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The development of sequence-tagged sites (STSs) in *Lolium perenne* L.: the application of primer sets derived from other genera

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Abstract Genetic analysis, particularly the development of genetic linkage maps in forage grass species, lags well behind other members of the *Poaceae*. Comparative mapping within this family has revealed extensive conservation in gene and marker synteny among chromosomes of diverse genera. Recently, the ability to transfer mapped STS markers between barley and wheat has been demonstrated. The transfer of mapped STS markers between cereals and forage grasses could provide PCR-based markers for comparative mapping in these species providing they amplify homologous sequences. In this study, primers derived from three barley genes of defined function and a gene from *Phalaris coerulescens* were used to amplify homologous fragments in *Lolium perenne*. Primers derived from two barley and two oat cDNA clones were also tested along with eight barley and two *Triticum tauchii* STS markers. Twenty one primer pairs derived from 18 loci were tested. Eleven primer pairs (52%) amplified homologous sequences in *L. perenne* from ten (55%) of the loci targetted. Thirteen new STS markers were generated in *L. perenne*, of which ten have been mapped in barley or rye and amplify homologous sequences in *L. perenne*.

Keywords Sequence tagged sites · PCR · Homology · Wheat · Barley · *Lolium* · Forage grasses

Introduction

The genera *Lolium* and *Festuca* belong to the family *Poaceae* subfamily *Festucoideae*, tribe *Festucaceae*. Perennial ryegrass (*Lolium perenne* L., *2*n=*2x=14*) is one of the most-widely cultivated forage and amenity grasses in temperate climates while Italian ryegrass (*Lolium multiflorum* L., *2*n=*2x=14*) is used widely for hay and silage production. Meadow fescue (*Festuca pratensis* Huds., *2*n=*2x=14*) and tall fescue (*Festuca arundinaceae* Schreb., *2*n=*6x=42*) are also widely used as fodder.

Comparative mapping based on RFLP markers has revealed extensive conservation of synteny within the *Triticeae* (Nelson et al. 1995a, b, c; Van Deynze et al. 1995a; Marino et al. 1996). Importantly, conservation of gene and marker colinearity has been extended to the genomes of other *Poaceae* species (Ahn and Tanksley 1993; Ahn et al. 1993; Kurata et al. 1994; Sherman et al. 1995; Van Deynze et al. 1995b; Devos et al. 1998) shedding light on genome structure and evolution. Comparative genetics within the *Poaceae* has also allowed the identification of putative orthologous loci controlling agronomic traits (Paterson et al. 1995; Laurie 1997; Devos and Gale 1997; Sarma et al. 1998), thereby opening up the possibility of cross-species, map-based approaches to the cloning of genes of agronomic importance.

A number of genetic linkage maps have been published for various forage grass species. Hayward et al. (1994) presented a linkage map of *Lolium* incorporating restriction fragment length polymorphic (RFLP) markers, randomly amplified polymorphic DNA (RAPD) markers, and a number of isozyme loci. This map has recently been extended and now includes 83 RFLP markers of which a small number derive from members of the *Triticeae* (Hayward et al. 1998). More recently, Bert et al. (1999) have described the generation of a high-density linkage map of *Lolium* based upon amplified fragment length polymorphism (AFLP) markers. Both maps are likely to be merged in the near future thereby providing a high-density linkage map suitable for the analysis of quantitative trait loci (QTLs).

As genetic linkage mapping in forage grass species progresses, it will be important to utilise molecular markers which will allow the maps generated to be

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aligned with each other as well as with those already derived for other grass species, specifically those of rice and barley. This will enable an assessment of gene and marker colinearity, as well as the wealth of mapping data in these species to be accessed by forage grass researchers and breeders. To-date, comparative mapping studies have relied upon RFLP markers, principally those derived from complementary DNA (cDNA) probes due to their ability to detect homologous sequences in distantly related genomes. Van Deynze et al. (1998), for example, have assembled a set of cDNA probes previously mapped in various species of the *Poaceae* which hybridize well across a diverse range of species, are low-copy and provide good genome coverage. Inclusion of these markers is the most-logical means for allowing the alignment of *Lolium*/*Festuca* maps with those of barley and rice.

While RFLP probes are well suited to comparative genetic mapping studies, screening large numbers of heterologous RFLP markers across many diverse *Lolium* and *Festuca* mapping populations may not be feasible. PCR-based marker systems have the benefit of being faster, cheaper and less technically demanding. However, of the more-widely used PCR-based markers (i.e. AFLPs, SSRs), few are capable of cross-species amplification of homologous sequences (Westman and Kresovich 1998). Sequence tagged site (STS) markers are generated by designing oligonucleotide primers from a known sequence which specifies a unique locus within the target genome. Typically, sequences used for the generation of STS markers include genomic DNA clones and complementary DNA (cDNA) clones previously characterised via Southern analysis (i.e. RFLP probes). In contrast to AFLP and SSR markers, primer binding sites are more-likely to be conserved across species for STS markers derived from cDNA RFLP clones (Blake et al. 1996; Mano et al. 1999). Erpelding et al. (1996), for example, have shown that STS markers can be readily transferred between wheat and barley. Consequently, STS markers may offer a reliable PCR-based system for mapping orthologous loci across distantly related species and, hence, aid in the alignment of genetic linkage maps from divergent species.

