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Use of genome-specific repetitive DNA sequences to monitor chromatin introgression from *Festuca mairei* into *Lolium perenne*

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Abstract Repetitive DNA sequences contribute considerably to an understanding of the genomes of higher plants. Repetitive DNA sequences tend to be genome-specific due to the rate of amplification and extent of divergence. Two genome-specific probes from the genomic DNA library of *Festuca arundinacea* var. *genuina* Schreb. were selected and characterized. TF521 was found to be P genome-specific since it was able to hybridize with *Festuca pratensis* Huds. (PP) and *Festuca arundinacea* var. *genuina* ($PPG_1G_1G_2G_2$), but not, or only weakly, with tetraploid *Festuca* species. TF521 hybridized only with the diploid *Festuca* and not with the *Lolium* species (LL). TF436 was specific to tetraploid species of *Festuca*, such as *F. arundinacea* var. *glaucescens* Boiss. ($G_1G_1G_2G_2$) and *Festuca mairei* St. Yves ($M_1M_1M_2M_2$). By means of Southern hybridization, TF436 was used to detect chromatin introgression of *F. mairei* in the progenies of the hybrid *F. mairei*×*Lolium perenne* L. Potential addition and translocation lines were identified in the BC_1F_1 derivatives of *F. mairei*×*L. perenne*. In situ hybridization was used to confirm the genetic identity of these lines. Sequence analyses indicated that TF436 and TF521 were two novel DNA sequences as no homologous sequences were found in Genebank.

Keywords *Festuca* · *Lolium* · Repetitive DNA sequences · In situ hybridization

Introduction

Repetitive DNA sequences contribute considerably to our understanding of the evolution of plant genomes. The rapid amplification and divergence of these sequences have had a substantial impact on speciation (Bedbrook et al. 1980a; Flavell 1980). The repetitive DNA sequences include tandem and dispersed repeats. Tandem repeats consist of short repeat units that are arranged in tandem and clustered in specific chromosome regions, such as the centromeres (Harrison and Heslop-Harrison 1995) and telomeres (Bedbrook et al. 1980b). Dispersed repeats are widely dispersed throughout plant genomes (Heslop-Harrison 1991).

The molecular characterization of repetitive DNA sequences provides further insights into the organization and evolution of plant genomes. Many types of repetitive DNA sequences exist. Microsatellites may have only 2–4-bp repeat motifs, while other repeat units, such as rDNA, may be 10 kbp long. Species-specific repetitive sequences, with variable lengths of the repeat unit, have been extensively documented (Appels et al. 1981; Ganal et al. 1988; Zhao et al. 1989; Iwabuchi et al. 1991; Perez-Vicente et al. 1992; Anamthawat-Jonsson and eslop-Harrison 1993; Li et al. 1995). Repetitive DNA sequences represent at least 20% and sometimes more than 90% of the total genome, but the function of most repetitive sequences seems to be complex and difficult to elucidate.

Intergeneric hybridization involving *Festuca* and *Lolium* has been extensively studied (Thomas and Humphreys 1991; Jauhar 1993). With the goal of introducing persistence and drought resistance from *Festuca mairei* St.Yves (2n=4*x*=28, $M_1M_1M_2M_2$) into *Lolium perenne* L. (2n=2*x*=14, LL), initial crosses were made (Chen et al. 1995) and the genomic relationships between the two species were studied (Cao et al. 2000). The RFLP markers from the *Pst*I genomic library of hexaploid *Festuca arundinacea* var. *genuina* Schreb. have been used to trace the introgression of *F. mairei* chromatin into *L. perenne* (Chen and Sleper 1999). Compared with unique and low-copy DNA sequences, repetitive DNA sequences are more variable and tend to be more species-specific. Although the function of repetitive sequences remains largely unsolved, genome-specif-

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ic repetitive sequences have been used as molecular markers to trace alien chromatin introgression into cultivated crops (Schwarzacher et al. 1992). Similar investigations, however, have seldom been undertaken with *Lolium* and *Festuca* species. Several repetitive *Lolium multiflorum* Lam. and *F. arundinacea* specific DNA sequences have been isolated by Perez-Vicente et al. (1992). These sequences were used in dot analyses to study the genomic makeup of *Festulolium* hybrids.

