A survey of restriction fragment length polymorphisms in tall fescue and its relatives[1]

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Restriction fragment length polymorphisms (RFLPs) have several advantages over conventional genetic markers and as a result have received increased attention from plant breeders and geneticists. The objective of this study was to construct a tall fescue (*Festuca arundinacea* Schreb.) genomic library and to survey RFLPs in tall fescue and its relatives. Using plasmid pUC19 as a vector and *Escherichia coli* XL1-Blue cells as hosts, the first reported *Pst*I genomic DNA library has been established from hexaploid ($2n = 6x = 42$) tall fescue. The genomic clones were evaluated using nine genotypes from three species of *Festuca* and three restriction enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII). One hundred and seventy-four probes gave readable results, of which 21% were repetitive and 79% single-copy. The single-copy probes revealed good polymorphism in tall fescue. Approximately 21% of the probes did not cross hybridize to any of the diploids or tetraploids or both and, therefore, represented genome-specific clones.

Key words: tall fescue, genome-specific probes, genomic library, polyploids, genomic relationships

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Le polymorphisme des longueurs de fragments de restriction présente plusieurs avantages sur les marqueurs génétiques conventionnels et, par voie de conséquence, il a reçu une attention accrue des améliorateurs de plantes et des généticiens. Le but de la présente étude a été de constituer une banque génomique de la fétuque élevée (*Festuca arundinacea* Schreb.) et d'investiguer le polymorphisme des longueurs de fragments de restriction (PLFR) chez cette espèce et les génotypes d'espèces apparentées. Utilisant le plasmide pUC19 comme vecteur et des cellules de l'*Escherichia coli* XL1-Blue comme hôtes, la première banque d'ADN génomique *Pst*I rapportée a été établie à partir d'une fétuque élevée hexaploïde ($2n = 6x = 42$). Les clones génomiques ont été évalués à l'aide de neuf génotypes dérivés de trois espèces de *Festuca* et trois enzymes de restriction (*Bam*HI, *Eco*RI et *Hin*dIII). Cent soixante-quatorze sondes ont donné des résultats lisibles, desquels 21% étaient des répétitions et 79% des copies individuelles. Ces dernières ont révélé l'existence d'un bon polymorphisme chez la fétuque élevée. Environ 21% des sondes ne se sont pas hybridé à aucune des plantes diploïdes ou tétraploïdes ou les deux; elles représentent donc des clones spécifiques à un génome.

Mots clés : fétuque élevée, sondes spécifiques à un génome, banque génomique, polyploïdes, relations génomiques.

[Traduit par la rédaction]

Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is one of the major cool-season forage grasses produced in the United States. It is a perennial hexaploid ($2n = 6x = 42$) and belongs to the genus *Festuca*, which includes approximately 80 species. It is native to the temperate and cool climates throughout Europe, North Africa, and west and central Asia and Siberia (Terrell 1979). Tall fescue has been cultivated in the United States for less than 100 years. Use of cultivars such as 'Kentucky-31' has expanded livestock production in the transition zone of the United States since the 1940s (Sleper 1985). Development of new cultivars is dependent upon knowledge of genetics and genetic relationships of germ plasm. The polyploid, open-pollinated, and usually self-incompatible features of tall fescue hamper genetic studies.

Seal (1983) estimated the DNA contents of Feulgen-stained nuclei of tall fescue by microdensitometry. The C-value (DNA content of the unreplicated haploid genome) was 1.98 pg for the diploid ($2n = 2x = 14$) meadow fescue (*Festuca pratensis* Huds.) and 5.27–5.83 pg for tall fescue. From these results, the genome size of *F. pratensis* is approximately 1.98×10^6 kb and that of *F. arundinacea* Schreb. is approximately $5.27–5.83 \times 10^6$ kb.

Molecular markers, especially restriction fragment length polymorphisms (RFLPs), have received increased attention from plant geneticists and breeders. RFLPs are different fragment lengths of restriction endonuclease digested DNA detected by a defined probe between individuals. They can be used as genetic markers and possess several advantages over conventional genetic markers including being universal, abundant, stable, and convenient. RFLPs are expected to show Mendelian codominant inheritance and minimal pleiotropic effects, and are detectable in all living tissues and at all stages of development (Helentjaris 1987; Beckmann and Soller 1986).

RFLPs are therefore useful in genetic mapping, germplasm evaluation, cultivar identification, and breeding programs as indirect selection criteria (Beckmann and Soller 1986). Work with RFLP analysis has been reported in crops such as maize (*Zea mays* L.) (Helentjaris 1987; Hoisington

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TABLE 1. Genotypes used in library evaluation and their genomes

No.	Genotype	Species	$2n$
1	PI 237707	*F. pratensis* Huds.	14
2	PI 311046	*F. pratensis* Huds.	14
3	D1334-1	*F. pratensis* Huds.	14
4	Bn354-2	*F. arundinacea* var. *glaucescens* Boiss.	28
5	Bn354-5	*F. arundinacea* var. *glaucescens* Boiss.	28
6	Bn574-5	*F. arundinacea* var. *glaucescens* Boiss.	28
7	'Kentucky-31'	*F. arundinacea* var. *genuina* Schreb.	42
8	'Martin'	*F. arundinacea* var. *genuina* Schreb.	42
9	'Mozark'	*F. arundinacea* var. *genuina* Schreb.	42

