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Development and characterisation of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.)

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Abstract Enrichment methods were optimised in order to isolate large numbers of simple sequence repeat (SSR) markers for perennial ryegrass (*Lolium perenne* L.), with the aim of developing a comprehensive set of loci for trait mapping and cultivar identification. Two libraries were constructed showing greater than 50% enrichment for a variety of SSR-motif types. Sequence characterisation of 1853 clones identified 859 SSR-containing clones, of which 718 were unique. Truncation of flanking sequences limited potential primer design to 366 clones. One-hundred selected SSR primer pairs were evaluated for amplification and genetic polymorphism across a panel of diverse genotypes. The efficiency of amplification was 81%. A relatively high level of SSR polymorphism was detected (67%), with a range of 2–7 alleles per locus. Mendelian segregation of alleles detected by selected SSR-locus primer pairs was demonstrated in the F_1 progeny of a pair cross. Cross-species amplification was detected in a number of related pasture and turfgrass species, with high levels of transfer to other *Lolium* species and members of the related genus *Festuca*. The identity of putative SSR ortholoci in these related species was confirmed by DNA sequence analysis. These loci constitute a valuable resource of ideal markers for the molecular breeding of ryegrasses and fescues.

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Introduction

Perennial ryegrass (*Lolium perenne* L.) is the most important forage grass species in temperate regions, and is also widely used as a turf grass (Holmes 1980). It is an obligate outbreeder with a genetically determined gametophytic self-incompatibility system (Cornish et al. 1979). Ecotypes and cultivars therefore typically show high levels of genetic variability.

Molecular-marker systems provide the means for marker-based breeding in perennial ryegrass, allowing marker-assisted selection (MAS) of key agronomic target traits such as herbage quality characters, disease resistance and resistance to abiotic stress. Implementation of molecular markers in breeding programs will permit efficient early selection of desirable genotypes, which is particularly important due to the high levels of genotype x environment interaction seen for agronomic characters in this species (Hayward et al. 1994). In addition, molecular markers may be used for DNA profiling in order to provide supporting information for varietal identification and seed-purity certification (Morell et al. 1995).

Molecular marker systems based on RFLP and RAPD (Hayward et al. 1994, 1998) as well as AFLP (King et al. 1998; Bert et al. 1999) have been developed for perennial ryegrass and have been used to construct genetic linkage maps. However, RFLP analysis is highly labour intensive, while RAPDs are dominant markers with serious reproducibility problems (Jones et al. 1997). AFLP analysis detects multiple loci with high reproducibility, but also produces dominant markers (Powell et al. 1996). Due to the likely genetic complexity of mapping crosses and population structures in this outbreeding species, the ideal marker system for both MAS and DNA profiling is simple sequence repeat polymorphism (SSRP). SSR markers are highly reproducible, genetically co-domi-

nant and multiallelic. SSRs are suitable for framework mapping as they map to the same location in different crosses, and are amenable to automation for high-throughput genotyping (Rafalksi et al. 1996).

SSR loci arise due to the local repetition of small sequence motifs in short tandem arrays. The length of the basic repeat may conventionally vary from 1 to 6 bp (Tautz 1989). Genetic polymorphism arises due to variation in the number of repeated units, probably as a consequence of slippage during DNA replication (Levinson and Gutman 1987). The variation in array length may be detected by the design of conserved PCR primers to non-repetitive flanking regions (Litt and Luty 1989; Weber and May 1989). SSR markers and SSR-based genetic linkage maps have been developed for a large number of plant taxa, including many economically important species (Akkaya et al. 1992; Wu and Tanksley 1993; Saghai Maroof et al. 1994; Roder et al. 1995; Chin et al. 1996; Taramino et al. 1997).

SSR technology has been relatively little developed for forage crop and turfgrass species, although SSR isolation for genetic diversity analysis has been reported for the turfgrass seashore paspalum (Liu et al. 1995) and SSR markers have been developed for trait mapping and genotype identification in tetraploid alfalfa and other perennial and annual *Medicago* species (Diwan et al. 1997). The isolation and characterisation of SSR loci from eukaryotic genomes is a labour-intensive and costly process. The average frequency of SSR loci is variable between plant genomes (Morgante and Olivieri 1993; Wang et al. 1994; Pfeiffer et al. 1997), limiting the efficiency of recovery from randomly cloned DNA fragments when the frequency is low. For perennial ryegrass, Kubik et al. (1999) report the isolation of 16 *bona fide* SSR-containing clones from a primary hybridisation screen of 13000 plasmid clones This apparently low rate of recovery corresponds to a large number of SSR loci within the genome as a whole, which could be accessed with a more-efficient isolation procedure. The construction of small genomic libraries enriched for SSR loci allows for recovery frequencies in excess of 50%, with considerable cost and time savings (Karagyzov et al. 1993; Kijas et al. 1994 Edwards et al. 1996; Hamilton et al. 1999). However, standard methodologies may require modification in order to obtain the highest levels of enrichment (Cordeiro et al. 1999).

Here we describe the optimisation of SSR enrichment conditions for perennial ryegrass and the structural variants of SSR loci detected in this discovery process. A set of SSR loci have been sequence-tagged by primer design, and screened for the ability to detect genetic polymorphism across a range of perennial ryegrass genotypes in order to identify a set of loci suitable for framework mapping. A sub-set of the perennial ryegrass SSR loci have been tested for the detection of conserved ortholoci in other related species of pasture and turf grass.