The first objective of the present study was to investigate whether oligonucleotide primers derived from barley genes cloned in related programmes within our laboratory could be used to PCR-amplify orthologous loci from *L. perenne*. A primer pair derived from a novel thioredoxin-encoding gene cloned from *Phalaris coerulescens* was also tested. The cloned PCR products were sequenced in order to determine whether the primers used amplified homologous sequences in *L. perenne*. STS markers were then developed either by using the original barley primers or by designing *Lolium*-specific primers. Initial success also led us to test whether selected barley and oat cDNA RFLP probes could be used to develop primer pairs which would amplify homologous sequences in *L. perenne*. Finally, a number of published STS markers from barley and *Triticum tauchii* were screened across *L. perenne*. PCR fragments from selected markers were cloned and sequenced to assess the ability of primer pairs to target homologous sequences in *L. perenne*.

Material and methods

DNA isolation

Genomic DNA was isolated from *L. perenne* leaf tissue as described by Taylor et al. submitted.

Source of oligonucleotide primers

Heterologous primers

Oligonucleotide primers derived from genomic and complementary DNA clones encoding barley L-asparaginase (ASN; GenBank accession no. AF308474), asparagine synthetase 1 (AS1; GenBank accession no. AF307145) and $HS1^{vul}$ (GenBank accession no. AF307333) were used to screen in *L. perenne*. In addition, primers derived from a novel thioredoxin-encoding gene isolated from *P. coerulescens* (Li et al. 1997; Langridge et al. 1999) were also used to screen in *L. perenne*.

Triticeae *group-5 RFLP clones*

Two barley and two oat RFLP clones previously mapped to the long arm of barley chromosome 5H were selected for analysis. Sequence data was already available for clones BCD450 and CDO1508 (Van Deynze et al. 1998). The remaining two clones (BCD183 and CDO504) were cycle-sequenced using the Ampli*Taq* BigDye Deoxy Terminator sequencing kit (PE Biosystems, Denmark) and the M13 forward (-40) and reverse sequencing primers (Promega, Madison, Wis., USA) according to the manufacturer's instructions. Sequences were analysed on an Applied Biosystems International (ABI) 310 capillary DNA sequencer. Oligonucleotide primers were designed using the Oligo ver. 5.0 primer analysis software package (NBI, Plymouth, Minn., USA) and commercially synthesised (DNA Technology, Aarhus, Denmark).

Barley and T. tauchii *STS markers*

Barley STS markers were obtained from Dr. Y. Mano. Primer sequences and map positions for all markers used are given in Mano et al. (1999). *T. tauchii* STS markers were obtained from Dr. L. Talbert. Primer sequences and mapping information for a number of these markers is given in Erpelding et al. (1996) while information for the complete set can be accessed over the World Wide Web (WWW) in GrainGenes (http://wheat.pw.usda.gov/).

Polymerase chain reaction (PCR)

Preliminary screening

PCR reactions were carried out in a total volume of 10 µl. Each reaction contained 0.5–1.0 U of *Taq* (Promega, Madison, Wis., USA), Ampli*Taq* (PE Biosystems, Denmark) or *BIO-X-ACT* polymerase (Bioline, London, UK), 1–6 pmol of each primer (1 pmol for heterologous and *Triticeae* group-5 primers, 3 pmol for barley STS primers and 6 pmol for *T. tauchii* STS primers), 200 µM of dNTPs, 1.0, 1.5, 2.0, 2.5 or 3.0 mM of $MgCl_2$, 1× PCR buffer and 50–100 ng of total genomic DNA. Annealing temperatures ranged from 45°C (*T. tauchii* STS markers), 50°C (barley STS markers) and 53–55°C (heterologous and *Triticeae* group-5

markers). PCR cycling parameters were typically 36 cycles of 30–60 s denaturation, annealing and extension (heterologous and *Triticeae* group-5 markers) or 36 cycles of 30–60 s denaturation and annealing and 120 s extension (barley and *T. tauchii* STS markers). PCR reactions involving "GenBar" primers and "KM" primers required 1 U of *BIO-X-ACT* polymerase and a 1×high-specificity buffer in order to amplify the correct target sequence (S. Borg and K. Madsen, personal communication).

PCR optimisation

Heterologous primers and Triticeae *group-5 primers*

Preliminary screening allowed the determination of appropriate PCR conditions (i.e. $MgCl_2$ concentration and cycling paramenters) for all primer pairs under analysis. Reactions were carried out using a PTC-100 (MJ Research Inc., Watertown, Mass., USA) or a Biometra T3 (Biometra biomedizinische Analytik GmbH, Goettingen, Germany) PCR machine as described above.