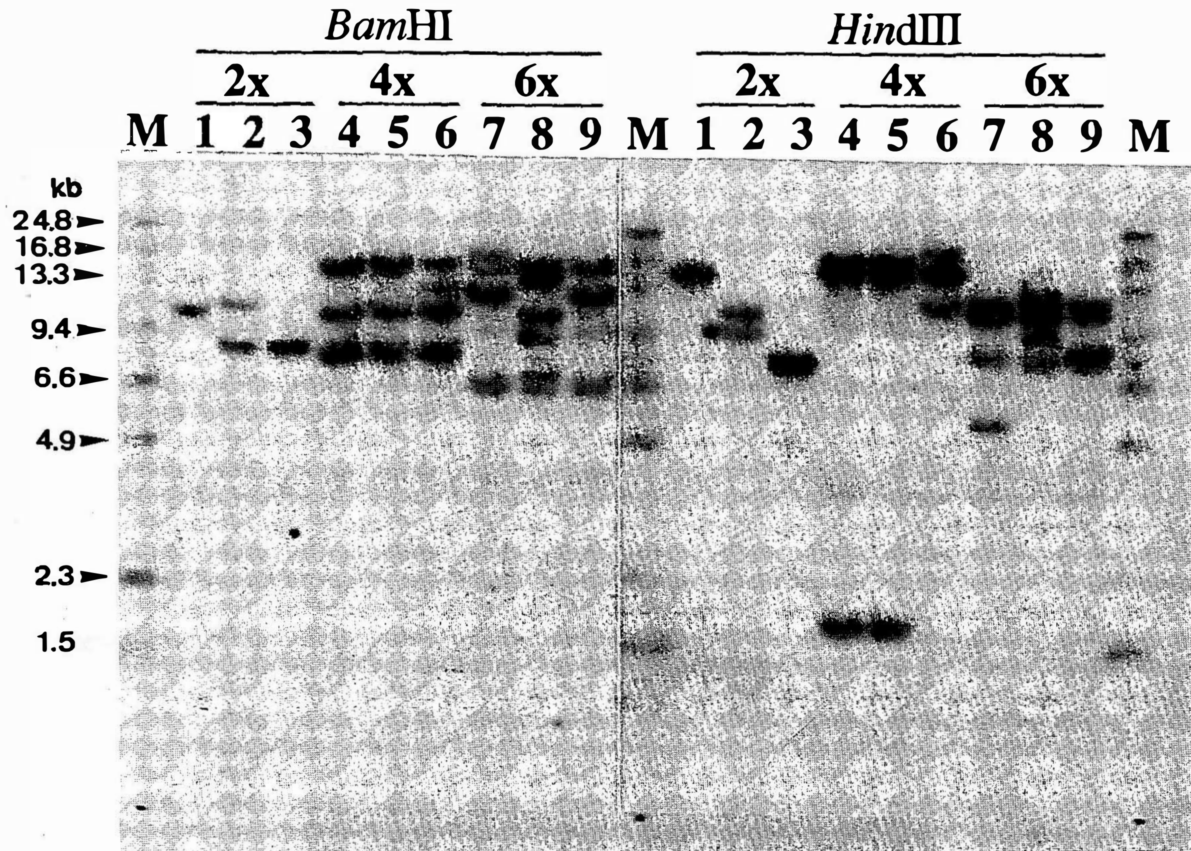

FIG. 1. Autoradiogram from hybridization with clone TF531 to DNA from nine genotypes digested by enzymes *Bam*HI and *Hin*dIII. Note the high polymorphism among genotypes. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1-3, diploid ($2x$); 4-6, tetraploid ($4x$); 7-9, hexaploid ($6x$). Exposed for 12 h at −80°C.

and Coe 1989), lettuce (*Lactuca sativa* L.) (Landry *et al.* 1987), and rice (*Oryza sativa* L.) (McCouch *et al.* 1988). It is hoped that RFLPs of tall fescue may provide new information useful for breeding and genetic investigations.

The objective of this research was to construct a tall fescue *Pst*I genomic library and to survey RFLPs in tall fescue and its relatives.

Materials and methods

Plant genotypes

Three genotypes (i.e., three individual plants) from each of three ploidy levels for a total of nine different genotypes were used in this study (Table 1). The ploidy levels examined were diploid ($2n = 2x = 14$) *F. pratensis*, tetraploid ($2n = 4x = 28$) *F. arundinacea* var. *glaucescens*, and hexaploid ($2n = 6x = 42$) *F. arundinacea* var. *genuina*.

Extraction of plant genomic DNA

Genomic DNA was extracted from lyophilized ground leaf tissues with CTAB buffer (0.1 M Tris at pH 7.5, 0.7 M NaCl, 0.01 M EDTA at pH 8.0, 0.14 M β-mercaptoethanol, and 0.03 M mixed alkytrimethylammonium bromide) (Saghai-Mahoof *et al.* 1984) and purified with 1 volume phenol and 1 volume of chloroform–octanol (24:1).