Materials and methods

Plant material

SSR enrichment libraries were constructed from genomic DNA extracted from a single genotype of the perennial ryegrass cultivar Ellett, and barley (*Hordeum vulgare* L. cv. Galleon) was chosen as a control to test the species-specificity of enrichment. Screening *L. perenne* SSR (LPSSR) primers for genetic polymorphism was performed using single genotypes from the following eight *L. perenne* accessions: ecotype North African (Morocco); cultivars Aries (New Zealand), Aurora (Switzerland), Victorian (Australia), Ellett (New Zealand), Yatsyn (New Zealand) and Vedette (New Zealand), and the doubled-haploid DH297. Herbage from all plants was supplied by Dr. Kevin F. Smith of the Pastoral and Veterinary Institute, Agriculture Victoria-Hamilton, except for DH297 which was supplied as a sterile meristem culture by Dr. Sue Dalton of the Institute of Grasslands and Environmental Research (IGER), Aberystwyth, U.K. Mendelian segregation of alleles detected by LPSSR primer pairs was evaluated using the heterozygous parent and selected F_1 progeny from the p150/112 family (Bert et al. 1999), supplied by Dr. Mervyn Humphreys, IGER, U.K.

Detection of putative SSR ortholoci in related species was performed using single genotypes from the following species: annual ryegrass (*Lolium rigidum* Gaud. ecotype VLR-1), Italian ryegrass (*Lolium multiflorum* Lam. cv Concord), tall fescue (*Festuca arundinacea* Schreb. cv Demeter), meadow fescue (*Festuca pratensis* Huds.), red fescue (*Festuca rubra* L.), Kentucky bluegrass (*Poa pratensis* L.), phalaris (*Phalaris aquatica* L. cv Scirocco) and oats (*Avena sativa* L.). DNA from all genotypes was extracted using a 1×CTAB method (Fulton et al. 1995). Genomic DNA from meadow fescue and red fescue was supplied by Dr. Odd-Arne Rognli of the Agricultural University of Norway.

Construction of SSR-enriched libraries and sequencing

SSR-enriched libraries of *L. perenne* were constructed using the procedure of Edwards et al. (1996), with modifications as described in the results section. Multiplex enrichment was performed with the oligonucleotides $(CA)_{20,}$ $(CT)_{15,}$ $(ACT)_{14,}$ $(AGA)_{14,}$ $(CAA)_{14,}$ $(CTA)_{14,}$ $(CTT)_{14,}$ $(GAC)_{14,}$ $(CAG)_{10,}$ $(AGC)_{14,}$ $(CAT)_{14}$ and $(ACA)_{14}$ bound to the selection filter. Dinucleotide SSR selection was performed with $(GA)_{15,}$ $(GC)_{15,}$ $(GT)_{15,}$ $(CA)_{15,}$ $(CT)_{15,}$ $(CG)_{15,}$ $(AT)_{15}$ and $(TA)_{15}$ oligonucleotides, and $(CA)_n$ enrichment was carried out with $(CA)_{15}$ oligonucleotides alone. SSR-enriched fragments were cloned into the *Bss*HII site of a modified pUC19 vector (pJV1) provided by Dr. K.J. Edwards, IACR-Long Ashton Research Station, Bristol, U.K..

Plasmids were transformed into Max Efficiency DH5α competent cells (Life Technologies), plated onto LB agar plates (Sambrook et al. 1989), containing 50 µg/ml of ampicillin, 50 µg/ml of X-galactosidase and 0.5 mM of IPTG, and incubated for 16 h at 37°C. DNA from recombinant colonies was extracted using the Wizard Plus SV (Promega Co., Madison, Wis., USA) or the QIAprep turbo (Qiagen) purification kit, and sequenced on an ABI automated sequencer (PE Applied Biosystems) using the M13 forward primer and the BIGDYE TERMINATOR (PE Applied Biosystems) cycle sequencing kit.

Classification of SSRs and primer design

Sequences containing at least five di-, tri-, tetra-, penta- or hexanucleotide repeats were selected. SSR structure was defined in terms of four categories: pure repeats of the form $(N_1N_2)_x$ or $(N_1N_2N_3)_x$; imperfect repeats of the form (e.g. $N_1N_2N_1N_2N_2N_1N_2N_2N_2N_1N_2$.....etc.); interrupted repeats of the form $(N_1N_2)_x(N)_y(N_1N_2)_z$ or $(N_1N_2N_3)_x(N)_y(N_1N_2N_3)_z$, and compound repeats of the form $(N_1N_2)_x(N_3N_4)_y$, $(N_1N_2N_3)_x(N_4N_5N_6)_y$ or $(N_1N_2)_x(N_3N_4N_5)_y$. This nomenclature resembles that of Weber (1990) except that the term 'interrupted' is used here to denote lo-

cus structures previously termed 'imperfect'. The same definition of interrupted repeats was adopted by Peakall et al. (1998). Redundant sequences were defined as clones containing the same repeat sequence, or a close variant (>95% similarity), in the 5′- and 3′- flanking sequences.

Primers were designed to the flanking regions of the SSR using the PrimerPremier 4 program (Premier Biosoft International, Palo Alto, Calif., USA) based on criteria of GC content, melting temperature and absence of secondary structure. Primers were designed in the 16–27 nucleotide range to yield amplification products of 70–400 bp, and were synthesised by Pacific Oligos (Lismore, NSW, Australia).

PCR amplification and product electrophoresis

PCR amplifications were performed in a 20-µl volume containing 25 ng of genomic DNA, 1×PCR buffer (Finnzyme, Espoo, Finland), 0.2 mM of each dNTP, 0.5 µM of each forward and reverse primer and 0.4 U of DyNAzyme II (Finnzyme) DNA polymerase. The forward primer was end-labelled with γ-^{33}P-ATP (400 Ci/mmol, Geneworks, Adelaide, S.A., Australia). PCR was performed in a MJ PT-200 (MJ Research Inc., Waltham, Mass., USA) thermocycler using one of the following touch-down profiles, depending on the T_m value of the primer pairs: (1) 10 cycles of 60 s at 94°C, 30 s at 65°C, 60 s at 72°C with a reduction of the annealing temperature of 1°C by every cycle, followed by 20 cycles of 30 s at 94°C, 30 s at 55°C and 30 s at 72°C; (2) a similar profile as (1) with an initial annealing temperature of 60°C and a final annealing temperature of 50°C; (3) a similar profile as (1) with an initial annealing temperature of 55°C and a final annealing temperature of 45°C, and (4) a similar profile as (1) with an initial annealing temperature of 50°C and a final annealing temperature of 40°C. The PCR products were denatured by adding 15 µl of a denaturing gel loading buffer (Sambrook et al. 1989) and heating at 94°C for 5 min. SSR alleles were separated by running PCR products on a denaturing 6% (w/v) acrylamide gel (19:1 acrylamide:bis-acrylamide, Amresco, Solon, Ohio, USA) in 1×TBE (Sambrook et al. 1989) at 80 W for 5000 Vh using a Biomax STS 45i DNA sequencing unit (Kodak). A 100-bp size ladder (Promega Co., Madison, Wis., USA) was included on each gel and the size of amplification products was estimated by extrapolation. Gels were transferred onto Whatman 3 MM paper and dried in a gel dryer (Bio-Rad 583) at 80°C for 45 min. Banding patterns were visualised using a Phosphorimager 400b (Molecular Dynamics, Sunnyvale Calif., USA) or by exposing gels for 48 to 72 h to X-ray film (Biomax MR, Kodak).

