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Anchored simple-sequence repeats as primers to generate species-specific DNA markers in *Lolium* and *Festuca* grasses

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Abstract Simple-sequence repeats (SSRs) comprising three tetranucleotide repeat sequences with two-base 'anchors', namely 5´-$(AGAC)_4$GC, 5´-AC$(GACA)_4$ and 5´-$(GACA)_4$GT, were used in PCR reactions as primers to develop inter-SSR DNA fingerprints of the outbreeding grass species *Lolium multiflorum*, *L. perenne*, *Festuca pratensis* and *F. arundinacea*. Each species was represented by DNA samples from 3 to 6 varieties. In all four species distinctive species-specific DNA profiles were produced that were common across a number of varieties despite their diverse origin. While the fingerprints of the two ryegrasses, *L. multiflorum* and *L. perenne*, were the most similar, a number of inter-SSR DNA markers were generated that enabled them to be distinguished from each other. Some slight variations were found between varieties, which provided putative variety-specific markers for cultivar identification. In addition, variations in the DNA profiles of the genotypes of *L. multiflorum* and *F. pratensis* were examined, and the results showed that variety-specific fingerprints are integrated patterns made up from the profiles of individual genotypes. Amongst the primers used, AC$(GACA)_4$ generated the best distinction between *Lolium* and *Festuca* individuals and provides an effective new tool for genome identification. A number of species-discriminating sequences, ranging in size between 550 bp and 1,600 bp, were cloned: three clones for *F. pratensis*, one clone for *L. multiflorum* and one clone for *F. arundinacea.* A *F. pratensis* fragment pFp 78H582 was sequenced. Southern hybridization confirmed the presence of this fragment in *F. arundinacea* (which contains one genome of *F. pratensis*), but no homology was found with *L. multiflorum*. However, a *F. arundinacea* clone amplified with $(GACA)_4$GT, pFa 104H1350, was found to be unique to the *F. arundinacea* genome.

Key words *Lolium* · *Festuca* · Inter-SSR fingerprinting · DNA markers

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Introduction

The genera *Lolium* and *Festuca* belong to the family *Poaceae* subfamily *Festucoideae*, tribe *Festucaceae.* Four outbreeding species, *Lolium multiflorum*, *L. perenne*, *Festuca pratensis* (diploid and tetraploid) and *F. arundinacea* (hexaploid), are widely used as major components of pasture and amenity grasslands. Close relationships and considerable genome homology are found between the ryegrasses and fescues in this group (Jaughar 1975; Thomas et al. 1994; Pašakinskienė et al. 1998), and this has allowed breeders to make hybrids and to develop new types of forage grasses called *Festuloliums.* The amphiploid between *L. multiflorum*×*F. pratensis* [=*Festulolium braunii* (K. Richter) A. Camus] is a product of such a breeding programme, and genomic in situ hybridization (GISH) studies have revealed high levels of recombination between the genomes (Zwierzykowski et al. 1998; Canter et al. 1999). *Festulolium* provides new combinations of agronomically important characters: high fodder quality derived from *Lolium* and resistance to environmental stresses from *Festuca*. Introgression breeding, involving the transfer of small alien segments of *Festuca* chromatin into the background genome of *L. multiflorum*, is another way of combining the desired characters together. Using this breeding approach, together with GISH as a marker for identifying recombined chromosomes, Humphreys and Pašakinskienė transferred *Festuca* genes into *L. multiflorum* to produce drought-resistant lines (Humphreys and Pašakinskienė 1996).

Molecular markers have proved useful for establishing genetic maps for marker-assisted selection and for quantitative trait locus (QTL) analysis in both *Lolium* (Hayward et al. 1998) and *F. arundinacea* (Xu et al. 1995). Constructing genetic maps, however, is expensive, laborious and time-consuming and for the mapping of QTLs, particularly in outbreeding species such as *Lolium* and *Festuca* where no 2 genotypes are identical, has practical limitations for breeding purposes.