Twenty one percent of the clones were found to be repetitive in the *Pst*I genomic DNA library of hexaploid *F. arundinacea* (Xu et al. 1991). In this report, two genome-specific repetitive sequences (TF436 and TF521) were characterized and the tetraploid *Festuca*-specific sequence TF436 was used to detect alien chromatin introgression from *F. mairei* in the progenies of the hybrid *F. mairei*×*L. perenne*.

Material and methods

Plant materials

Two genotypes of each ploidy level of *Festuca* and *Lolium* were used in this study (Table 1). The ploidy levels examined include the diploids *F. pratensis* (2n=2*x*=14) and *L. perenne* (2n=2*x*=14), the tetraploids *F. arundinacea* var. *glaucescens* (2n=4*x*=28) and *F. mairei* (2n=4*x*=28), and the hexaploid *F. arundinacea* (2n=6*x*=42). All plant materials were maintained in the greenhouse at the University of Missouri-Columbia.

DNA isolation and Southern blotting

RFLP procedures, such as plant DNA extraction, restriction digestion, blotting and Southern hybridization, were conducted as described by Xu et al. (1991). Probes were prepared by mini-preparation and radioactively oligo-labeled with α-^{32}p-dCTP (NEC). Both plasmid DNA and polymerase chain reaction (PCR) products were suitable for oligolabeling. After hybridization, washes were conducted as follows: (1) 2×SSC, 0.5% SDS at room temperature (RT) for 10 min, (2) 0.1×SSC, 0.1% SDS at RT for 10 min, and (3) 0.1×SSC, 0.1% SDS at 65°C from at least 30 min up to 2 h. The autoradiograms were obtained by exposing X-ray film (Kodak X-OMAT AR).

DNA sequencing and analysis

Automated cycle sequencing of TF436 and TF521 using an ABI model 377 sequencer were accomplished at the DNA Core Facility, University of Missouri-Columbia. The forward and reverse M13 sequencing primers were used to sequence both strands of the TF436 and TF521 DNA clones. Alignment of the sequences and further analyses were conducted using the DNASTAR program and GCG version 9.1 (Genetics Computer Group, Madison, Wis.). BLASTN (version 2.0.2) was accessed through the NCBI (The National Center for Biotechnology Information Bethesda, Md.) and the DDBJ (DNA Data Bank of Japan) web server, to search for homologs in the databases.

Chromosome preparation and in situ hybridization

Chromosome preparations and in situ hybridization protocols were conducted as previously described (Cao et al. 2000).

Table 1 Plant materials used for repetitive probe screening

No.	Genotype	Species	2n[a]
1	Citation II	*L. perenne*	14
2	Calypso	*L. perenne*	14
3	Stella	*F. pratensis*	14
4	303.1–1	*F. pratensis*	14
5	Bn354–2	*F. arundinacea* var. *glaucescens*	28
6	Bn586–86–2	*F. arundinacea* var. *glaucescens*	28
7	PI 283313–2	*F. mairei*	28
8	F. mairei#2	*F. mairei*	28
9	Kentucky-31	*F. arundinacea* var. *genuina*	42
10	HD28–56	*F. arundinacea* var. *genuina*	42