Primer evaluation

All primer pairs were screened on the set of eight diverse genotypes for their ability to yield an amplification product of the expected size and to detect polymorphism. For primers that detected polymorphism, the number of alleles and the polymorphism information content (PIC) of the SSR was calculated as described by Saal and Wricke (1999), based on expected heterozygosity (Hedrick 1985):

$$\mathrm{PIC} = H = 1 - \sum_{i=1}^{k} p_i^2 \qquad (1)$$

where p_i is the frequency of the i-th allele out of the total number of alleles, and k is the number of different alleles in the sample.

Cloning and sequencing of PCR amplification products

Products of cross-species amplification were cloned and sequenced. Two microliters of unlabelled PCR product were cloned into the pGEM-T Easy Vector (Promega), transformed using XL 10-Gold ultracompetent cells (Stratagene, La Jolla, Calif., USA), plated on LB agar plates (Sambrook et al. 1989), containing 50 µg/ml of ampicillin, 50 µg/ml of X-galactosidase and 0.5 mM of IPTG, and incubated for 16 h at 37°C. DNA from eight recombinant colonies from each PCR reaction was extracted and sequenced as described above. Sequences containing SSRs were compared to each other using the multiple-sequence alignment procedure Clustal W (Thompson et al. 1994).

Results

Optimisation of SSR enrichment in perennial ryegrass

Initial construction of SSR-enriched libraries according to the method of Edwards et al. (1996) gave unacceptably low levels of enrichment (<10%; data not shown). Modifications to the protocol were made in order to improve SSR enrichment levels for perennial ryegrass. The restriction enzyme used in the primary digestion, hybridisation temperature and the temperature and stringency of the post-hybridisation washes were varied, along with the oligonucleotide complexity on the selection filter.

Between five and eleven clones were sequenced from each of the libraries. SSR enrichment for barley was 100% efficient, showing that the standard protocol may be highly successful for some species. For perennial ryegrass, a reduction in the wash temperature improved multiplex enrichment, as did a reduction in the number of selective oligonucleotides to a single $(CA)_{15}$ oligonucleotide. A reduction in the hybridisation temperature did not improve enrichment, nor did a change in the restriction enzyme (Table 1).

The library based on selection for $(CA)_n$ repeats (approximately 50% SSR enrichment) was designated LPSSRH. The library based on multiplex selection for a number of dinucleotide and trinucleotide repeats with a reduced wash temperature (approximately 60% SSR enrichment) was designated LPSSRK. These libraries were selected for large-scale SSR discovery in perennial ryegrass.

Characterisation of SSR loci

DNA sequences were obtained from a total of 1853 clones, of which 859 (46%) contained SSR loci (Table 2). The level of redundancy across all libraries was 16% (141 clones). Redundancy was most commonly found within the same library, although duplicates across libraries were also observed. Redundancy may be attributable to clone duplication, allelism (since an outbred multiple heterozygous genotype of *L.perenne* was used as the source of DNA for library construction), or to locus duplication. The 718 unique SSR clones were further classified into truncated and non-truncated categories. The criterion for truncation was the presence of less than 25 bp of either 5′- or 3′-flanking sequence. A total of 366 unique SSRs were non-truncated and were directly accessible to PCR primer design. The two main libraries differed in terms of redundancy and levels of truncation, with LPSSRH showing a higher level of redundancy

Table 1 SSR enrichment levels in libraries constructed using various modifications to the standard method

Species	Restriction enzyme	Filter[a]	Hybridisation temperature (°C)	Wash temperature (°C)	[SSC][b]	% SSRs[c]
L. perenne	*Rsa*I	Multiplex	50	50	0.5×	16
L. perenne	*Rsa*I	Multiplex	40	50	0.5×	0
L. perenne	*Rsa*I	Multiplex	50	45	0.5×	60 (K[d])
L. perenne	*Rsa*I	Multiplex	50	50	1.0×	16
L. perenne	*Ssp*I	Multiplex	50	50	0.5×	0
L. perenne	*Hinc*I	Multiplex	50	50	0.5×	0
L. perenne	*Rsa*I	Di	50	50	0.5×	14
L. perenne	*Rsa*I	CA	50	50	0.5×	50 (H[d])
H. vulgare	*Rsa*I	Multiplex	50	50	0.5×	100

[a] Oligonucleotide repeats bound to filter. Multiplex=mixture of di- and tri-nucleotide repeats; Di=di-nucleotide repeats alone; CA=CA repeat alone
[b] Concentration of SSC used in final three washes. All libraries had an initial five washes at 2×SSC. All washes contained 0.01% (w/v) SDS
[c] Percentages based on sequencing 5–11 clones per library
[d] Selected LPSSR library