Barley and T. tauchii *STS markers*

STS markers were optimised using a temperature-gradient PCR machine (Biometra TGradient, Biometra biomedizinische Analytik GmbH, Goettingen, Germany). All reactions were carried out using *Taq* polymerase and 2.0 mM of $MgCl_2$. Cycling parameters consisted of: 94°C 5 min, followed by 36 cycles consisting of 94°C 30 s, 50°C (gradient ±10°C) 30 s, 72°C 2 min, with a final extension step of 72°C for 5 min. Seven samples per primer pair were included across the gradient at temperature points of 44.0, 46.4, 48.8, 51.2, 53.6, 55.9 and 58.1°C.

Optimised PCR-cycling parameters

All optimised PCR reactions included an initial denaturation step of 94°C for 5 min for reactions using *Taq* (*T*) and *BIO-X-ACT* (*B*) polymerase, or 94°C for 7 min for reactions using Ampli*Taq* (*A*) polymerase. The following cycling parameters were then applied: A 94°C 30 s, T_A 30 s, 72°C 30 s×36; B as for A, ×40; C 94°C 15 s, T_A 15 s, 72°C 30 s×40; D 94°C 30 s, T_A 30 s, 72°C 45 s×36; E 94°C 30 s, T_A 30 s, 72°C 90 s×36; F 94°C 30 s, T_A 30 s, 72°C 120 s×40; G 94°C 60 s, T_A 60 s, 72°C 60 s×36; H 94°C, 60 s, T_A 60 s, 72°C 120 s×36; I 94°C 30 s, T_A 30 s, 72°C 60 s×38; J as for I, ×40; K 94°C 15 s, T_A 15 s, 72°C 120 s×40 and L 94°C 20 s, T_A 20 s, 72°C 60 s×36. All PCR reactions were completed with a final extension step of 72°C for 5 min.

Cloning, sequencing and analysis of *L. perenne* PCR products

Where PCR reactions resulted in the amplification of a single fragment, PCR products were TOPO-TA-cloned directly into pCR2.1-TOPO (Invitrogen BV, Groningen, The Netherlands) according to the manufacturer's instructions. For primer pairs which resulted in the amplification of >1 fragments, those corresponding most closely in size to the amplification product/s observed in barley or wheat and/or the most intensely staining fragments were excised from a 1% TAE agarose gel and purified using a silica matrix as described by Boyle and Lew (1995). Purified fragments were then incubated with 0.5 U of *Taq* polymerase, 50 mM of dATP, 2 mM of $MgCl_2$ in 10 µl at 72°C for 20 min, prior to TOPO-TA cloning as described above.

Two independent clones were isolated for each primer set analysed except for GenBar12/GenBar6 (five clones), BCD183-F/BCD183-R1 (one clone) and MWG694 (one clone). Clones were cycle-sequenced as described previously. Where necessary, an additional sequence was generated using sequencing primers designed from intial M13 and subsequent flanking sequences. Sequences were edited and analysed using Factura v 2.0 feature identification software and AutoAssembler v 2.0 DNA sequence assembly software (PE Biosystems, Denmark) and deposited at GenBank (Accession no. AF290444–AF290471).

Similarity searches were performed using BLAST algorithms (Altschul et al. 1990). BLASTX was used to compare sequences to the protein sequence databases while BLASTN was used to compare sequences to the non-redundant DNA sequence databases and EST sequence databases. For all searches, default parameters were employed. Data analysis was conducted using the WWW interface. In addition, flanking sequences of the RFLP probes from which oligonucleotide primers were derived were retrieved from the GenBank and GrainGenes databases and aligned with their putative *L. perenne* homologues using the computer software package DNASIS v 2.5 genetic analysis software (Hitachi Software, Calif., USA). Sequence analysis and the calculated percent sequence identity included primer sequences in all cases. For all sequences other than those derived from the MWG-series STS markers, the percent sequence identity was calculated from putative coding regions (i.e. sequence comparison was to cDNA sequences or sequences where exons/introns had been identified). Since the MWG-series STS markers are derived from genomic DNA clones, sequence comparison was over the complete length of each sequence.

L. perenne STS primers

It was not possible to optimise heterologous primer pairs for the asparaginase and thioredoxin loci sufficiently in order to use them as STS markers in *L. perenne*. New primers were therefore derived from *L. perenne* sequence data and optimised as described above.

BLASTN analysis identified a clone derived from primer set 4 which had homology to barley glutamine synthetase. Sequence data derived from this clone was aligned with the barley glutamine synthetase (Gln2) cDNA sequence (GenBank accession no. X53580) and primers designed from regions of conserved sequence. Both primers had a single base-pair mis-match relative to the barley sequence.

Restriction endonuclease digestion of *L. perenne* PCR products

Optimised primer pairs were used to screen *L. perenne* individuals 1–6. Where necessary, PCR conditions were further optimised by either increasing the annealing temperature slightly or by substituting Ampli*Taq* for *Taq*. PCR reactions were carried out using a Biometra T3 PCR machine (Biometra biomedizinische Analytik GmbH, Goettingen, Germany).