Construction of genomic DNA library

Purified genomic DNA from a hexaploid tall fescue clonal population was digested with *Pst*I (3.5 units/μg DNA) for 4 h, and fractionated using a 10–40% sucrose gradient by centrifuging at 20°C and 25 000 rpm for 20 h in a Beckman L8-M ultracentrifuge. The 1- to 2-kb fractions were precipitated, dissolved in distilled H_2O, and used for ligation. The library was constructed in pUC19 and *E. coli* XL-Blue (Stratagene) as host. The ligation was performed using T4 DNA ligase at 14°C overnight. Before ligation the vector DNA was digested with *Pst*I and dephosphorylated with

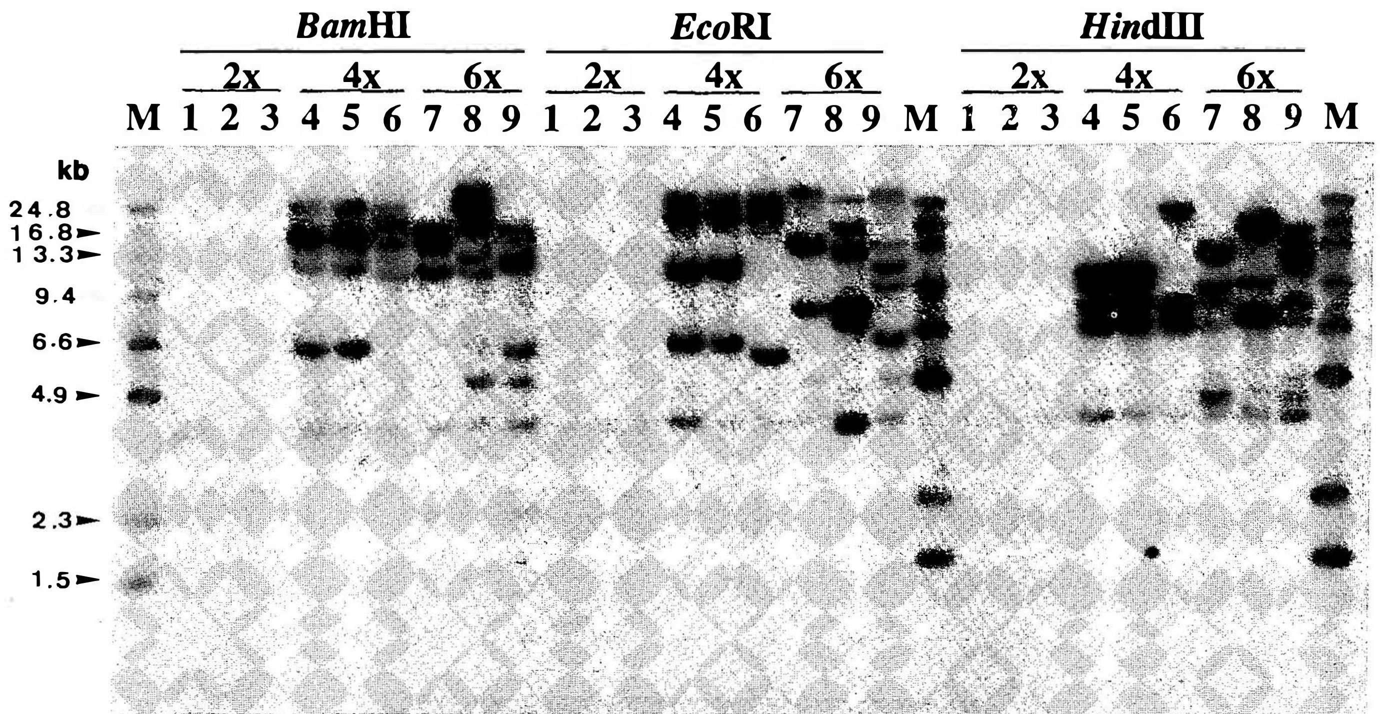

FIG. 2. Clone TF515 only hybridized to DNA of *F. arundinacea* var. *glaucescens* Boiss. and *F. arundinacea* var. *genuina* Schreb., indicating that *F. pratensis* Huds. lacks the homologous DNA sequence to clone TF515. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1–3, diploid (2*x*); 4–6, tetraploid (4*x*); 7–9, hexaploid (6*x*). Exposed for 12 h at −80°C.

TABLE 2. Probe-screening results of clones giving readable blots

				Probes not hybridized to ploidies		
	Total	Repetitive	Single copy	2*x*	4*x*	2*x* and 4*x*
No.	174	36	138	31	1	4
%		20.7	79.3	17.8	0.6	2.3

NOTE: 2*x*, diploid; 4*x*, tetraploid; 6*x*, hexaploid.

calf intestinal alkaline phosphatase (Boehringer Mannheim). Ligation and transformation were based on the manufacture's procedures. The transformed cells were plated on media containing ampicillin, X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside), and ITPG (isopropylthio-β-galactosidase) (Sigma).

Restriction digestion, electrophoresis, and Southern blotting

Samples of genomic DNA were restricted separately with three enzymes (*Bam*HI, *Eco*RI, and *Hin*dIII). Digested DNA (10 or 15 μg/lane) was subjected to electrophoresis through a 0.7% agarose gel. The gel was denatured for 30 min in 0.4 M NaOH and 0.6 M NaCl, and neutralized for 30 min in 0.5 M Tris-HCl at pH 7.5 and 1.5 M NaCl solution. DNA was then Southern blotted to a nylon membrane (Micron Separations Inc.) in 25 mM $NaPO_4$ buffer, pH 6.5. The damp filters were then treated for 120 000 μJ with a Stratagene UVlinker™1800 and baked at 92°C for 2 h.