Table 2 Large-scale SSR discovery in different libraries from *L. perenne*

Item	Library[a]			Total
	LPSSRH	LPSSRK	Other	
Number of clones sequenced	510	1314	29	1853
Clones containing SSR loci[b] (percentage of clones sequenced)	197 (38%)	648 (49%)	14 (48%)	859 (46%)
Redundant SSR clones[c] (percentage of clones containing SSRs)	48 (24%)	92 (14%)	1 (7%)	141 (16%)
Unique SSR clones (percentage of clones sequenced)	149 (29%)	556 (42%)	13 (45%)	718 (39%)
Truncated (<25 bp flanking sequence) SSR clones (percentage of unique SSR clones)	96 (64%)	248 (45%)	8 (62%)	352 (49%)
Unique, non-truncated SSR clones (percentage of clones sequenced)	53 (10%)	308 (23%)	5 (17%)	366 (28%)

[a] For description of libraries, see Table 1
[b] The operational definition of an SSR was the presence of at least five repeats of a di-, tri-, tetra-, penta- or hexa-nucleotide repeat
[c] Duplicate SSRs with >95% sequence similarity

Table 3 Frequency and type of di-, tri- and ≥tetra-nucleotide repeats isolated from the *L. perenne*-enriched libraries LPSSRH and LPSSRK

Repeat	LPSSRH[a]					LPSSRK[a]				
	Perfect[b]	Interrupted	Imperfect	Compound	Total	Perfect	Interrupted	Imperfect	Compound	Total
Dinucleotide	49%	28%	4%	9%	90%	25%	17%	7%	3%	52%
Trinucleotide	4%	<1%	3%	0%	8%	22%	9%	10%	4%	45%
≥Tetranucleotide	<1%	<1%	<1%	0%	2%	<1%	0%	2%	0%	3%
Total	54%	30%	8%	9%	100%	47%	26%	20%	7%	100%

[a] Percentages based on a total of 149 unique SSR clones from LPSSRH and 556 unique SSR clones from LPSSRK. See Table 1 for details of library construction
[b] See Materials and methods for definitions of repeat structure types

(24%) and truncation (64%) than LPSSRK (14% redundancy and 45% truncation).

SSR loci were classified in terms of repeat type and structure (Table 3). The LPSSRH library was almost exclusively (90%) composed of dinucleotide repeats, although a number of trinucleotide (8%) and tetranucleotide (2%) repeat types were co-selected. The LPSSRK library contained similar numbers of dinucleotide (52%) and trinucleotide (45%) repeats and a low level (3%) of ≥tetranucleotide repeats. Although the majority of SSR loci from both libraries had a perfect repeat structure, significant numbers of interrupted, imperfect and compound loci were also identified.

Table 4 shows the proportion of different repeat motifs in each of the structural categories by library. In both the LPSSRH and LPSSRK libraries, the predominant dinucleotide repeat was of the type $(CA)_n$. Figure 1 shows the distribution of motif repeat length for the perfect repeat type $(CA)_n$ in each library. LPSSRH shows a variation from n=5 to n=53 with a mean value of n=19, while

Table 4 Frequency of SSR motif types in LPSSRH and LPSSRK libraries

SSR motif type	Library	
	LPSSRH[a]	LPSSRK[a]
Dinucleotide SSRs[b]		
(CA), (AC), (TG), (GT)	78%	33%
(GA), (AG), (CT), (TC)	3%	16%
(AT), (TA)	0%	< 1%
(CG), (GC)	0%	0%
Compounds	9%	3%
Trinucleotide SSRs[b]		
(GAA), (AAG), (AGA), (TTC), (TCT), (CTT)	5%	15%
(CAA), (AAC), (ACA), (TTG), (TGT), (GTT)	< 1%	13%
(CAT), (ATC), (TCA), (ATG), (TGA), (GAT)	0%	7%
Other	3%	6%
Compounds	0%	4%
≥Tetranucleotide SSRs[b]	2%	3%

[a] See Table 1 for details of library construction. Percentages are based on a total of 149 unique SSR clones from LPSSRH and 556 unique SSR clones from LPSSRK

[b] Includes perfect, interrupted and imperfect SSR types. SSRs are divided into motif types with similar nucleotide structures

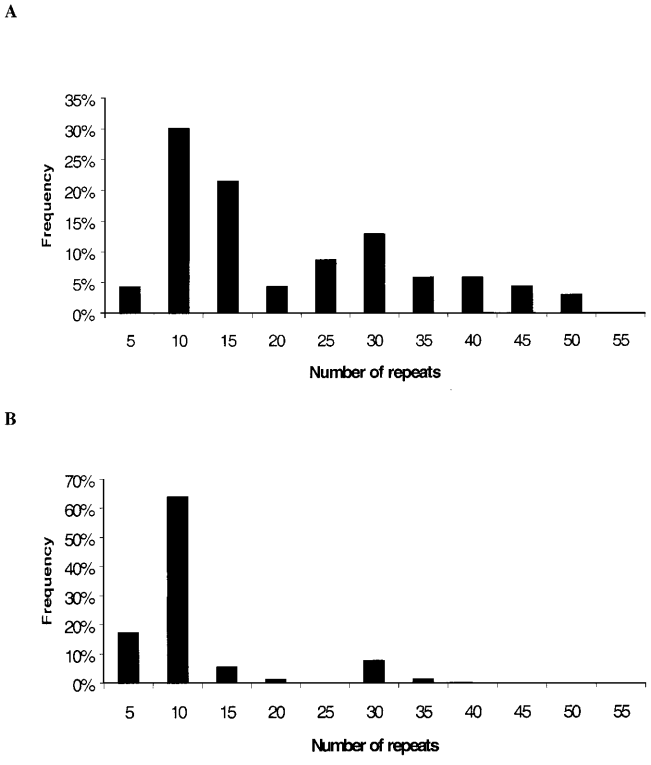

Fig. 1A, B Frequency distributions of SSR loci by repeat array number for $(CA)_n$ repeats. **A** Analysis of the LPSSRH library, based on 70 SSR loci. **B** Analysis of the LPSSRK library, based on 94 SSR loci

LPSSRK shows a variation from *n*=5 to *n*=51 with a mean value of *n*=11. Across all dinucleotide repeat types, the average motif repeat length was higher in LPSSRH (*n*=19; range of *n*=5–53) than LPSSRK (*n*=12; range of 5–51; data not shown). For the trinucleotide SSRs, the mean repeat length was again higher in LPSSRH (*n*=17; range of *n*=7–27) than LPSSRK (*n*=12; range of *n*=5–35; data not shown).