In the work presented here we have focused on a more economical and comparatively simple DNA marker system for the grasses based on simple-sequence repeats (SSRs), or microsatellites. This is known as inter-SSR fingerprinting and involves the use of anchored-SSRs as primers in polymerase chain reaction (PCR) amplification (Zwietkiewicz et al. 1994; Gupta et al. 1994). Inter-SSR fingerprinting has been successful for a wide range of agronomically important crops, e.g. maize, oil seed rape and sugar beet (Kantety et al. 1995; Charters et al. 1996; Lorenz et al. 1997). Closely spaced SSRs oppositely oriented on the DNA template serve as primer binding sites to initiate amplification of the intervening DNA sequences of different sizes. The PCR product is separated on an agarose gel thereby providing a DNA fingerprint, or DNA profile. Studies in *Bromus* and *Agropyron* grasses have previously shown that inter-SSRs yield considerably more polymorphism than randomly amplified polymorphic DNA (RAPD) analysis (Gyulai et al. 1997). In the study reported here, we have used inter-SSR fingerprinting for developing DNA markers within the *Lolium/Festuca* complex both as a tool for marker-assisted selection and for genome identification.

Materials and methods

Plant material, DNA extraction

The grass varieties listed in Table 1 were obtained from the Genetic Resources collections at the Institute of Grassland and Environmental Research, UK, and at the Lithuanian Institute of Agriculture. Variety DNA samples were extracted from 2-week-old seedlings germinated on filter paper following the DNA extraction protocol of Doyle and Doyle (1990). Pooled DNA samples taken from each variety comprised 50–100 genotypes. DNA of the individual genotypes was extracted from the leaves of grown plants.

PCR amplification and electrophoresis

Oligonucleotide primers were synthesized by VHBio (Newcastle-upon-Tyne, UK) (Table 2) using sequences suggested in Gyulai et al. (1997). Fifty-microlitre aliquots or PCR reaction mixtures contained 10 m*M* TRIS-HCl (pH 8.0), 50 m*M* KCl, 1.8 m*M* $MgCl_2$, 0.2 m*M* dNTP mix, 0.2 μ*M* primer, 50 ng of template DNA and 1.0 U of DyNAzyme II polymerase (Finnzyme OY) or *Taq* DNA polymerase (Promega). PCR was carried out on a Perkin Elmer 2400 thermocycler under the following conditions: 1 cycle of 95°C for 2 min, 50°C for 1 min, 72°C for 30 s; 39 cycles of 95°C for 30 s, 50°C for 1 min, 72°C for 30 s; 1 cycle 72°C for 6 min. Amplification products were separated on 1.5% agarose/TAE gels.

Table 1 List of plant material

Species	Variety	Chromosome number	Country of origin
L. multiflorum	Bartolini	2*n*=2x=14	The Netherlands
	Gordo	2*n*=2x=14	The Netherlands
	Zenith	2*n*=2x=14	The Netherlands
	RvP	2*n*=2x=14	Belgium
	Tribune	2*n*=2x=14	United Kingdom
	Macho	2*n*=4x=28	The Netherlands
	Tetrone	2*n*=4x=28	The Netherlands
	Atos	2*n*=4x=28	Poland
	Lotos	2*n*=4x=28	Poland
L. perenne	Sodrė	2*n*=4x=28	Lithuania
	Žvilgė	2*n*=4x=28	Lithuania
	Solen	2*n*=4x=28	Poland
F. pratensis	Dotnuva I	2*n*=2x=14	Lithuania
	Kaita	2*n*=2x=14	Lithuania
	Skra	2*n*=2x=14	Poland
	Skawa	2*n*=2x=14	Poland
	Westa	2*n*=4x=28	Poland
F. arundinacea	Barundi	2*n*=6x=42	The Netherlands
	Stef	2*n*=6x=42	Poland
	Terros	2*n*=6x=42	Poland

Table 2 Nucleotide sequence of tested primers

Primer code	Nucleotide sequence	Abbreviation
77H	5´-AGACAGACAGACAGACGC	$(AGAC)_4GC$
78H	5´-ACGACAGACAGACAGACA	$AC(GACA)_4$
104H	5´-GACAGACAGACAGACAGT	$(GACA)_4GT$

Cloning and sequencing

Amplified inter-SSR fragments were excised and purified using the QIAquick gel extraction kit (QIAGEN). Fragments were cloned using the pGEM-T Easy PCR cloning kit (Promega) and transformed into *Epicurian Coli* ultracompetent cells (Stratagene). Plasmid DNA was extracted from overnight cultures using the QIAprep Spin Miniprep kit (QIAGEN).

The nucleotide sequence was determined using the Thermo-Sequenase dye-terminator cycle sequencing kit (Amersham Pharmacia Biotech.) and a 373A DNA sequencer (Applied Biosystems).