[a] Somatic chromosome number

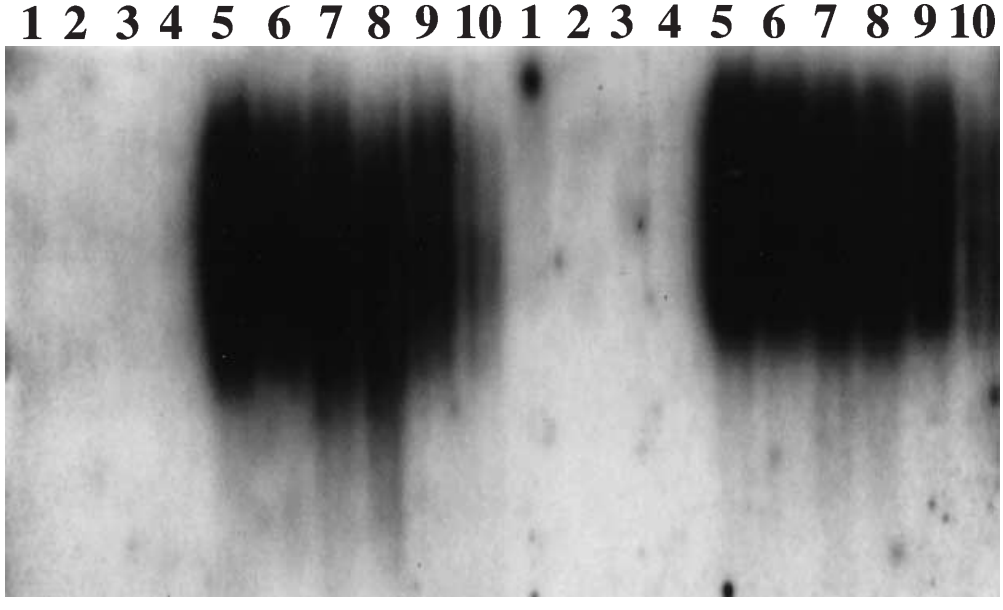

Fig. 1 Southern hybridizations of the *Hin*dIII and *Eco*RI-digested genomic DNA were probed with repetitive sequence TF436. Plant materials 1–10 are listed in Table 1

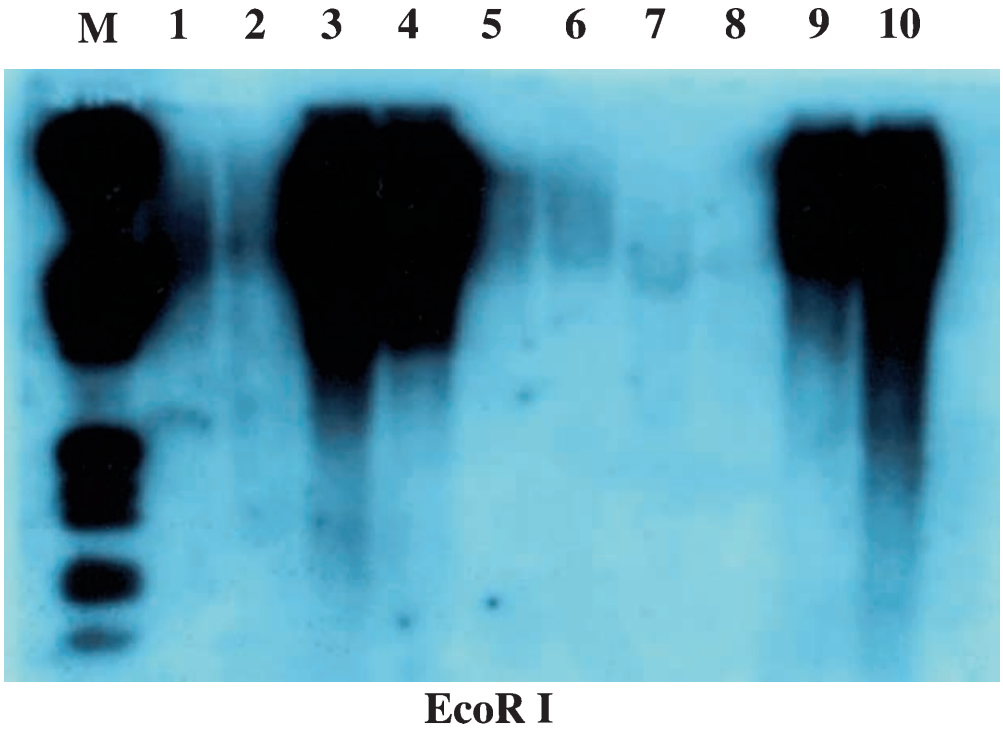

Fig. 2 Southern hybridization of the *Eco*RI-digested genomic DNA was probed with repetitive sequence TF521. Plant materials 1–10 are listed in Table 1. *M* is a λDNA *Hin*dIII molecular-weight marker