SSR polymorphism

PCR primers were designed for 101 LPSSR loci. Primer length varied from 16 to 27 nucleotides (average 20 nucleotides), with a range of G/C contents from 28% to 72% (average 50%, data not shown). The expected sizes of amplification products were estimated from the cloned sequences and varied from 70 bp to 400 bp (average 185 bp, data not shown). The predicted optimal annealing temperatures varied from 46°C to 58°C (average 52°C, data not shown). Primer pairs were tested for their ability to amplify DNA and detect polymorphism across eight perennial ryegrass genotypes. Details for ten representative primer pairs are shown in Table 5. From 101 primer pairs, 82 (81%) produced a clear amplification product within the expected size range and of these 67% (55) detected polymorphism (Table 6). Between two and seven alleles were detected across the eight genotypes (average 3.5), with polymorphism information content (PIC) values of between 0.2 and 0.8 (average 0.56). A typical amplification profile is shown in Fig. 2A.

The frequency of amplification was found to be uncorrelated with either primer length, primer G/C content, the calculated annealing temperature or expected fragment size (data not shown). A lower than average level of amplification was found for SSRs with >20 repeats when analysed across different SSR structural groups (Table 6). When the level of polymorphism within the different SSR categories was examined, polymorphism was found to be lowest for imperfect SSRs, trinucleotide SSRs, and SSRs with <10 motif repeats (Table 6). LPSSRK had a higher proportion of each of these categories compared to LPSSRH, leading to lower overall polymorphism.

The capacity of LPSSR loci to detect segregating polymorphism in a perennial ryegrass pair cross was evaluated. Co-dominant polymorphic genetic markers show either a AB×BB or AB×CC cross structure in the p150/112 family, allowing a full classification of two genotypic classes in the F_1 progeny. LPSSRH01H06 de-

Table 5 A selection of primer sequences designed for SSR loci that yielded amplification products of the expected size across eight *L. perenne* genotypes

SSR	Primer sequence (5′–3′)		Repeat motif/ repeat class	Expected size (bp)	Polymorphic	No. of alleles	PIC[a]
LPSSRH01A02	F	AAAGACCGCATACGAAGT	$(CA)_{27}$	131	Yes	5	0.78
	R	AACCAAAGCCTCAAGACA	Perfect				
LPSSRH01A07	F	TGGAGGGCTCGTGGAGAAGT	$(GT)_9$	77	Yes	3	0.59
	R	CGGTTCCCACGCCTTGC	Imperfect				
LPSSRH01A10	F	GAGGCACCGGCCATGGAG	$(CTT)_{20}$	152	Yes	4	0.72
	R	AGGACGAGCCACTCACTTG	Imperfect				
LPSSRH01D09	F	CAAGTGCCACCATAGATACAA	$(AG)_8$	262	No	1	–
	R	CGTGAAGATCACTATAAACACGA	Imperfect				
LPSSRH01E10	F	CGCAGCTTAATTTAGTC	$(CA)_{10}$	103	Yes	4	0.71
	R	GCTTTGAGTATGTAAAGTT	Perfect				
LPSSRH01F02	F	TCTGTGGGTCCTTCTGGAT	$(TCGC)_6$	145	No	1	–
	R	TCGGGTGATGATGTTGACTT	Imperfect				
LPSSRH01H06	F	ATTGACTGGCTTCCGTGTT	$(CA)_9$	150	Yes	4	0.59
	R	CGCGATTGCAGATTCTTG	Perfect				
LPSSRH02C11	F	TGGAATAACGATGAAAAG	$(CA)_4$ TA $(CA)_4$	198	Yes	7	0.82
	R	CATCACGAATTAACAAGAG	Interrupted				
LPSSRK01A03	F	GGACGAACTGCCGAGACA	$(CTT)_7$	247	No	1	–
	R	CGGGCATGGTGAGAAGGA	Perfect				
LPSSRK01A11	F	CGGCCACCCTTGATAGAG	$(CA)_{21}$	205	Yes	4	0.65
	R	TCGTCAAGGATCCGGAGA	Imperfect				

[a] Polymorphism information content as described in Materials and methods

Table 6 Amplification and polymorphism data for different SSR categories across eight *L. perenne* genotypes

Category	Amplification efficiency (%)	Polymorphism in screening panel[a]	Range of allele number detected	Average polymorphism information content (PIC)
All SSRs	81% (82/101)	67%	2–7	0.56
By SSR class[b]:				
Perfect SSRs	79% (41/52)	73%	2–7	0.60
Interrupted	81% (21/26)	67%	2–7	0.51
Imperfect SSRs	88% (15/17)	60%	2–4	0.55
By SSR types[b]:				
Dinucleotide repeats	82% (67/82)	72%	2–7	0.56
Trinucleotide repeats	78% (14/18)	50%	2–4	0.55
By repeat number[b]:				
5–10 repeats	89% (33/37)	58%	2–5	0.53
11–20 repeats	89% (32/36)	72%	2–7	0.57
21–30 repeats	50% (16/32)	69%	2–5	0.61
By library				
LPSSRH	85% (50/59)	78%	2–7	0.56
LPSSRK	78% (31/40)	52%	2–5	0.57

[a] Percentage of successful amplifications

[b] Structural classes from which <10 primer pairs were tested (compound, ≥tetranucleotide repeats, SSRs with >30 repeats) are not described

tects three alleles in a sub-set of p150/112 F_1 progeny (Fig. 2B), with a monomorphic allele contributed by the homozygous DH290 parent and the two segregating alleles showing a ratio in close agreement with Mendelian expectation (1:1).