Southern hybridization

Digoxigenin-labeled probes were prepared by PCR of 200 pg cloned insert SSRs using T7 and SP6 primers under conditions described in Dalton et al. (1998) except that the cycling conditions were 2 min at 95°C followed by 25 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 5 min. Hybridization, blocking and detection were as described in Dalton et al. (1998).

Results

Fingerprints for species and varieties

DNA fingerprints produced with the tetranucleotide repeats $(AGAC)_4GC$, $AC(GACA)_4$ and $(GACA)_4GT$ are illustrated in Figs. 1 and 2. All primers gave rise to PCR products observed as discrete bands of different sizes

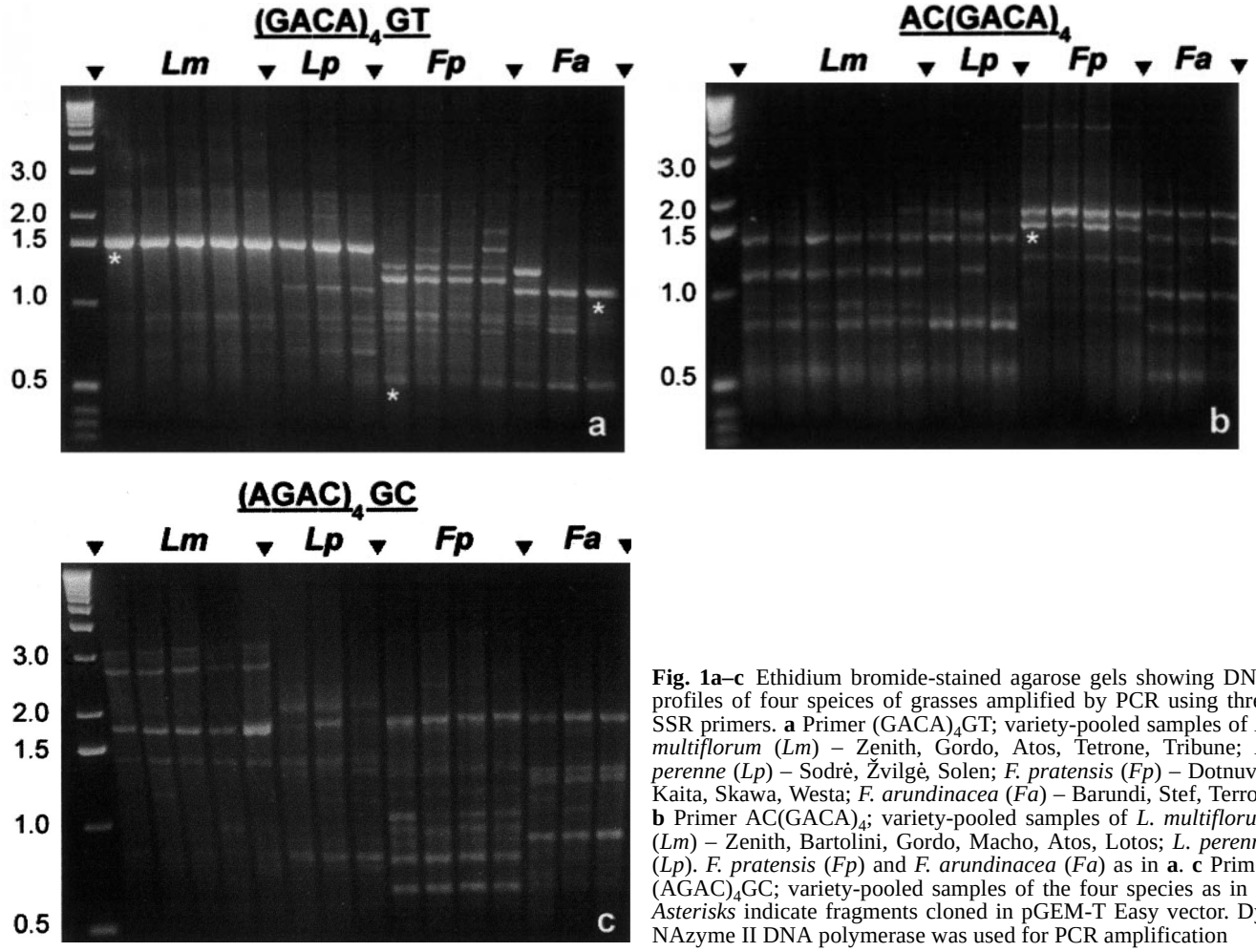

Fig. 1a–c Ethidium bromide-stained agarose gels showing DNA profiles of four speices of grasses amplified by PCR using three SSR primers. **a** Primer $(GACA)_4GT$; variety-pooled samples of *L. multiflorum* (*Lm*) – Zenith, Gordo, Atos, Tetrone, Tribune; *L. perenne* (*Lp*) – Sodrė, Žvilgė, Solen; *F. pratensis* (*Fp*) – Dotnuva, Kaita, Skawa, Westa; *F. arundinacea* (*Fa*) – Barundi, Stef, Terros. **b** Primer $AC(GACA)_4$; variety-pooled samples of *L. multiflorum* (*Lm*) – Zenith, Bartolini, Gordo, Macho, Atos, Lotos; *L. perenne* (*Lp*). *F. pratensis* (*Fp*) and *F. arundinacea* (*Fa*) as in **a**. **c** Primer $(AGAC)_4GC$; variety-pooled samples of the four species as in **a**. *Asterisks* indicate fragments cloned in pGEM-T Easy vector. DyNAzyme II DNA polymerase was used for PCR amplification

that were generally prominent and consistently species-specific throughout all *Lolium* and *Festuca* cultivars despite their diverse origin (Fig. 1a–c). Changing the two anchor bases for the $(GACA)_4$ repeat from $(GACA)_4GT$ to $AC(GACA)_4$ resulted in a completely different fingerprint (Fig. 1a, b).