Probe preparation and DNA hybridization

Clonal plasmid DNA was isolated from transformed bacteria according to Birnboim (1983). Inserts were isolated by digesting plasmid DNA with *Pst*I and running through 1.1% low-melting agarose gel. The insert was excised from the gel and labelled to high specific activity with α-^{32}P dCTP (New England Nuclear) (Feinberg and Vogelstein 1984). Hybridization was based on the method of Helentjaris *et al.* (1985). The filter was prehybridized with 0.17 mL/cm^2 of prehybridization solution (5× SSC (1× SSC: 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's, 0.05 M Tris-HCl pH 8.0, 0.2% SDS, 1 mg/mL denatured salmon sperm (SS) DNA) for approximately 5 h at 65°C and then hybridized with 0.014 mL/cm^2 of the hybridization solution (10% dextran sulfate, 5× SSC, 1× Denhardt's, 0.2% SDS, 0.05 M Tris-HCl pH 8.0, 0.01 M EDTA, 0.1 mg/mL SS DNA) overnight at 65°C. The filters were washed in 2× SSC, 0.5% SDS solution and 0.1× SSC, 0.1% SDS solution at room temperature for 10 min, and then in 0.1× SSC, 0.1% SDS solution at 65°C for 2 h. The autoradiograms were obtained by exposing X-ray film (Kodak X-OMAT™AR) with one intensifying screen for up to 5 days at −80°C.

Probe nomenclature

Clones were identified with the prefix TF and numbers following TF referred to the sequence in which recombinant plasmids were isolated.

Results and discussion

Genomic clone evaluation

A *Pst*I genomic DNA library consisting of 700 clones was constructed from hexaploid tall fescue. The genomic clones were randomly chosen and hybridized to nine genotypes. These genotypes belong to three species of the genus *Festuca* with three different ploidy levels (Table 1). Most of the genomic clones from the hexaploid readily cross hybridized to genomic DNA of the diploids and tetraploids (Fig. 1); however, some did not (Fig. 2). A clone was considered to have repetitive sequences when it detected three or more bands in diploids (usually very complicated); otherwise it was considered as single or low copy. So far 174 clones have been evaluated and gave readable results. Among them, 36 (21%) were found to be repetitive, and 138 (79%) were single or low copy (Table 2). Genomic libraries contain clones from both coding and noncoding sequences of the original cellular DNA and many genomic clones contain

TABLE 3. Degree of polymorphism detected between genotypes within a species or between species

	Diploid (2x)			Tetraploid (4x)			Hexaploid (6x)			Between species		
	1 vs. 2	1 vs. 3	2 vs. 3	4 vs. 5	4 vs. 6	5 vs. 6	7 vs. 8	7 vs. 9	8 vs. 9	2x vs. 4x	2x vs. 6x	4x vs. 6x
*Bam*HI	25.8	34.1	28.7	4.7	57.8	61.9	79.5	78.8	54.2	88.1	92.5	91.1
Mean		29.5			41.5			70.8			90.6	
*Eco*RI	26.9	38.0	37.7	10.1	59.7	59.8	79.8	81.5	54.3	93.0	93.2	93.2
Mean		34.2			43.2			71.9			93.1	
*Hin*dIII	24.6	36.4	32.1	8.7	56.0	57.9	75.4	72.9	52.3	85.3	90.9	89.4
Mean		31.0			40.9			66.9			88.5	
Grand mean	25.8	36.2	32.5	7.8	57.8	59.9	78.2	77.7	53.6	88.8	92.2	91.2

TABLE 4. Ratios of bands shared among three ploidy levels

	Ploidy	Total no. of bands	Bands present in higher ploidy (%) 4x	6x	Average no. of bands detected by an enzyme–probe combination
*Bam*HI	2x	183	35.1	52.5	1.3
	4x	352	—	60.8	2.6
*Eco*RI	2x	179	30.1	45.8	1.3
	4x	339	—	54.9	2.5
*Hin*dIII	2x	170	29.4	50.0	1.2
	4x	350	—	59.7	2.5

repetitive sequences (Sutherland and Mulley 1989). Complete genomic libraries are usually not a very efficient source of clones used in RFLP mapping (Murray *et al.* 1989); therefore, cDNA libraries or *Pst*I genomic libraries have been used to enrich single-copy clones. cDNA libraries, constructed from mRNAs isolated from a given tissue, represent only the coding sequences that are transcribed in that tissue. Most of the cDNA clones contain single copy, based on the fact that most functional genes are present in low copy number in higher eukaryotes (Goldberg *et al.* 1973). Construction of *Pst*I genomic libraries is another way to enrich low-copy sequences. Cytosine residues in plant repetitive sequences are frequently methylated. By using the C methylation sensitive restriction enzyme *Pst*I, and the methylation insensitive enzyme *Eco*RI, two libraries were constructed in tomato with inserts having the same sizes (1.5–3.0 kb). Approximately 92% of the clones from the *Pst*I library were single copy, whereas 36% of the *Eco*RI clones were single copy (Tanksley *et al.* 1988). Our results also showed that the *Pst*I genomic library was efficient in providing single- or low-copy fragments in tall fescue.