Results and discussion

Distribution of TF436 and TF521 in *Festuca-Lolium* species

TF436 and TF521 are two repetitive sequences derived from a hexaploid *F. arundinacea Pst*I genomic library (Xu and Sleper 1994). TF436 hybridized with the tetraploids *F. arundinacea* var. *glaucescens* ($G_1G_1G_2G_2$) and *F. mairei* ($M_1M_1M_2M_2$) as well as the hexaploid *F. arundinacea*, but not with the diploids *F. pratensis* (PP) and *L. perenne* (LL) (Fig. 1). The genomes of *F. pratensis* (PP) and *F. arundincea* var. *glaucescens* ($G_1G_1G_2G_2$) are believed to be the donors of the hexaploid *F. arundinacea* ($PPG_1G_1G_2G_2$) (Sleper 1985). RFLP hybridization patterns clearly showed that TF436 was specific to tetraploid *Festuca* species with no signals, or only relatively weak signals, found in diploid *Festuca* species. By contrast, TF521 hybridized with diploid and hexaploid *Festuca*, but not or only weakly with the tetraploid *Festuca* (Fig. 2), which suggests that TF521 is a P genome-specific probe. Both probes showed a smear hybridization pattern throughout the track. This kind of hybridization pattern is typical of dispersed repetitive sequences (Mouras et al. 1987).

TF436 and TF521 showed similar hybridization patterns in *F. mairei* and *F. arundinacea* var. *glaucescens*. Previous studies suggested that the two tetraploids may have diverged from the same progenitor or at least shared one homologous genome (Malik and Thomas 1967; Bowman and Thomas 1976; Bulinska-Radomaska and Lester 1986). Of 54 non-repetitive RFLP probes from the *F. arundinacea Pst*I genomic library used in our study, 50 probes showed hybridization signals in both *F. mairei* and *F. arundinacea* var. *glaucescens*. Another four probes (TF121, TF153, TF212 and TF416) that did not hybridize with *F. mairei* also did not hybridize with *F. arundinacea* var. *glaucescens*. An attempt to use genomic in situ hybridization (GISH) by using genomic *F. mairei* DNA as a probe to differentiate *F. mairei* and *F. arundinacea* var. *glaucescens* failed. These results indicated that there was no significant DNA structural differentiation in the genomes of the two tetraploid species. However, GISH has been successfully used to differentiate *F. pratensis* from *F. arundinacea* var. *glaucescens* (Humphreys et al. 1995).

Features of DNA sequences

TF436 and TF521 DNA sequences were cloned into the *Pst*I site in pUC19 (Xu et al. 1991). The complete sequences of both TF426 and TF521 clones are shown in Fig. 3A and B and have been submitted to GeneBank with the accession numbers AF087933 and AF087934, respectively. Sequence analysis of TF436 revealed a 1,098-bp insert with a 59.53% overall AT content. Direct and inverted repeats were located using the GCG program. There were 11 perfect direct repeats with lengths of more than 7 bp in TF436. Conspicuously, there was a long direct repeat with 23 bp starting from position 28, and a 9-bp direct repeat located nearby (Fig. 3A). No short tandem repeat unit was present in this sequence. Three perfect inverted repeats with lengths of more than 8 bp were present throughout the whole sequence of TF436, but no direct repeats were found to flank them.

Transposable elements make up over 50% of the nuclear DNA in many higher plants with large and complex

(A)