Overall *L. multiflorum* and *L. perenne* had the most bands in common, but distinct *L. perenne* markers of 1.2 kb, amplified using $(GACA)_4GT$ (Fig. 1a) and of 0.78 kb amplified by $(AGAC)_4GC$ (Fig. 1c), were observed. In addition a 2.8-kb sequence and a group of stutter bands above it obtained with a $(AGAC)_4GC$ primer were found with *L. multiflorum* DNA but not in *L. perenne* DNA. Diploid varieties of *L. multiflorum* had apparently the same DNA profiles as tetraploid varieties, for example Zenith, Bartolini and Gordo (all diploids) and Macho, Atos and Lotos (all tetraploids), in the $AC(GACA)_4$ amplicon (Fig. 1b). Lithuanian *L. perenne* var. Žvilgė differed from 2 other varieties, Solen (Polish) and Sodrė (Lithuanian), by a 1.2-kb fragment which was consistently found in the $AC(GACA)_4$ profile of another ryegrass, *L. multiflorum* (Fig. 1b). On the other hand, *L. multiflorum* Polish var. Lotos had a 2.0-kb marker characteristic of *L. perenne*.

For *F. pratensis*, a distinct and consistent pair of bands was amplified by $(GACA)_4GT$, 1.3 kb and 1.4 kb long (Fig. 1a), and by $AC(GACA)_4$, 1.5 kb and 1.7 kb (Fig. 1b). A short specific fragment of 0.7 kb was obtained in the $(AGAC)_4GC$ amplicon (Fig. 1c). A distinct band of around 5 kb was found for diploid *F. pratensis* vars. Dotnuva, Kaita and Skawa using $AC(GACA)_4$ but not for tetraploid *F. pratensis* var. Westa (Fig. 1b). On the other hand, Westa had unique bands of 1.5 kb and 2.0 kb in the $(GACA)_4GT$ amplicon (Fig. 1a) which distinguished it from the other 3 *F. pratensis* varieties.

F. arundinacea profiles with the $(AGAC)_4GC$ primer were the same for all varieties, each with the species-specific band of 0.9 kb (Fig. 1c). The $(GACA)_4GT$ primer provided a prominent band of 1.4 kb as a marker for var. Barundi (Fig. 1a).