Degree of polymorphism

Genomic clones identified considerable amounts of polymorphism among tall fescue accessions. Many clones distinguished most of the cultivars used, as shown in the autoradiogram of clone TF531 (Fig. 1). The level of polymorphism was calculated using pairwise comparisons of the banding patterns obtained from the 138 single-copy clones. Each band was given a letter (regardless of signal dosages). A pairwise comparison was done on the hybridization patterns for each probe–enzyme combination. When comparing genotypes within a species, the two genotypes compared were considered to be different if they had at least one different band. For the comparison among species, the two species were considered different if one genotype in a species showed a different band from a genotype in another species. The percentage of pairwise comparisons with different hybridization patterns over the total number of comparisons was used as an indicator for the level of polymorphism. It was shown that hexaploids provided more polymorphism than tetraploids, which in turn exhibited more variation than diploids. Among diploids, an average of 29.5, 34.2, and 31.0% of the polymorphism was detected by *Bam*HI, *Eco*RI, and *Hin*dIII, respectively; 41.5, 43.2, and 40.9% for 4x and 70.8, 71.9, and 66.9% for the 6x species. The three enzymes used showed similar trends (Table 3). The diploid had the least amount of polymorphism. This may be due to the diploid genotypes being closely related or having low amounts of genetic variation. The low polymorphism in the diploid will make it difficult to do RFLP mapping in this species.

The 138 single-copy probes gave a total of 183 and 352 different bands in 2x and 4x digested with enzyme *Bam*HI, 179 and 339 with *Eco*RI, and 170 and 350 with *Hin*dIII, respectively (Table 4). For enzyme *Bam*HI, 35.1 and 52.6% of the bands in the 2x were also shown in the 4x and 6x, respectively; while 60.8% of the bands in the 4x existed in the 6x. *Eco*RI and *Hin*dIII gave similar results. Each enzyme–probe combination gave an average of 1.3 and 2.6 different bands in the 2x and 4x, respectively. Data in Table 4 imply that the P genome may be closely related to G_1 or G_2 and suggest that this relationship needs further study.

Extent of genetic relatedness among genotypes

The genetic relatedness among genotypes can be estimated by RFLP analysis. This was expressed in two instances from our data. First, among hexaploids, 78.2 and 77.7% hybridization patterns were polymorphic between genotypes