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  1  CTGCAGCACA CACATGGTCC TTGTGAAGGA CAGCATCCTT GACTGCTTTT
 51  AGAGCAGCAT CTTGACTGCT TTTAGAGCAG CATCTTAGAC TAGCATCTAG
101  CATATTCGTG GCTGGCTAGC AGCCCTATAA ATATGTAACC CCAACCCCTC
151  AGGTTGGTAT GGCATTGTGT GAGAAGTGTG AGAAATAAAC CAACGAAAAT
201  TGCCCCAACT CTCTTAGTGT CATCCTCTTC TTCATGAAAG TGAGGCTAGA
251  GATACTAACA GCTTTTGCCA AATATTAGTG CTTGGTGTTG ACATCCTTAT
301  ATAATTGATT TTTCAAGGCA CTGTCGGAGT AAGTTTGGAT TAGGGTTTTC
351  ATCTGTTGTG CATTCATGCA ATTATGTAAA GTATTTTGTT GGAAATGCAA
401  GAACCACCTT GTGCCTTTAC CTTTCACGCA TGTTAAGATT ACAGGAGTAC
451  CGTTTGTAAC ATTTGCAATA CTTTTATTCT CAGGCTGCAA AAGGAGCAGG
501  TGTGCCTGTT ATCTTGGATG CAGGTGGCAT GGATGCTCCT GTCCCTGGAG
551  AATTACTGGG GCTGGTAGAT ATTTTTAGTC CAAATGAAAC AGAGCTAGCA
601  CGTTTGACTG GAATGCCTAC CAAAACTTTT GAACAGATCA GCCAGCAGCA
651  GGAGCATGCC ATAAAATGGT GAGATGTCTA AATTGGACAT TTTTATGTAG
701  TTCGTTTCTG CATTTCTTCT ATATTCAGCT ATACCTTCTA ACATCACTCT
751  TTTGTTTCTT TTGGTTAGCC ATGACCGTGG ATCTTAATAT CTTTATTTAA
801  GAAACTCAAT TAATGATGTT GGGTTACGGC GATCATCTGA TAGTAGCCAA
851  CTATAAGGAA TGTGTTGCTT TCCCTTCTTT CTCCCTTAGT GTAGGTCTGT
901  GTGAATGTGT TTCCCCTACA CCTATTAGGA CATCCGCCTC TTACTCTTCT
951  TGTTATATAA AAGTTTCCAA GGGTGTCGAT TGTACCTATG GATAAATATG
1001 CTAGATGAAC TGTAAACATA TATGGAGTGT GCGTACAAGT GATTTTTTCT
1051 GATTTGCAAA AACTATATGA ACTCTGGATC TTTATTTCTT ACCTGCAG
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(I) (II) (III) (I) (II) (III)

(B)

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  1  CTGCAGCGTG GAAGTGCTGG TGGTTGGTGC GGCTGATGCC AGCAAGGAAG
 51  CTGCGGCGGA AGGCGCTGGA CAGACGAGGT CTACGCGCAC GGCCAAGCCA
101  CCGACCGAGC GTGACCAGGG AGCTGCGGGT ACATCTGCAG
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Fig. 3A, B Nucleotide sequence of the two repetitive DNA clones. **A** TF436 sequence, GeneBank accession no. AF087933. CTGCAG is the restriction site of *Pst*I. *Bold and italic bold letters* indicate direct duplication. *Underlined letters* indicate inverted repeats. **B** TF521 sequence, GeneBank accession no. AF087934

genomes (San Miguel and Bennetzen 1998). Transposable elements play a major role in rearranging genomes and altering individual gene structures and regulation (Bennetzen 2000). However, no direct evidence has suggested the presence of transposable elements in *Lolium* and *Festuca*. Computer-assisted data analyses of interspersed repetitive DNA sequences may reveal features attributed to mobile elements, such as LTR-retrotransposons (Bennetzen 2000). The sequence of clone TF436 showed no similarity with the terminal inverted repeats of Ac/Ds transposable elements. No significantly identical DNA sequences were found for TF436 after searching Genebank.

TF521 was found to be a clone with an insert of 140 bp. It has a 66.43% GC content. Short sequences, GGAAG and GCTGG, were present three times (Fig. 3B). By searching the DNA Data Bank of Japan (DDBJ), a 72.4% similarity in a 58-bp overlap was detected between TF521 (positions 8–65) and an activator (Ac)-like element Pac1 gene (position 1,386–1,445) (accession number U02300), cloned from pearl millet [*Pennisetum glaucum* (L.) R. Br.] (MacRae et al. 1994). This correlation is probably coincidental because TF521 is too short compared to the 4,585 bp of U02300. Chromosome in situ hybridization indicated that TF521 was located in subtelomeric regions in diploid *F. pratensis* (data not shown).

A 19-nt primer was designed (5′-CAACCTGAGGGGTTGGGGT-3′, ordered from Integrated DNA Technologies, Inc.) which was complementary to the sequence from positions 138 to 156 in TF436 (Fig. 3A). This primer was matched with the M13 reverse primer (5′-GGAAACAGCTATGACCATG-3′) to amplify a sequence containing the 23 bp-long direct repeat. The PCR product was used as a probe to produce the RFLP hybridization pattern shown in Fig. 4. This probe was able to hybridize with all the *Lolium* and *Festuca* species, with tetraploid *Festuca* species having two bands each. TF436 might have more copies in tetraploid *Festuca* which would make TF436 a species-specific sequence.