Genotype fingerprinting

Primers $(AGAC)_4GT$ and $AC(AGAC)_4$ were used to determine DNA profile variation amongst individual genotypes of *L. multiflorum* and *F. pratensis*. Very little variation for *L. multiflorum* was revealed using $(GACA)_4GT$

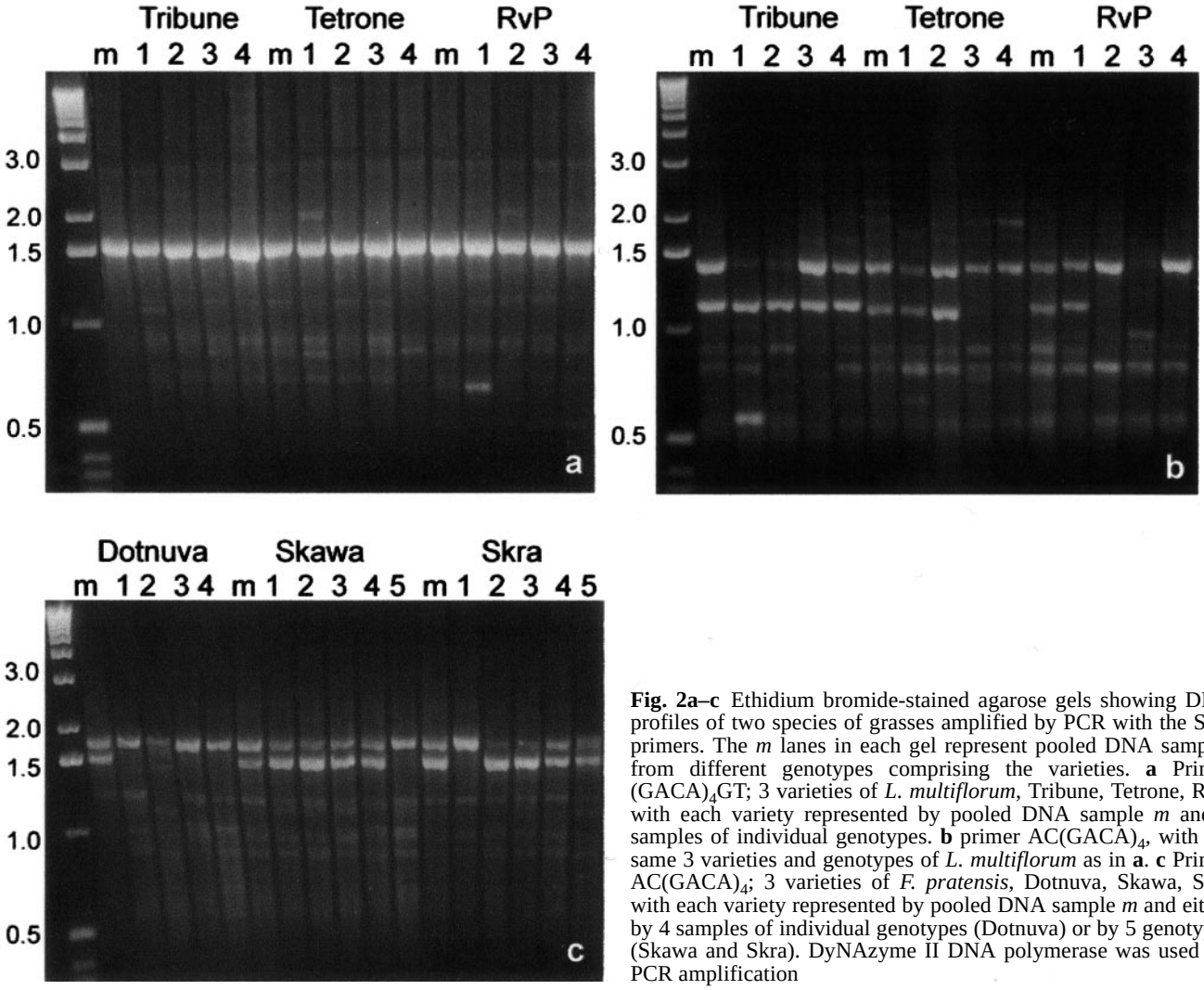

Fig. 2a–c Ethidium bromide-stained agarose gels showing DNA profiles of two species of grasses amplified by PCR with the SSR primers. The *m* lanes in each gel represent pooled DNA samples from different genotypes comprising the varieties. **a** Primer $(GACA)_4GT$; 3 varieties of *L. multiflorum*, Tribune, Tetrone, RvP, with each variety represented by pooled DNA sample *m* and 4 samples of individual genotypes. **b** primer $AC(GACA)_4$, with the same 3 varieties and genotypes of *L. multiflorum* as in **a**. **c** Primer $AC(GACA)_4$; 3 varieties of *F. pratensis*, Dotnuva, Skawa, Skra with each variety represented by pooled DNA sample *m* and either by 4 samples of individual genotypes (Dotnuva) or by 5 genotypes (Skawa and Skra). DyNAzyme II DNA polymerase was used for PCR amplification

