phleum* L. Euphytica, **16**: 267–282.

Nielsen, E. L., and Smith, D. C. 1959. Chlorophyll inheritance patterns and extent of natural self pollination in timothy. Euphytica, **8**: 169–179.

Nordenskiold, H. 1941. Cytological studies in triploid *Phleum*. Bot. Not. pp. 12–32.

———. 1945. Cytogenetic studies in the genus *Phleum*. Acta Agric. Suec. **1**: 1–137.

———. 1949. Synthesis of *Phleum pratense* L. from *P. nodosum* L. Hereditas, **35**: 190–202.

———. 1953. A genetical study in the mode of segregation in hexaploid *Phleum pratense*. Hereditas, **39**: 469–488.

———. 1954. A genetical study in the mode of segregation in hexaploid *Phleum pratense*. Caryologia, **6**(Suppl.): 704–707.

Riley, R. 1965. Cytogenetics and the evolution of wheat. *In* Essays on crop plant evolution. *Edited by* J. Hutchinson. Cambridge University Press, Cambridge. pp. 103–122.

Stebbins, G. L., Jr. 1950. Variation and evolution in plants. New Columbia University Press, New York.

Teppner, H. 1980. Karyologie und Systematik einiger Gefäßpflanzen der Ostalpen. Botanische Studien im Gebiet der Planneralm (Niedere Tauern, Steiermark). VII. Phyton (Horn, Austria), **20**: 73–94.

Waines, J. G., and Kimber, G. 1973. Satellite number and size in *Triticum monococcum* L. and the evolution of the polyploid wheats. Can. J. Genet. Cytol. **15**: 117–122.

Wexelsen, H. 1941. Chlorophyll deficient seedlings in timothy (*Phleum pratense* L.). J. Hered. **32**: 227–231.

Wilton, A. C., and Kiebesadel, L. J. 1973. Karyology and phylogenetic relationships of *Phleum pratense*, *P. commutatum*, and *P. bertolonii*. Crop Sci. **13**: 663–665.