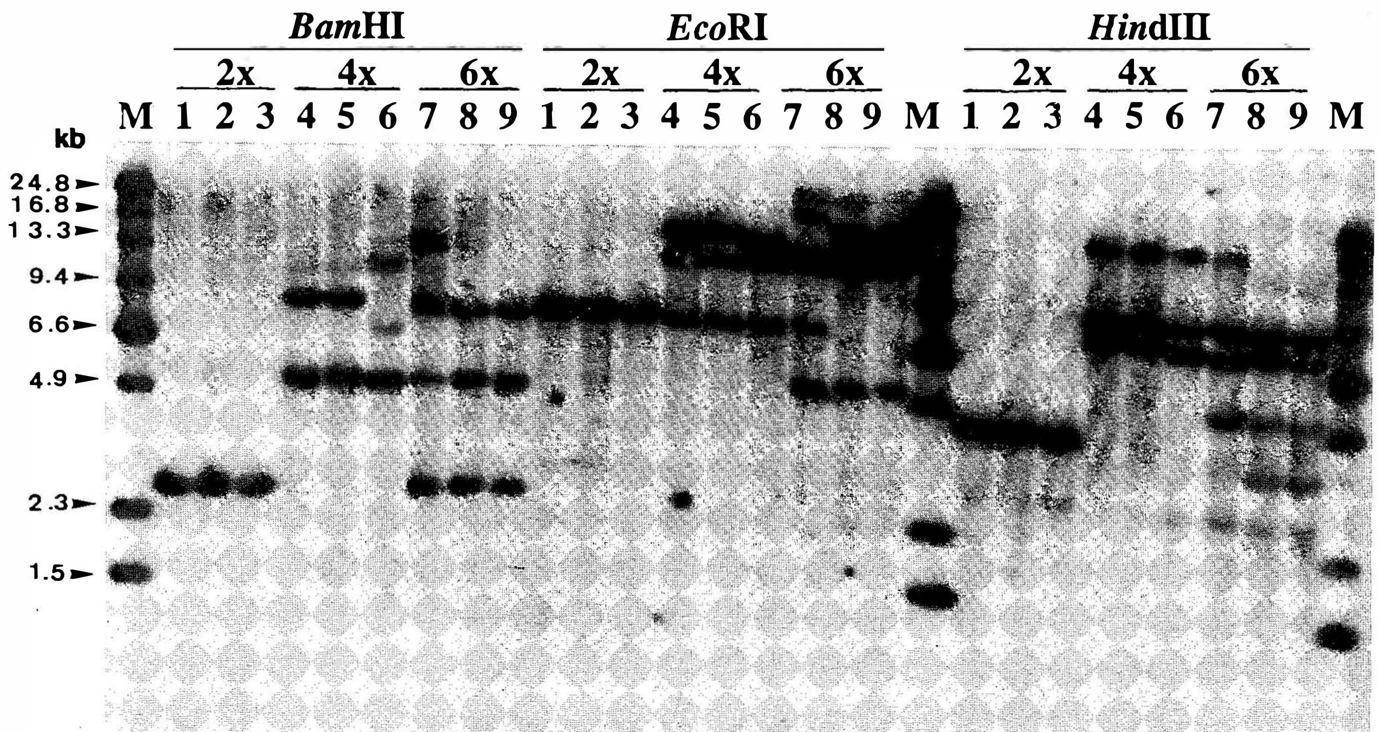

FIG. 3. Autoradiogram of clone TF171 probed onto DNA of nine genotypes digested by enzymes *Bam*HI, *Eco*RI, and *Hin*dIII. Note the different banding patterns in three species. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1–3, diploid (2*x*); 4–6, tetraploid (4*x*); 7–9, hexaploid (6*x*). Exposed for 48 h at −80°C.

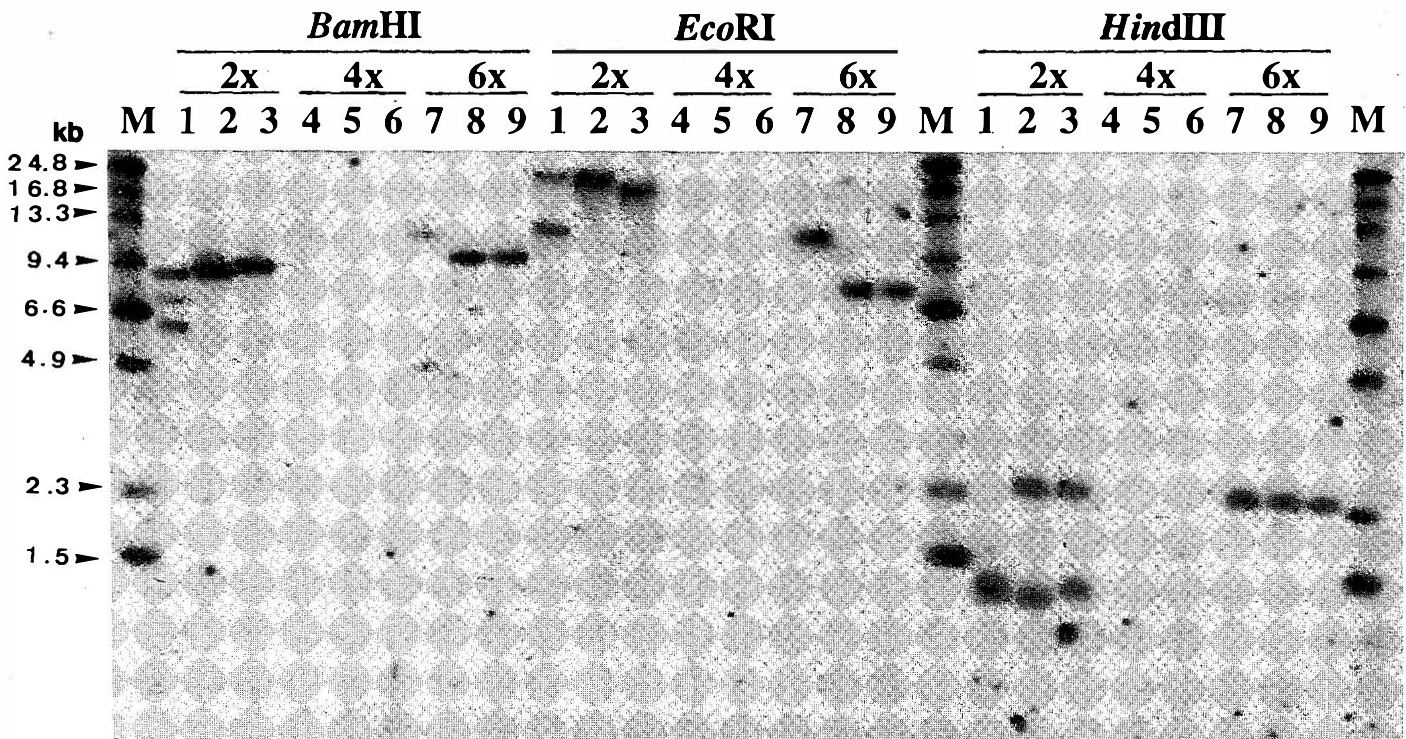

FIG. 4. Clone TF153 did not hybridize to DNA of *F. arundinacea* var. *glaucescens* Boiss. (4*x*) genotypes. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1–3, diploid (2*x*); 4–6, tetraploid (4*x*); 7–9, hexaploid (6*x*). Exposed for 63 h at −80°C.

from cultivars 'Kentucky-31' and 'Martin' and between 'Kentucky-31' and 'Mozark', but only 53.6% were polymorphic between genotypes of 'Martin' and 'Mozark' (Table 3). 'Martin' and 'Mozark' tall fescue cultivars were originally developed at the University of Missouri—Columbia from two different broad-based populations but were not related to the cultivar 'Kentucky-31'. Two 4*x* genotypes, Bn354-2 and Bn354-5, showed the lowest polymorphism, ranging from 4.7% for *Bam*HI to 8.7% for *Hin*dIII. These two genotypes were from the same half-sib family. Second, genotypes within a species showed more similar banding patterns than genotypes from different species. The three genotypes of one species were clearly distinguished from those of the other species by the hybridization patterns, as