as a primer (Fig. 2a). Both the variety mixture and the individual genotypes showed a uniform pattern, with a main prominent band at 1.5 kb. Nevertheless, a few fragments could be considered as potential genotype markers, namely in Tetrone genotype No. 1 (2.0 kb) and in RvP genotype No. 1 (0.75 kb). Profiles of *L. multiflorum* generated with $AC(GACA)_4$ were more complex (Fig. 2b). Pooled DNA samples of the 3 varieties Tribune, Tetrone and RVP had identical profiles, with two prominent bands of 1.2 kb and 1.4 kb. All possible combinations of absence or presence of these two bands was found amongst individual *Lolium* genotypes, namely: (1) both the 1.2-kb and 1.4-kb bands present; (2) the 1.2-kb band only; (3) the 1.4-kb band only; (4) both absent (genotype No. 3 of RvP). In certain genotypes, for example No. 1 of Tribune, other genotype-specific bands were present (0.6 kb) which have potential for use as specific genotype markers.

The same $AC(GACA)_4$ primer gave rise to genotype-specific fingerprints in *F. pratensis* (Fig. 2c). There was within-cultivar genotype variation, with either one or other of the 1.5-kb or 1.7-kb bands absent in certain individuals.

Table 3 Designation of cloned inter-SSR fragments

Name	Species	Primer	DNA polymerase
pLm 104H 1500	*L. multiflorum*	$(GACA)_4GT$	DyNAzyme II, *Taq*
pFp 104H 550	*F. pratensis*	$(GACA)_4GT$	DyNAzyme II, *Taq*
pFp 78H 1500	*F. pratensis*	$AC(GACA)_4$	DyNAzyme II, *Taq*
pFp 78H 582	*F. pratensis*	$AC(GACA)_4$	*Taq*
pFa 104H 1350	*F. arundinacea*	$(GACA)_4GT$	DyNAzyme II, *Taq*

Cloning and sequencing

Some inter-SSRs were cloned in the pGEM-T Easy vector (Table 3; Fig. 1a, b, asterisks) to produce sufficient amounts of probe DNA to: (1) test the sequence homology of PCR products across different species by Southern blot hybridization, and (2) in future to develop fluorescence in situ hybridization (FISH) probes to locate SSR-enriched sites on the chromosomes, as carried out in barley (Pedersen and Linde-Laursen 1994; Pedersen et al. 1996).

The cloning experiment resulted in three inter-SSR clones for *F. pratensis*, one clone of *L. multiflorum* and one clone of *F. arundinacea* (Table 3). Two different

Fig. 3 A 582-bp insert-SSR sequence found in *Festuca pratensis* and *F. arundinacea* but absent in *Lolium multiflorum* generated by PCR amplification with the $AC(GACA)_4$ primer. The primer sequence is in *bold* and *italics* (*underlined*); the intra-repeats are in *bold*

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1    ACGACAGACA GACAGACAGA GAGCAACCAC ACGTGTGAAT GAAATAAAAT
51   GCTGTGGATT CGATCCATCG GACGGCACCG CCGCCTCCCT GAAAAGTGAA
101  AACAAAATAA GCTAAGCAAC CACCGCTCTG CCGTCGAAAA CGACGGCCAC
151  CCACATCATA GCCAGTGCTG CTATGACGGC CAAAAATCAC GATTCTAAAT
201  TAACCACAGA GACAACAACA ACCACTCAAA GTGAACTAAT AAACAGAATA
251  CTCTCGAGTT CAAGCCAGCT GCAGACAGTT TACAATATCC ACACAATGAC
301  CCAAAGAATC TTGTAGAGAT AGTACACACT CTGAGTTGGA TTTACCGGTA
351  CGATCATGAT AATGGCGATG TCAATCAGTG GTTGAAAATC TTGCAAATCC
401  AGCAAAATCT CTGGAGCAAA AGAGACAAAA AGGGATTCCA CATATTCACT
451  TGGTAAGAAT CAAGAGCTCT TTCCACAGCT GTCCTAAAGT ATACAGCACC
501  ACAATAACAT TGTAAAACTC GTTCATCCTC CATCACAGGC AGAACAATTA
551  CAATATCTTC CGAATGTCTG TCTGTCTGTC GT
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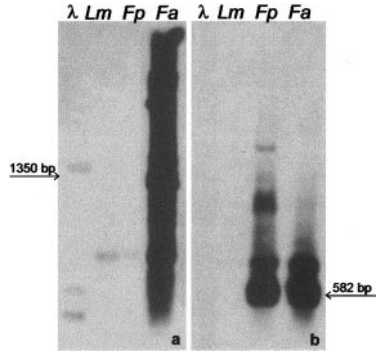