PCR products amplified from all six individuals were monomorphic for all primer pairs used. Consequently, PCR products were digested with *Taq*I, *Rsa*I and *Hsp*92II (Promega, Madison, Wis., USA) in a preliminary analysis to detect polymorphism. Five microlitres of each PCR reaction was digested with 5.0 U of restriction enzyme in 10 µl according to the manufacturer's directions. Digestion products were analysed by electrophoresis in 2–3% 0.5×TBE agarose gels.

Chromosomal assignment of STS markers

PCR products derived from heterologous primers and *Triticeae* group-5 RFLP clones were assigned to individual barley chromosomes using the wheat-barley (cv "Chinese Spring" – cv "Betzes") chromosome addition lines (CALs; Islam et al. 1981; Islam 1983). Monomorphic PCR products between Chinese Spring and Betzes were digested with four-base cutter restriction endonucleases as described above. Informative restriction enzymes were then used to digest PCR products derived from the CALs.

Results

Primer sets

Table 1 shows the origin and sequence of primer sets used in this study while Table 2 shows the PCR conditions used to screen these primer sets in wheat, barley and *L. perenne*, and the size of amplification products generated. Primer sets 12, 13 and 14 were derived from sequence data obtained from *L. perenne* PCR products generated by primer sets 3, 7 and 4, respectively. Barley and *T. tauchii* STS markers selected for temperature gradient PCR analysis are shown in Table 3. Table 4 gives the results from sequencing and similarity search analysis for all fragments analysed.

Cloning and sequence analysis of *L. perenne* PCR products

Heterologous primers

Optimised PCR conditions resulted in the amplification of a single PCR product of the expected size from *L. perenne* genomic DNA for primer sets derived from the AS, ASN, HS1 and Trx loci (Table 2, primer sets 1, 2, 3, 6 and 7). All fragments amplified in *L. perenne* using these primer sets showed high levels of homology to their barley and *P. coerulescens* counterparts (Table 4). In addition, these fragments possessed the same structure as their barley counterparts. For example, primer sets 2 and 3 direct the amplification of overlapping fragments from the barley L-asparaginase gene. In barley, this gene is comprised of two exons of 396 bp and 606 bp, respectively, interrupted by a single intron of 1,657 bp (K. Madsen, personal communication). Comparison between the assembled *L. perenne* sequences with the barley asparaginase gene reveals conservation in gene structure; the *Lolium* gene has two exons of 396 bp and 600 bp, respectively, interrupted by a single intron of 1,672 bp. Overall sequence identity in coding regions is 89% (data not shown).

Primer set 4 derived from the barley HS1^{vul} locus could not be optimised to amplify a single band while primer set 5 failed to amplify in both *L. perenne* and wheat DNA even at reduced stringency. None of the five clones derived from primer set 4 were homologous to

Table 1 Oligonucleotide primer pairs used in this study. The origin of primers (locus/clone and species), primer name and sequence is shown. *Lolium*-specific primers were designed from sequence data obtained from PCR fragments amplified and cloned in initial studies using heterologous primers

Locus/Clone	Species	Primer		Primer sequence (5′→3′)
		Name	Set	
Heterologous primers				
Asparagine synthetase (AS1)	*H. vulgare*	RT1	1	ATGACAGACGTTCCATTCGG
		RT2		CCAGAGATGACATCTTGACC
L-asparaginase (ASN)	*H. vulgare*	KM51	2	CATGGCGCGCTGGGCCATTG
		KM38		CATGATGCTGGCCTCCATCTC
		KM29	3	GCACCGTCGAGATGGAGGCCA
		KM45		TGGGTACCTCACTCCCAGATGCCGACCTC
HS1^{vul}	*H. vulgare*	GenBar12	4	CCTCGAGGCCACACCCG
		GenBar6		CTGAGATGAATCACTATAGG
		GenBar54	5	GACCCGGACGATTTCCTGC
		GenBar8		CGGCAGTAGTTTGGGTCGG
		GenBar54	6	GACCCGGACGATTTCCTGC
		GenBar55		GTCTTGGCGGCGTCGAGG
Thioredoxin (Trx)	*P. coerulescens*	XUP	7	AAGCAAACAAGGATGGGAAAA
		XLP		GCGGAAAAGACACGGAAACTG
Triticeae group-5 primers				
BCD183	*H. vulgare*	BCD183-F	8	TCGTCATCGGAGGATTGTA
		BCD183-R1		CTGTCATTACACGGCTGAT
BCD450	*H. vulgare*	BCD450-F	9	GGTACATCATTCTCAGTCCC
		BCD450-R1		ACCGGCCTATGAAAGTTGAGA
CDO504	*A. sativa*	CDO504-F	10	ACGACGACCCGAAGAG
		CDO504-R		GCGGTGTGGACGAGA
CDO1508	*A. sativa*	CDO1508-F	11	ATGCGGGCGTTGCT
		CDO1508-R		GGCGGATATGGGAGGTG
Lolium STS primers				
L-asparaginase (ASN)	*L. perenne*	LpASP-F	12	GGCGTGGACCCGAACCTG
	H. vulgare	KM45		TGGGTACCTCACTCCCAGATGCCGACCTC
Thioredoxin (Trx)	*L. perenne*	LpTHIO-F	13	GACTTATCCCCAACTCA
	L. perenne	LpTHIO-R		CCATCGCCAATAGC
Glutamine synthetase (Gln2)	*L. perenne*	LpGS-F	14	GGGTTGGAGGGTCTGGAAT
	L. perenne	LpGS-R		TGACGAAGTGAAAGGTTCAG