Detection of SSR ortholoci in related species

In order to evaluate cross-species amplification by perennial ryegrass SSR-locus primer pairs, 50 LPSSR loci were selected from the set of 100 loci screened for polymorphism in perennial ryegrass. The efficiency of cross-species amplification varied from 80% for annual ryegrass to 12% for oats (Fig. 3A). Representative results of

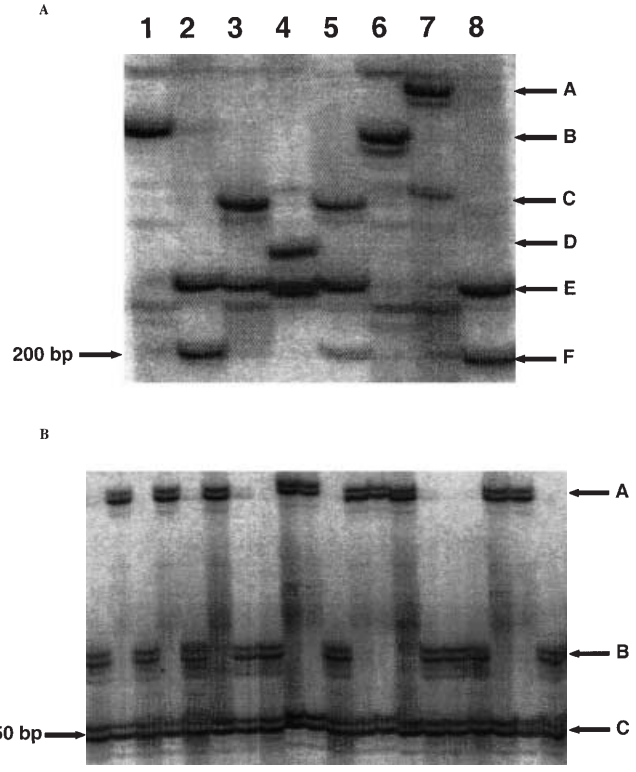

Fig. 2A SSR amplification products amplified by locus LPSSRH02D12 for eight genotypes of *L. perenne*. *1* North African; *2* Aries; *3* Aurora; *4* Victorian; *5* Ellett; *6* Yatsyn; *7* Vedette; *8* DH297. The SSR structure in the cloned sequence is $(CA)_{12}$ and the predicted amplification product size is 219 bp. Six alleles designated *A* (largest) to *F* (smallest) are indicated. **B** Segregating polymorphism for SSR alleles detected by locus LPSSRH01H06 in 20 F_1 progeny from the p150/112 mapping population. Three alleles are indicated, designated *A* (largest) to *C* (smallest). The segregating alleles *A* and *B* are derived from the heterozygous parent, while the monomorphic *C* allele is inherited from DH290. The SSR structure in the cloned sequence is $(CA)_9$ and the predicted amplification product size is 150 bp

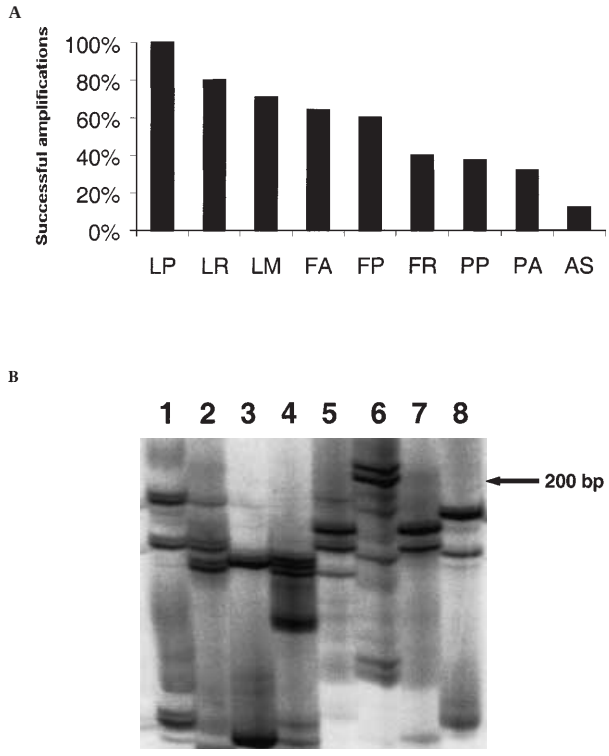

Fig. 3A Frequency of cross-species amplification by *L. perenne* SSR loci in related species. *LP*: *L. perenne*; *LR*: *L. rigidum*; *LM*: *L. multiflorum*; *FA*: *F. arundinacea*; *FP*: *F. pratensis*; *FR*: *F. rubra*; *PP*: *P. pratensis*; *PA*: *P. aquatica*; *AS*: *A. sativa* **B** SSR ortholocus detection among Poaceae species related to *L. perenne*. Amplified products were obtained for locus LPSSRH01H06. *1*: *L. rigidum*; *2*: *L. multiflorum*; *3*: *F. pratensis*; *4*: *F. arundinacea*; *5*: *F. rubra*; *6*: *P. aquatica*; *7*: *P. pratensis*; *8*: *A. sativa*

amplification across each of the related species are shown (Fig. 3B).

In order to demonstrate the presence of *bona fide* SSR ortholoci in the related species, PCR products were cloned from amplifications using primer pairs which showed substantial cross-species transfer. Figure 4 shows a DNA sequence alignment for locus LPSSRH01H06, using a single representative clone from each species. The cloned LPSSRH01H06 sequence from enrichment library LPSSRH shows a very high degree of identity with a PCR-derived clone from perennial ryegrass, with divergence confined to the SSR array $[(GT)_{10}$ and $(GT)_6]$ and several individual nucleotide positions in the 3′-region flanking the SSR. Both *L. perenne* and *L. multiflorum* contain perfect (GT)*n* repeats, while the remaining species contain interrupted SSRs of variable length. The *L.perenne* sequences contain an approximately 30-bp deletion relative to the other sequences immediately downstream from the SSR. Some clone to clone variation was observed among sequences derived from single species, which may be attributable to PCR errors and/or allelic variation due to heterozygosity (data not shown).