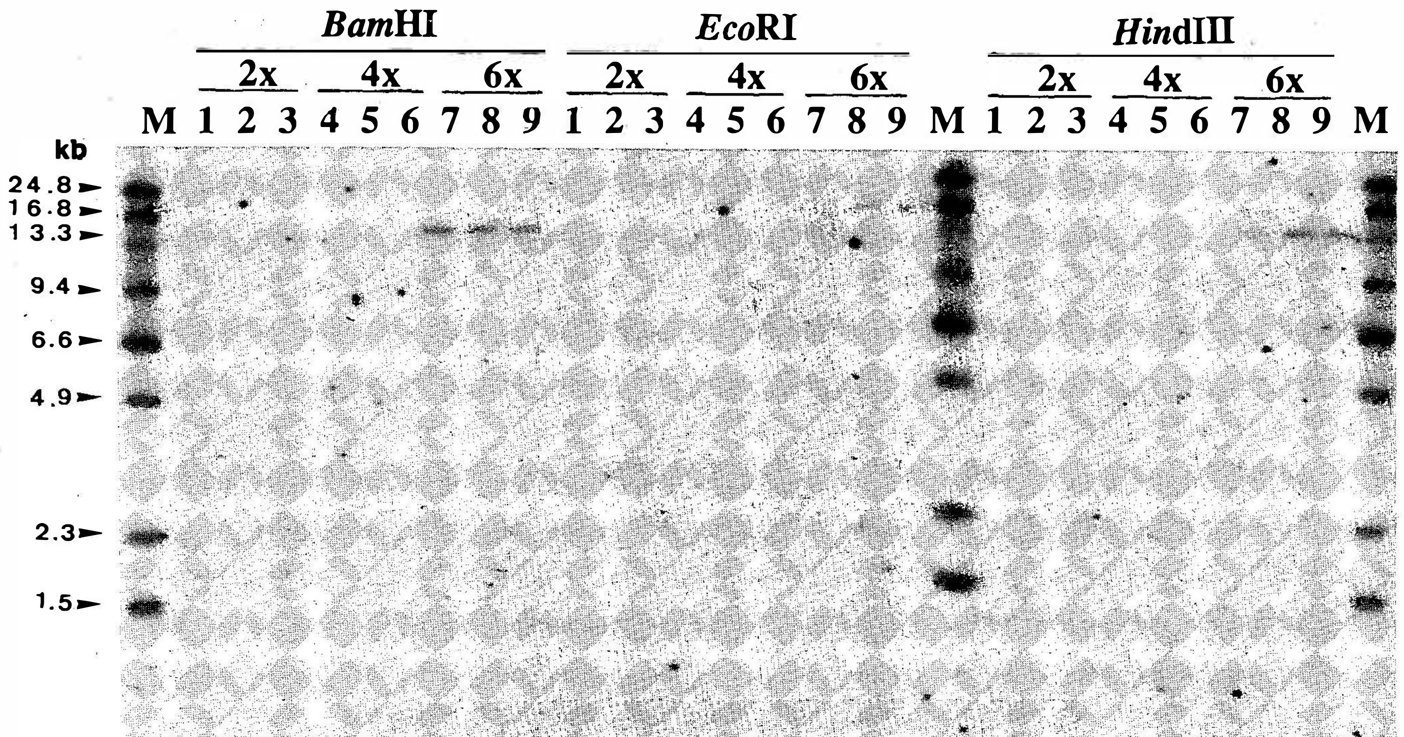

FIG. 5. Autoradiogram of clone TF173 probed onto DNA of nine genotypes digested by enzymes *Bam*HI, *Eco*RI, and *Hin*dIII. TF173 only hybridized to the hexaploid species. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1–3, diploid (2*x*); 4–6, tetraploid (4*x*); 7–9, hexaploid (6*x*). Exposed for 53 h at −80°C.