Table 2 PCR conditions and product size for primer pairs used in this study. ND: not determined, – PCR product not observed

Primer set [a]	Size (bp) [b]			PCR conditions [c]
	Wheat	Barley	*L. perenne*	
1	520	521, 534	527	55°C, 2.5, *T*, A
2	250	256	256	56°C, 1.5, *B*, B
3	2,200 1,900, 1,200	2,441	2,449	55°C, 2.5, *B*, H
4	2,200 1,900, 700	1,634	1,500, 1,800, 2,200	55°C, 1.0, *B*, H
5	–	968	–	55°C, 2.5, *B*, A
6	450	452	444	55°C, 1.5, *B*, A
7	ND	1,500	1,574	55°C, 2.0, *T*, G
8	1,150	1,150, 900 [d]	1,200	45°C, 3.0, *A*, J
9	750	750	795	53°C, 2.5, *A*, A
10	400	–	395	60°C, 1.5, *A*, C
11	–	–	216, 516	58°C, 2.0, *A*, C
12	ND	2,629	2,637	65°C, 2.5, *B*, H
13	ND	ND	702	55°C, 2.0, *T*, A
14	2,200	2,200	2,200	55°C, 2.5, *B*, F

[a] See Table 1
[b] Product sizes in bold derived from sequence data. All other sizes determined by gel electrophoresis
[c] Optimised PCR conditions used to screen *L. perenne*. The annealing temperature, $MgCl_2$ concentration (mM), *Taq* polymerase (*T*=Promega, *B*=*BIO-X-ACT*, *A*=Ampli*Taq*) and cycling parameters (see Materials and methods) used are shown for each primer set
[d] This band was not observed under more stringent PCR conditions; the PCR product was not observed for these primer pairs

Table 3 Selected barley and *T. tauchii* STS markers analysed via temperature-gradient PCR. The chromosomal location of markers is shown along with product sizes in barley (MWG-markers) or wheat (F15, G44 and G49) and *L. perenne*. The penultimate annealing temperature (PT_A) before PCR failure is shown for each marker. In a number of instances, markers amplified the product from *L. perenne* at the highest annealing temperature (T_A) employed (58.1°C). Markers shown in bold italic were selected for cloning and sequencing analysis. Markers were considered to amplify multiple bands if more than one band was visible either at the penultimate annealing temperature (PT_A) before PCR failure or at the highest T_A used (58.1°C). Sizes shown for *L. perenne* PCR products refer to those products most similar in size to the PCR products observed for wheat or barley, or for *L. perenne* products which were selected for further analysis

Marker	Primer Set	Chromosome [a]	Size (bp) [b]		PT_A (°C)
			Barley/wheat	*L. perenne*	
MWG502	15	5H	1,050	1,000+	55.9
MWG522	16	5H	1,300	400	53.6
MWG532	***17***	***2H***	***870***	***900***	***55.9***
cMWG694	***18***	***2H***	***1,500***	***1,300***	***53.6***
cMWG770	19	5H	580	600+	53.6
MWG848	***20***	***3H***	***700***	***300***	***58.1***
MWG858	21	2H	1,350	1,300+	48.8
MWG897	***22***	***6H***	***1,100***	***800***	***58.1***
MWG900	*23*	*5H*	*950*	*1,100+*	*46.4*
MWG912	24	1H	580	520+	53.6
MWG913	***25***	***1H***	***500***	***500***	***58.1***
MWG2033	***26***	***4H***	***1,000***	***950***	***53.6***
MWG2054	27	2H	720	650	55.9
MWG2076	28	2H	1,700, 1,200, 550, 300 [e]	1,700, 500, 300+	48.8
MWG2123	29	2H	2,000	1,800+	53.6
MWG2193	30	5H	1,900	500	58.1
MWG2230	31	5H	320	300+	58.1
MWG2249	32	5H	500	350	51.2
MWG2264	***33***	***6H***	***450***	***560, 400*** [c]	***51.2, 58.1***
F15	***34***	***2AB, 3 A, 6ABD***	***1,200, 700, 200*** [e]	***700***	***55.9***
G44	35	5BL, 5DL	600 [e]	700+	53.6
G49	***36***	***4 A, 7D***	***1400, 1200, 900, 700*** [e]	***900, 700***+[d]	***58.1, 53.6***