Discussion

The data presented here demonstrate that large numbers of SSR loci may be isolated from perennial ryegrass using the enrichment library technology. Previous studies on this species based on non-enriched libraries and colony hybridisation obtained only a small number of loci at a frequency of 0.12% of the clones screened (Kubik et al. 1999). The estimated prevalence of common SSR loci $[(GA)_n$ or $(CA)_n]$ in perennial ryegrass from this study (one per 350 kb on average) was towards the low end of the reported range for plant taxa (Condit and Hubbell 1991). Nonetheless, such an estimate of prevalence implies the presence of several thousand SSR loci for each common motif type.

The standard procedure of Edwards et al. (1996) required substantial modifications in order to obtain libraries showing greater than 50% SSR enrichment for perennial ryegrass. Similar problems were encountered for SSR enrichment in sugarcane (Cordeiro et al. 1999).

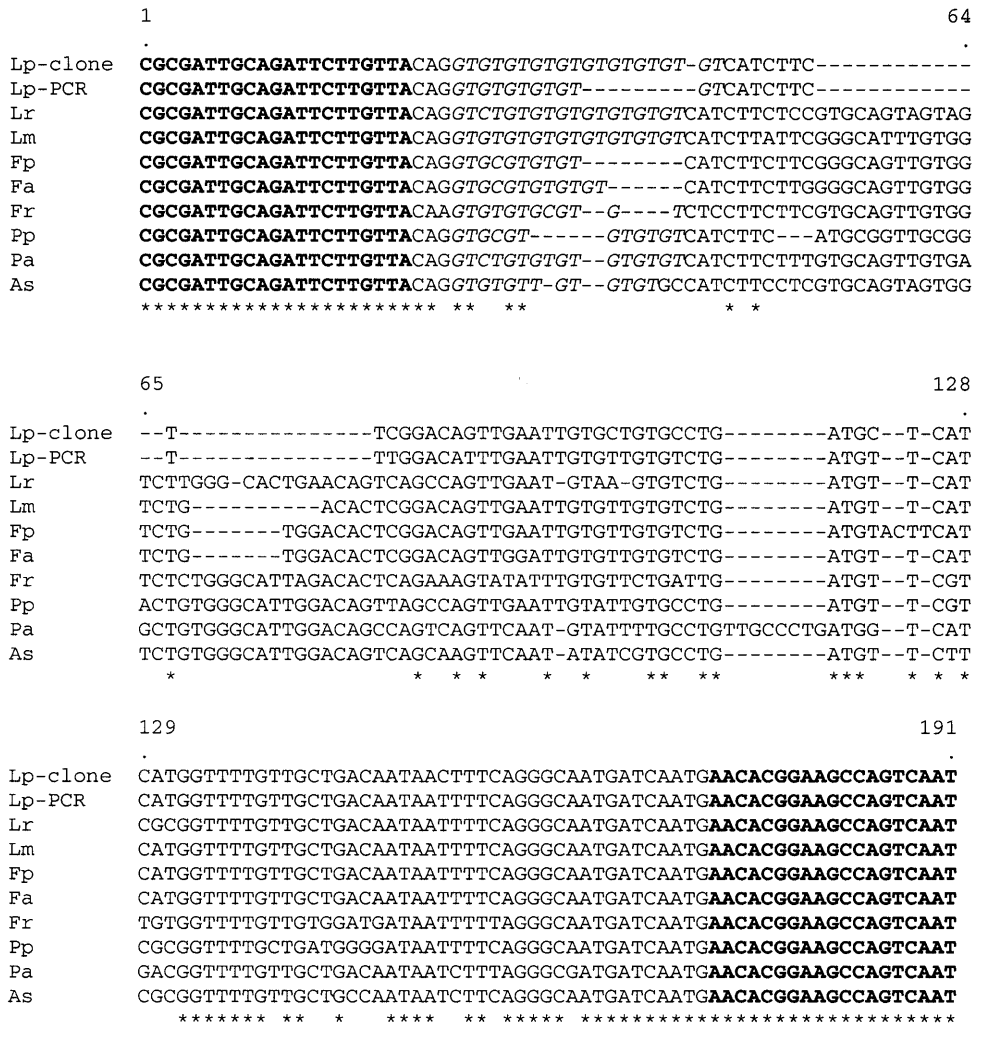

Fig. 4 Sequence comparison of SSR ortholoci. Primer pairs designed for locus LPSSRH01H06-amplified sequences from the following related species, detecting the indicated SSR structure (in *italics*): *Lp-clone*: *L. perenne* enrichment library H clone, $(GT)_{10}$; *Lp-PCR*: *L. perenne* amplification product clone, $(GT)_6$; *Lr*: *L. rigidum*, $(GT)CT(GT)_7$; *Lm*: *L. multiflorum*, $(GT)_9$; *Fp*: *F. pratensis*, $(GT)GC(GC)_3$; *Fa*: *F. arundinacea*, $(GT)GC(GC)_4$; *Fr*: *F. rubra*, $(GT)_3GC(GT)_2$.; *Pp*: *P. pratensis*, $(GT)G(GT)_4$; *Pa*: *P. aquatica*, $(GT)CT(GT)_6$; *As*: *A. sativa*, $(GT)_3T(GT)_3$. DNA sequences were aligned in 5′→3′ orientation and numbered from bp positions 1 to 191. The primer sequences are indicated in *bold*. Sequence identities across all taxa are indicated with an *asterisk* (*)

In both species, modifications of the post-hybridisation washing conditions contributed to substantial improvements in SSR recovery. In this study, reduction in the complexity of the selective oligonucleotide mixture also resulted in improved recovery. Multiplex selection may favour the binding of a single genomic fragment to multiple synthetic oligonucleotide sequences, allowing the capture of sequences which lack SSRs but may show high levels of cryptic simplicity (Tautz et al. 1986). The importance of SSR enrichment optimisation for each target species is clearly demonstrated.

Redundancy in SSR enrichment libraries limits the number of effective loci which may be developed. The value of 16% reported here is lower than reported in other studies (24% in tea tree: Rossetto et al. 1999). Clones were considered redundant if they contained >95% similarity in their 5′- and 3′-flanking sequence. Some of the redundant clones with a variation in their nucleotide composition may prove informative for the design of allele-specific single-nucleotide polymorphisms (SNPs) or for the detection of duplicated loci.