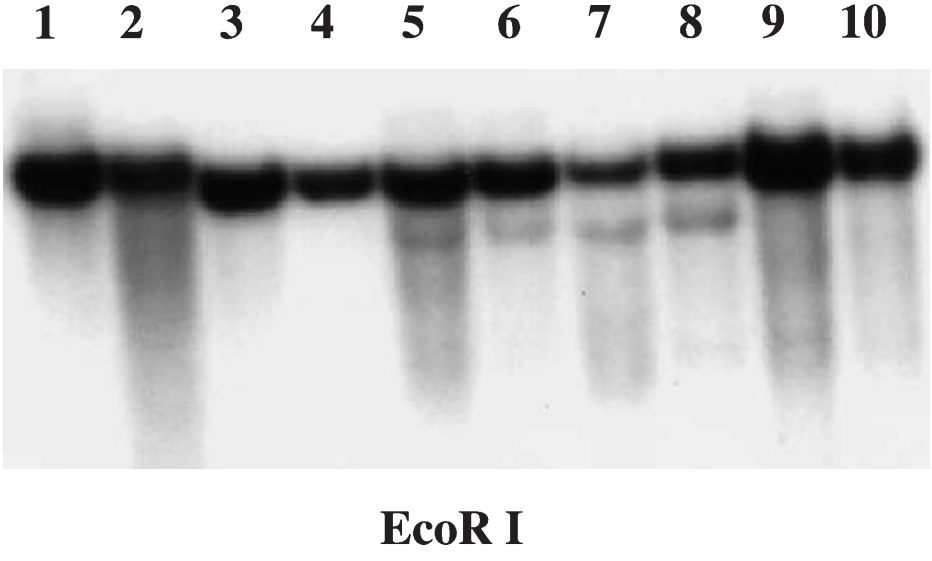

Fig. 4 Southern hybridization of *Eco*RI-digested genomic DNA labeled with a part of TF436 (the first 137 bp)

Table 2 Characteristics of introgression lines derived from *F. mairei* (Fm)×*L. perenne* (Lp)

Plant	2n	Inflorescence	Glume		Pollen stainability (%)	TF436
			Inside	Out		
Lp	14	Spike	0[a]	1[a]	90	–
Fm	28	Panicle	1	1	95	+
Fm×Lp	21	Panicle	1	1	0.5	+
BC_1F_1–6	21	Spike	0	1	32	–
BC_1F_1–11	14	Spike	0	1	50	+
BC_1F_1–14	14	Spike	0	1	50	–
BC_1F_1–15	28	Spike	1	1	60	+
BC_1F_1–16	18	Spike	1	1	10	±[b]
BC_1F_1–24	28	Panicle	1	1	62	±
BC_1F_1–26	28	Spike	1	1	35	±
BC_1F_1–27	28	Panicle	1	1	65	±
BC_1F_1–30	28	Panicle	1	1	65	±

[a] 0=absent, 1=present
[b] ±: intermediate Southern hybridization signals between + and –