[a] Chromosome location of STS markers follows Mano et al. (1999) and Erpelding et al. (1996)
[b] Product size as determined by Mano et al. (1999)
[c] Marker MWG2264 resulted in the amplification of a single band of about 400 bp at the highest annealing temperature (58.1°C). At lower temperatures (51.2°), a band of about 560 bp was also observed. Both bands were cloned and analysed via sequencing
[d] Marker G49 resulted in the amplification of 4 bands. Both the 1,400-bp and 1,200-bp bands were amplified strongly at all annealing temperatures. The 900-bp band and the 700-bp band were amplified faintly at 58.1°C and 53.6°C, respectively and were cloned and analysed via sequencing
[e] Size of PCR product was not available from the literature and was therefore determined from preliminary PCR analysis

Table 4 Sequence identity to putative homologues and similarity to known genes for *L. perenne* PCR fragments. Sequence identity (SI) and BLASTN/X results including BLAST scores, P-values, match accession numbers and descriptions are shown. A threshold of 100 was used for both BLASTN and BLASTX searches except for the sequence LpASN-1.ALL and sequences showing homology to their putative homologues. The search was conducted on July 24, 2000 using the default BLAST parameters. "F" and "R" indicate forward and reverse sequences, respectively. "ALL" indicates that the entire sequence was obtained. Sequence identity less than 50% is indicated by a dash (–). NSH: no significant homology detected via BLAST analysis

Sequence	SI (%)	Score [b]	P-value	Match	Description
LpAS1.ALL	94 (AS1) 87 (AS2)	101	2e-19	U55874	Asparagine synthetase (*G. max*)
LpASN-1.ALL	91	99	2e-21	P50287	L-asparaginase (*Arabidopsis thaliana*)
LpASN-2.ALL	88	210	1e-53	P50288	L-asparaginase (*Lupinus albus*)
LpHS1.ALL	91	301	4e-80	AA753930	Immature seed cDNA (*O. sativa*)
LpTrx.ALL	95–100 [a]	221	7e-55	AF159389	Thioredoxin-like protein (*P. coerulescens*)
		253	2e-64	AF159387	Thioredoxin-like protein (*L. perenne*)
Lp183.F	–	NSH			
Lp450.ALL	92	131	1e-28	AA231679	BCD450.F cDNA (*H. vulgare*)
	75	58	1e-06	AA231680	BCD450.R cDNA (*H. vulgare*)
LpV504.ALL	95	151	2e-34	AF236870	CDO504 (*H. vulgare*)
		196	4e-48	AF230517	Unknown gene CDO504(*Leymus cinereus*)
LpV1508.ALL	53	214	1e-53	AP000836	Genomic DNA, chromosome 1(*O. sativa*)
LpB1508.ALL	–	NSH			
LpGln2.F	87–95 [a]	157	6e-36	X53580	Glutamine synthetase 2 (*H.vulgare*)
LpGln2.R	89–97 [a]	121	3e-25	X53580	Glutamine synthetase 2 (*H.vulgare*)
Lp532.ALL	52	NSH			
LpV532.ALL	51	NSH			
Lp694.F	51	NSH			
Lp694.R	52	270	1e-70	AW925259	Pre-anthesis spike EST (*H. vulgare*)
Lp848.ALL	-	NSH			
Lp897.ALL	95	189	1e-45	AJ234631	MWG0897.uni (*H. vulgare*)
	82	68	4e-09	AJ234632	MWG0897.rev (*H. vulgare*)
Lp900.F	–	NSH			
Lp900.R	–	NSH			
Lp913.ALL	90	599	e-169	AJ234643	MWG0913 (*H. vulgare*)
		171	9e-65	P31023	Dihydrolipoamide dehydrogenase (*Pisum sativum*)
Lp2033.ALL	–	NSH			
Lp2264 A.ALL	84	256	6e-66	AJ234870	MWG2264 (*H. vulgare*)
Lp2264B.ALL	60	105	2e-20	AJ234870	MWG2264 (*H. vulgare*)
		105	6e-21	AI725280	PtIFG2 cDNA (*P. taeda*)
Lp15.ALL	79 (F15 U) 94 (F15R)	471	e-130	M60599	Dihydrodipicolinate synthetase (*T. aestivum*)
Lp49 A.F	78 [c]	NSH			
Lp49 A.R	–	126	3e-27	BE404328	Etiolated seedling root cDNA (*T. aestivum*)
Lp49B.ALL	–	157	2e-36	C26461	Rice callus cDNA (*O.sativa*)

[a] Sequence identity (%) determined from BLAST alignments
[b] Where identified, BLASTN similarities between *L. perenne* fragments and their barley, wheat, oat or *P. coerulescens* homologues are shown
[c] The sequence G49 U from which the primer G49L was derived extended only 8 bp beyond the primer binding site. Aligment between G49 U and Lp49 A.F identified 7/9 conserved bases

HS1^{vul}. However, BLASTN analysis revealed that one clone showed strong homology to glutamine synthetase from barley, rice and maize (Table 4). This clone was further sequenced and analysed (see below).

Triticeae *group-5 primers*

Primers derived from the barley cDNA clone BCD183 amplified poorly in *L. perenne* and wheat, even when reduced annealing temperatures and increased $MgCl_2$ concentrations (relative to barley) were used. A single *L. perenne* fragment analysed was not homologous to BCD183 (Table 4). In contrast, primers derived from the barley cDNA clone BCD450 were optimised to amplify a single band of approximately 750 bp in barley and wheat, and 800 bp in *L. perenne* (Table 2). Both clones analysed contained the same fragment which showed high homology to BCD450 (Table 4).