Fig. 4a, b Southern hybridization of inter-SSR clones to *L. multiflorum*, *F. pratensis* and *F. arundinacea* DNA profiles. **a** $(GACA)_4GT$ primer profile blot hybridized with pFa 104H1350, **b** $AC(GACA)_4$ primer blot hybridized with pFp 78H582. For each lane 10 µl of PCR product was loaded

DNA polymerases, *Taq* polymerase isolated from *Thermus aquaticus* (Promega) and DyNAzyme II polymerase isolated from *Thermus brockianus* (Finnzymes OY), were used in PCR for inter-SSRs amplification. Both polymerases gave almost identical DNA fingerprints with the constant pattern of main characteristic bands. However, DyNAzyme II produced bands of higher intensity, indicating a higher yield of the product. On the other hand, some of the species-specific fragments were generated with *Taq* polymerase only, for example pFp 78H582 (Table 3).

A *F. pratensis*-specific 582-kb band obtained from the $AC(GACA)_4$ amplicon was sequenced (Fig. 3). Adenine was the most abundant nucleotide found either singly or as $(A)_2$, $(A)_3$ and $(A)_4$ repeats and was widely found throughout the inter-SSR sequence. Dinucleotide intra-repeats were abundant, especially $(CA)_2$, and a few trinucleotide intra-repeats were found; however none of tetranucleotide repeats appeared within this clone. This leads to the conclusion that the tetramers of the tetranucleotides used in this work are rather scattered within the genome of the grasses, which consequently makes them the primers of choice for developing DNA markers.

Southern hybridization

Two clones, pFp 78H582 and pFa 104H1350, were used in Southern hybridization to test their homology with related species. The pFa 104H1350 clone as probe gave an intense hybridization signal with *F. arundinacea* DNA but not with *L. multiflorum* nor *F. pratensis* DNA (Fig. 4a). The hybridization signal covered all of the DNA lane from the largest fragments to the smallest, which indicates that this sequence is highly common throughout the entire genome of *F. arundinacea*. The pFp 78H582 clone hybridized to both *F. pratensis* and *F. arundinacea* DNA but not to *L. multiflorum* DNA (Fig. 4b). This sequence showed homology to another fragment of approximately 740 bp, but it does not appear to be common throughout the genome. Both clones were confirmed as *Festuca*-specific sequences that can be employed as potential markers for screening *Festuca* DNA in the *Lolium* genomic background.

Discussion

Earlier molecular studies based on seed proteins, chloroplast DNA, rDNA and repetitive DNA made on the two ryegrass species, *L. multiflorum* and *L. perenne*, and the two fescue species, *F. pratensis* and *F. arundinaecea*, revealed their very close relationship (Bulińska–Rodomska and Lester 1988; Lehväslaiho et al. 1987; Charmet et al. 1997; Pašakinskienė et al. 1998). They are close enough in evolutionary terms to be hybridized, and to be used for interspecific chromosome pairing and for promiscuous recombination; yet paradoxically their repetitive DNAs are divergent enough, at least in the case of the *Lolium* and *Festuca* species, for them to be discriminated by GISH (Thomas et al. 1994; Pašakinskienė et al. 1998). In the present study, inter-SSR fingerprints proved to be a highly discriminative tool, and one that can be used as another approach for studying genome organization in these species. Amongst the four species investigated here, the two *Lolium* species, *L. multiflorum* and *L. perenne*, have the highest homology (no GISH discrimination). Inter-SSR fingerprinting using the $(GACA)_4$ repeat, anchored with two bases either as $AC(GACA)_4$ or $(GACA)_4GT$, revealed clear differences and enabled discrimination between the DNAs of *L. multiflorum* and *L. perenne*.