Detection of addition and translocations lines

Neither TF436 nor TF521 hybridized with *L. perenne*. This suggests that TF436 can be used as a M_1 (M_2) genome-specific marker to monitor the chromatin introgression from *F. mairei* into *L. perenne*. The scheme for producing the BC_1F_1 derivatives from the cross between *F. mairei* (4*x*) and *L. perenne* (2*x*) was presented by Chen and Sleper (1999). The 4*x* F_1 hybrid of *F. mairei*× *L. perenne* was used as the female in backcrossing to *L. perenne*. BC_1 plants were inter-pollinated to produce BC_1F_1 progenies whose chromosome numbers varied from 2n=14 to 32. TF436 was used to detect *F. mairei* chromatin in BC_1F_1 progenies by Southern hybridization. Some plants displayed intermediate signals between *L. perenne* and *F. mairei*, which may suggest *F. mairei* chromatin-introgression (Table 2). Morphological characteristics and chromosome counting assisted the interpretation of the results of the TF436 screening. For example, BC_1F_1–26 had a spike inflorescence that resembled *L. perenne* but the plant had 28 chromosomes and showed an intermediate TF436 signal. This information suggests that BC_1F_1–26 might be a *L. perenne–F. mairei* addition line with some chromosomes incorporated from *F. mairei*. Chromosome in situ hybridization could have provided direct evidence that *F. mairei* chromatin has been incorporated. However, the effort to find the chromosome location of TF436 in the addition and translocation lines failed. GISH was used instead to detect *F. mairei* chromatin by labeling the total genomic DNA of *F. mairei*. This produced positive results. BC_1F_1–26 is an addition line with 21 chromosomes from *L. perenne* and seven chromosomes from *F. mairei*. (Fig. 5). Its genomic constitution must be LLLM. This is very possible because it is not unusual for the diploid *L. perenne* to produce unreduced gametes (Chen et al. 1995). Some other results need to be investigated further. BC_1F_1–11 might be a translocation line (or a substitution line) which had only 14 chromosomes but it still showed positive RFLP signals. BC_1F_1–16 (2n=18) may be another addition line. The high percentage of chromosome pairing between *F. mairei* and *L. perenne* (Cao et al. 2000) may cause a wide range of chromosome variation in BC_1F_1 plants (Chen and Sleper 1999).

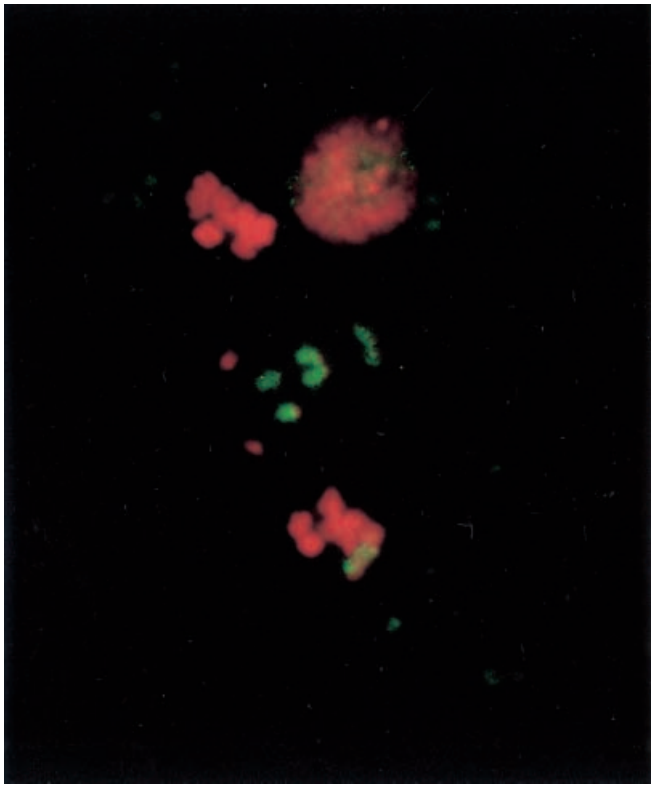

Fig. 5 Genomic in situ hybridization of BC_1F_1–26 (PMC AI, 2n=28) using *F. mairei* (2n=28) as a probe shows 21 *L. perenne* chromosomes (*red*) and seven *F. mairei* chromosomes (*green*)

In this report, the use of the repetitive sequence TF436 as a marker was both effective and informative in detecting *F. mairei* chromosome introgression. In situ hybridization produced accurate results even though species of *Lolium* and *Festuca* are close in their genomic relationships. The DNA content of each *Festuca* species is significantly distinct (Seal and Rees 1982) and the DNA structures or contents are distinct enough to be able to use GISH to differentiate the *Festuca* and *Lolium* genomes (Thomas et al. 1994; Canter et al. 1999; Cao et al. 2000). Direct use of genomic DNA as a probe (GISH) allows for differentiation between *Festuca* and *Lolium*

and the detection of introgressed alien chromatin in the *Festuca-Lolium* complex (Chen and Sleper 1999). However, the use of genome-specific repetitive DNA sequences as molecular markers should be both convenient and effective for screening progenies of large populations in *Festuca-Lolium* breeding projects.

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