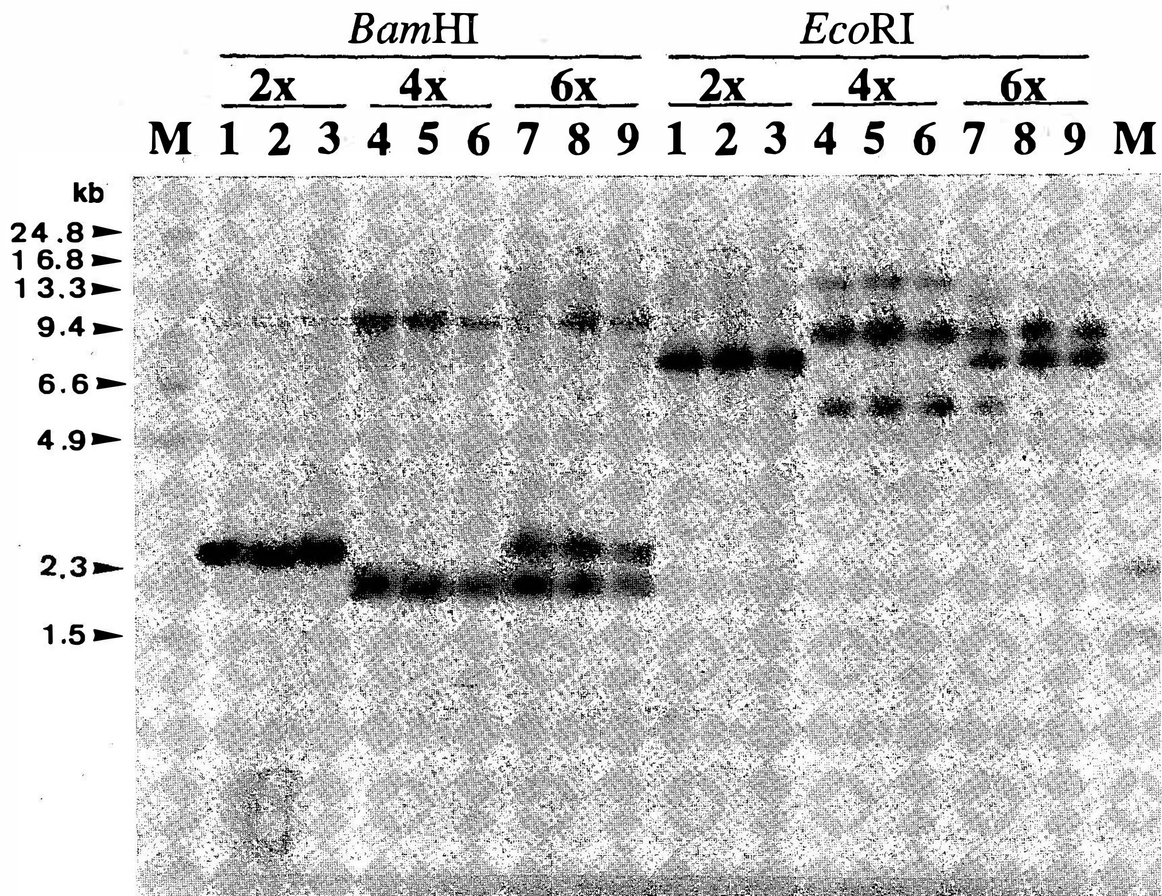

FIG. 6. Autoradiogram of clone TF524 probed onto DNA of nine genotypes digested by enzymes *Bam*HI and *Eco*RI. Note that *F. arundinacea* var. *genuina* Schreb. has the marked fragments unique either in *F. pratensis* Huds. or in *F. arundinacea* var. *glaucescens* Boiss. These unique bands were not polymorphic within a species. Lane M is the molecular mass marker, with size marked at the left of the first lane. Genotypes 1–3, diploid (2*x*); 4–6, tetraploid (4*x*); 7–9, hexaploid (6*x*). Exposed for 25 h at −80°C.

shown in the autoradiogram of clone TF171 (Fig. 3). Approximately 90% of the hybridization patterns were different among the three species (Table 3).

Genome-specific probes

Of the 174 probes, 37 (21.3%) did not hybridize to all three genotypes of either 2*x* or 4*x* or both species and seemed to be genome specific. Thirty-one (17.8%), such as TF515, did not hybridize to any genotypes of the 2*x* species (Fig. 2); one (TF153) did not hybridize to any of the 4*x* species (Fig. 4); and four similar to TF173 did not hybridize to either the 2*x* or 4*x* species (Fig. 5). From cytological and morphological studies, a phylogenetic relationship among tall fescue and its relatives has been proposed such that *F. pratensis* and *F. arundinacea* var. *glaucescens* are progenitors of the 6*x* species *F. arundinacea*, which has genomes $PPG_1G_1G_2G_2$, with the P genome from 2*x F. pratensis* and the G_1 and G_2 genomes from the 4*x F. arundinacea* var. *glaucescens* (Sleper 1985). Assuming the above genomic relationships are accurate, probes that do not hybridize to the 2*x* species should be specific to the G_1 or G_2 genomes. Those that do not hybridize with the 4*x* species should be specific to the P genome. Our results support this proposed phylogenetic theory. Clones such as TF524 showed shared bands that were present in the 2*x*, 4*x*, and 6*x* species (Fig. 6). When clone TF524 was probed to the DNA digested with *Bam*HI, the 2.5-kb fragment was present in the 2*x* and 6*x* but not in the 4*x*; the 2.0- and 10-kb fragments were present in the 4*x* and 6*x* but not in the 2*x*. The TF524-*Eco*RI combination also showed similar results. In most cases, these clones were not polymorphic within a species. These clones may be useful in the study of genomic relationships.

Results from our RFLP studies are encouraging. We are evaluating more probes, developing an RFLP linkage map, and looking for RFLP loci linked to important agronomic traits useful as selection criteria in a plant breeding program. Recurrent selection is thought to increase the frequency of desirable alleles and decrease the frequency of undesirable alleles in a population. It is expected that the frequency of an RFLP locus will be altered if the RFLP locus is linked to a selected trait locus. Therefore, one could find RFLP loci related to the morphological and quality traits selected by detecting allelic frequency changes. Because the heritability of an RFLP is 1, an RFLP locus closely linked to the trait selected may enable the plant breeder to use that RFLP locus (or loci) as an indirect selection tool(s) or marker(s) in a breeding program. By identifying desirable plants early in the selection process, the breeding cycle may be shortened. Perhaps greater emphasis can then be placed on other important agronomic traits and the breeding efficiency increased.

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