Difficulty was encountered using primers developed from oat cDNA clones CDO504 and CDO1508. Primer set 10 failed to amplify in *L. perenne* individuals 1 and 2 but amplified a band of about 400 bp in wheat and *L. perenne* individuals 3–6. This fragment showed high levels of homology (95%) to the oat clone CDO504, as well as to sequences homologous to CDO504 cloned from other grasses (Table 4). Multiple products were initially observed in wheat, barley and *L. perenne* for primer set 11. At increased stringencies, this primer set failed to amplify in wheat and barley while two bands of re-

Table 5 Assignment of PCR products to barley chromosomes. The loci assessed, the primer combinations and the PCR conditions employed are shown. Restriction enzymes used to identify polymorphisms between wheat and barley PCR amplification products are shown in parentheses

Locus	Primer Set [a]	RFLP [b]	CALs [c]	PCR conditions [d]
AS	1	5HL, 3HS	5HL, 3HS (*Taq*I, *Alu*I)	55°C, 2.5, *T*, A
ASN	2	2HL	2HL (*Cfo*I)	56°C, 1.0, *B*, A
ASN	3	2HL	2HL	58°C, 2.0, *B*, K
HS1	5	3HL, 6H	3HL, 6H	55°C, 1.5, *B*, A
BCD183	8	5HL	5HL	53°C, 2.0, *A*, A
BCD450	9	5HL	5HL (*Taq*I)	53°C, 2.5, *A*, A
Gln2	14	2HL	2HL (*Mbo*I)	55°C, 2.5, *B*, F

[a] See Table 1
[b] RFLP chromosomal positions for AS1 and AS2, ASN and HS1^{vul} (M. Moller, K. Madsen and S. Borg, personal communication), BCD183, BCD450 and Gln2 (GrainGenes, 2000)
[c] Chinese Spring-Betzes chromosome addition lines
[d] PCR conditions optimised for amplification of barley fragments. The annealing temperature, $MgCl_2$ concentration (mM), *Taq* polymerase (*T*=Promega, *B*=*BIO-X-ACT*, *A*=Ampli*Taq*) and cycling parameters (see Materials and methods) used are shown for each primer pair

duced intensity were observed in *L. perenne* (Table 2). Homology to CDO1508 was not observed for either of the fragments analysed.

Barley and *T. tauchii* STS markers

Nineteen barley and three *T. tauchii* markers demonstrating either simple banding profiles or the generation of amplification products similar in size to those generated in barley or wheat were selected from initial screening experiments (see Materials and methods). Optimisation of PCR conditions for selected markers was carried out using temperature-gradient PCR with a constant $MgCl_2$ concentration (Table 3). PCR conditions resulting in the generation of a single band were identified for 12 markers. Of these 12 markers, eight amplified a fragment in *L. perenne* which was similar in size to the corresponding barley or wheat products. The remaining ten markers generated more than one band at all temperatures employed prior to reaction failure. However, for all markers, at least one band was amplified in *L. perenne* which was similar in size to the corresponding barley or wheat product/s (Table 3).

Ten unique PCR fragments amplified in *L. perenne* using eight barley STS markers were analysed (Table 3). The marker MWG532 showed variability in its ability to amplify a single band of about 900 bp in *L. perenne*; the product amplified in individuals 3–6 appeared to possess a higher primer-annealing temperature than the product amplified in individuals 1 and 2. Amplification products from individuals 2 and 4 were cloned and sequenced and found to be 929 bp and 928 bp in length, respectively, with a sequence identity of 97%. Neither fragment was homologous to the barley clone MWG532 (Table 4). Similarly, fragments amplified by markers cMWG694, MWG848, MWG900 and MWG2033 were not homologous to the sequences from which the primers were derived.

In contrast, fragments amplified in *L. perenne* by markers MWG897, MWG913 and MWG2264 showed high levels of homology to their barley counterparts (Table 4). For example, MWG913 amplified a fragment of 496 bp in *L. perenne* compared to 500 bp in barley (Mano et al. 1999). Sequence identity between the *L. perenne* and barley fragments was 90% and BLASTX analysis indicates significant homology to dihydrolipoamide dehydrogenase from *Pisum sativum* (Table 4).

The *T. tauchii* marker F15 amplified a 729-bp fragment in *L. perenne* which showed 80% and 94% homology to sequences F15.uni and F15.rev, respectively. BLASTN analysis revealed significant homology to *Triticum aestivum* dihydrodipicolinate synthetase (Table 4). BLASTN analysis of F15.rev and F15.uni also revealed strong homology to *T. aestivum* dihydrodipicolinate synthetase and *Oryza sativa* dihydrodipicolinate synthetase (data not shown).