The successful conversion of SSR-positive clones into sequence-tagged LPSSR loci was highly limited by truncation of the cloned sequences (49%), either within the SSR array itself or within 25 bp of the 5′- or 3′-sequence flanking the SSR, such that primer design was difficult. Truncation has been observed in enrichment libraries derived from other species (Cordeiro and Henry 1999). The use of the restriction enzyme *Rsa*I may have contributed to the isolation of clones truncated within $(CA)_n$ SSR arrays, due to the presence of a 5′-AC-3′ motif within the enzyme recognition site (5′-GTAC-3′). The LPSSRH library showed particularly high levels of truncation, probably because this library was rich in $(CA)_n$ repeats with an average motif repeat length higher than for the LPSSRK library. Long SSR repeat arrays within small cloned fragments are more likely to be close to the end of insert sequence.

The predominant SSR motif isolated in this study was of the type $(CA)_n$. This was anticipated for the LPSSRH library, which was constructed using a $(CA)_{15}$ selection filter, but was also found to be the case for the multiplex-enriched LPSSRK library. Although $(CA)_n$ repeats have been reported to be rare in database surveys (Morgante and Olivieri 1993; Wang et al. 1994), they are apparently present in significant numbers in the perennial ryegrass

genome, as reported for other Poaceae genomes (Liu et al. 1995; Cordeiro and Henry 1999; Saal and Wricke 1999). The presence of substantial numbers of the SSR type $(GA)_n$ in the LPSSRK library reflects the abundance of this other common dinucleotide motif type. The $(AT)_n/(TA)_n$ motif, reported to be the most common type in plants (Powell et al. 1996), is normally excluded from enrichment libraries due to its self-complementary nature and is not significantly represented. The most common trinucleotide repeats in LPSSRK were of the type $(GAA)_n$ (15%) and $(CAA)_n$ (13%). Interrupted, imperfect and compound SSRs were substantially represented in both libraries.

The level of polymorphism detected across the panel of perennial ryegrass genotypes (67%) was relatively high, as expected for an allogamous species. Amplification efficiency was lowest for SSRs with >21 repeats, probably due to the inefficiency of in vitro DNA replication over long repetitive regions. Trinucleotide repeats showed lower levels of polymorphism than dinucleotide SSRs, as shown for ISSR markers in rice (Blair et al. 1999). The lower levels of polymorphism detected by interrupted and imperfect SSRs may be associated with the initial stages of mutational decay, so that replication slippage is less-likely to occur (Smulders et al. 1997). Increased levels of polymorphism were observed for >10 repeats, consistent with the association between higher repeat length and polymorphism described by Weber (1990). The higher proportion of short, imperfect and trinucleotide repeat types in the enriched library LPSSRK meant that the frequency of polymorphic SSRs that could be isolated was ultimately not much higher than from the LPSSRH library, despite the favourable attributes of LPSSRK in terms of enrichment levels and reduced truncation.

Previous studies have demonstrated limited transfer to distantly related species (Whitton et al. 1997) suggesting that effective cross-amplification may be limited to members of the same genus or closely related genera (Lagercrantz et al. 1993; White and Powell 1997; Peakall et al. 1998; Devey et al. 1999; Echt et al. 1999). In the present study, high levels of cross-amplification were seen in the two closely related ryegrass species *L.rigidum* (80%) and *L.multiflorum* (71%). Amplification was slightly lower in the *Festuca* species, followed by Kentucky bluegrass, phalaris and oats. Amplification levels reflected the phylogenetic relationships between these species. Both *L. rigidum* and *L. multiflorum* are capable of efficient interspecific hybridisation with perennial ryegrass, producing fertile F_1 hybrids (Naylor 1960). Kubik et al. (1999) also report efficient cross-amplification with these species, along with the inbreeding *Lolium* taxa *Lolium temulentum*, *Lolium remotum* and *Lolium persicum*. The genera *Lolium* and *Festuca* are closely related and are capable of forming intergeneric hybrids (Jenkins 1989). Meadow fescue (*Festuca pratensis*) is believed to be closely related to the outbreeding *Lolium* species (Darbyshire 1993; Xu and Sleper 1994; Stammers et al. 1995; Charmet et al. 1997), while some models for the evolution of the allohexaploid species *F. arundinacea* have invoked the presence of a *Lolium*-like genome (Borrill, 1976). *L. multiflorum*, *F. pratensis* and *F. arundinacea* have been reported to be much more closely related to one another than to *F.rubra* (Lehväslaiho et al. 1987). The genus *Poa* is believed to be allied to the *Lolium*/*Festuca* species complex (Yaneshita et al. 1993). Kubik et al. (1999) reported a 46% cross-amplification success rate from *L.perenne* to *P. pratensis*, close to the value of 38% in our study. The *Lolium*, *Festuca* and *Poa* species are all members of the tribe Poeae. Phalaris and oats are members of the tribe Aveneae which has been placed close to the tribe Poeae in modern phylogenies of the Poaceae (Devos and Gale 1997; Soreng and Davis 1998).

DNA sequence analysis of individual cloned amplification products confirmed the presence of SSR repeats in all of the test species for the locus LPSSRH01H06, although interrupted SSRs were detected in all of the taxa apart from *L. perenne* and *L. multiflorum*. A high degree of sequence conservation was detected in the 3′-flanking region, although single nucleotide differences and various pairwise insertion/deletion polymorphisms distinguished each of the species.

This paper reports the isolation and characterisation of a large number of polymorphic SSR markers for perennial ryegrass. The detection of LPSSR polymorphism in the p150/112 reference family will allow the construction of a framework SSR map of *L. perenne*. These markers will then be used to map and tag genes in trait-specific segregating populations for marker-assisted breeding, as well as for cultivar identification and seed-purity analysis. SSRs will provide an ideal marker system for molecular marker-based breeding in perennial ryegrass.

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