The different varieties of ryegrasses and fescues involved in this study were represented by pooled DNA samples, i.e. DNA mixtures taken from many individual genotypes. As these grasses are obligate outbreeding species, a variety represents a highly heterogeneous pop-

ulation with considerable intravarietal genotypic variation. The inter-SSR sequences described herein provide a useful marker system for discriminating between individuals within populations of *L. perenne* and *F. pratensis*. Some motifs are better suited for genotyping than others. Amongst the primers testes thus far, $AC(GACA)_4$ was found to offer the greatest potential for identifying genotypic variation based on differences in lengths of inter-SSRs. Since only three primers thus far have been used, it is likely that others will be found which allow still greater resolution. There are $(4)^4$ (=256) potential motifs of quadruplet repeats that can be designed. By attaching anchor bases, we will be able to further increase the discriminative capacity of these SSR primers. This has been found to be the case in maize and rice where SSRs combined with anchor bases increased the specifity of PCR amplification by reducing the number of target sites on the DNA template (Gupta et al. 1994; Akagi et al. 1996).

Southern hybridization proved that the 582-bp inter-SSR *Festuca*-specific sequence and the *F. arundinacea*-specific 1,350-bp sequence are not present in *L. multiflorum*. In addition to the *Festuca*-specific 582-bp sequence described, other insert-SSRs are being sequenced (unpublished data). There is no evidence thus far in *Lolium* and *Festuca* species that inter-SSR sequences contain any functional coding regions. However, clones of these sequences may be used as potential markers to follow introgression from *F. pratensis* and *F. arundinacea* into *Lolium*. Inter-SSRs such as the species-specific sequences described above have potential as markers to confirm the presence of closely linked *Festuca* genes. Clearly the suitability of such inter-SSR sequences as gene markers will depend on their frequency and distribution throughout the *Festuca* genome.

When SSRs are used as PCR primers for generating DNA profiles, numerous loci throughout the genome are generally targeted (Wu et al. 1993; Wang et al. 1994). It was demonstrated in a maize recombinant inbred population that DNA marker bands obtained by an $(ACAG)_4$ repeat are heritable, and mostly dominant (Gupta et al. 1994). Akagi et al. (1996) using an $(AG)_8$ anchored repeat produced a codominant DNA marker closely linked to the nuclear restorer gene (*Rf–1*) for fertility in rice. Disease resistance can also successfuly be tagged with inter-SSR markers, as demonstrated in chickpea resistance to fusarium wilt (Ratnaparkhe et al. 1998).

Humphreys et al. (1998) refer to potential difficulties in identifying small translocated chromosome segments of *Festuca* that may have introgressed into *Lolium* during intergeneric breeding programmes but due to their small size remain undetected by GISH. One of the *F. arundinacea* inter-SSRs described in the current work is being employed to screen for *F. arundinacea* genes amongst *L. multiflorum* drought élite lines developed from a hybrid between *L. multiflorum* (drought-sensitive) and *F. arundinacea* (drought-resistant) (Humphreys 1989; Humphreys and Thomas 1993). Humphreys and Pašakinskienė (1996), using GISH, found 3 lines out of 12 genotypes that contained *Festuca* introgressed segments. However, GISH resolution is currently considered ineffective for DNA segments smaller than 20 kb. Confirmation of the presence of the expected *Festuca* genes in the remaining 9 drought élite lines will require a more sensitive marker system than that of GISH. Here, inter-SSR sequences are shown as potential markers to follow the presence of *Festuca* DNA a *Lolium* genomic background.

Attempts have recently been made to locate SSR-enriched sites on chromosomes using the FISH technique. The GATA repeats were found to be unevenly distributed, with major sites around the centromeres in sugarbeet (Schmidt and Heslop-Harrison 1996) and chickpea (Gortner et al. 1998), while the GAA-satellite was useful for chromosome identification in barley and in related species of the *Triticeae* (Pedersen and Linde-Laursen 1994; Pedersen et al. 1996).

The work presented here identifies inter-SSR fingerinting as a valuable new marker system for genetic studies of the *Lolium/Festuca* complex. Inter-SSRs may be employed for (1) genotype and variety identification, (2) species-specific markers, (3) tracking of introgressed segments between *Festuca* and *Lolium*, (4) markers linked to agronomically important genes, (5) development of linkage maps and (6) probes for FISH to develop chromosome-specific markers.

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