STS marker development

Assignment of PCR products to barley chromosomes

PCR conditions were optimised for amplification in barley for primer sets 1, 2, 3, 5, 8 and 9 (Table 5; for discussion of results concerning primer set 14, see below). PCR products for primer sets 1 and 9 were monomorphic between wheat and barley. However, mappable polymorphisms were identified via restriction digestion of CAL PCR products. PCR amplification products for primer set 1 were mapped to barley chromosome arms 5HL and 3HS after digestion with either *Taq*I or *Alu*I. This is in agreement with the known chromosome locations for AS1 and AS2 in barley as established via RFLP analysis (M. Moller, personal communication). The product generated using primer set 2 was mapped to the long arm of barley chromosome 2H after digestion with *Cfo*I. The barley product generated by primer set 3 was mapped to the long arm of barley chromosome 2H without restriction digestion. L-asparaginase has been mapped to the long arm of barley chromosome 2H via RFLP analysis (K. Madsen, personal communication). PCR products for primer set 5 were mapped to barley chromosome 3HL and barley chromosome 6H. The chromosomal location

Table 6 Preliminary screening of STS markers in *L. perenne*. Optimised PCR conditions for *L. perenne* are shown for markers used in this study. Reactions were carried out using DNA from two individuals from the ecotype "NGB50", two from the variety "Veyo" and two from the variety "Borvi". PCR products were digested with *Taq*I, *Rsa*I and *Hsp*92II. Polymorphism was assessed by comparing digestion patterns individually, disregarding genotype, and expressed as a percentage of the total (6)

Marker	Chromosome [a]	Primer Set [b]	PCR conditions [c]	Polymorphism (%)		
				*Taq*I	*Rsa*I	*Hsp*92II
AS1	5HL	1	55°C, 2.5, *T*, A	16.6	0.0	0.0
BCD450	5HL	8	53°C, 2.5, *A*, L	33.3	33.3	0.0
CDO504	5HL	10	58°C, 1.5, *A*, C	16.6	16.6	16.6
ASN	2HL	12	65°C, 2.5, *B*, H	100.0	100.0	33.3
Trx	1RL	13	55°C, 2.0, *T*, A	16.6	0.0	0.0
Gln2	2HL	14	55°C, 2.5, *B*, F	100.0	100.0	100.0
MWG532	2H	17	55°C, 2.0, *T*, I	50.0	100.0	16.6
MWG694	2H	18	50°C, 2.0, *T*, E	33.3	66.6	0.0
MWG848	3H	20	55°C, 2.0, *T*, A	0.0	0.0	0.0
MWG897	6H	22	57°C, 2.0, *T*, D	33.6	16.6	66.6
MWG913	1H	25	57°C, 2.0, *T*, D	33.6	16.6	16.6
MWG2264	6H	33	55°C, 2.0, *T*, A	16.6	0.0	0.0
F15	2H	34	55°C, 2.0, *A*, I	0.0	0.0	0.0

[a] Chromosomal location of markers as determined in this study, GrainGenes (2000; Trx), Mano et al. (1999; MWG-series STS markers) and Erpelding et al. (1996; F15). Note that markers MWG532, MWG694 and MWG848 apparently do not target homologous sequences as determined via sequencing of *L. perenne* PCR products

[b] See Tables 1 and 3

[c] PCR conditions optimised to amplify across three *L. perenne* genotypes. The annealing temperature, $MgCl_2$ concentration (mM), *Taq* polymerase (*T*=Promega, *B*=*BIO-X-ACT*, *A*=Ampli*Taq*) and cycling parameters (see Materials and methods) used are shown for each primer pair

[d] Primer pair 10 failed to amplify in individuals 1 and 2 under the conditions shown. Consequently, this marker identifies a dominant polymorphism for these two individuals also

determined for both bands is in agreement with RFLP analysis (S. Borg, personal communication). PCR products generated for primer sets 8 and 9 were mapped to barley chromosome arm 5HL. Both BCD183 and BCD450 have been mapped as RFLPs to the long arm of barley chromosome 5H (GrainGenes 2000).

Lolium STS primers

Primer sets 3 and 7 successfully amplified homologous sequences from *L. perenne* while primer set 4 amplified a fragment showing homology to barley glutamine synthetase 2 (Table 5). Since these primer sets did not amplify efficiently enough in *L. perenne* to allow their use as STS markers, new primers were designed from *L. perenne* sequence information (see Table 1). Primer set 12 combined a new *L. perenne* primer with the original barley primer KM45 and resulted in the amplification of a fragment of about 2,600 bp in *L. perenne*. Based on sequence data, this primer set was expected to amplify a fragment of 2,637 bp from the L-asparaginase locus (Table 2). Primer set 13 resulted in the amplification of a 700-bp fragment from *L. perenne* genomic DNA which corresponded to the expected product of 702 bp (Table 2).

Primer set 14 amplified a fragment of approximately 2,200 bp from wheat, barley and *L. perenne* genomic DNA (Table 2), and the plasmid clone (data not shown). Polymorphism between wheat and barley amplification products was detected after digestion by *Mbo*I allowing the barley PCR product to be mapped to the long arm of barley chromosome 2H using the CALs (Table 5).

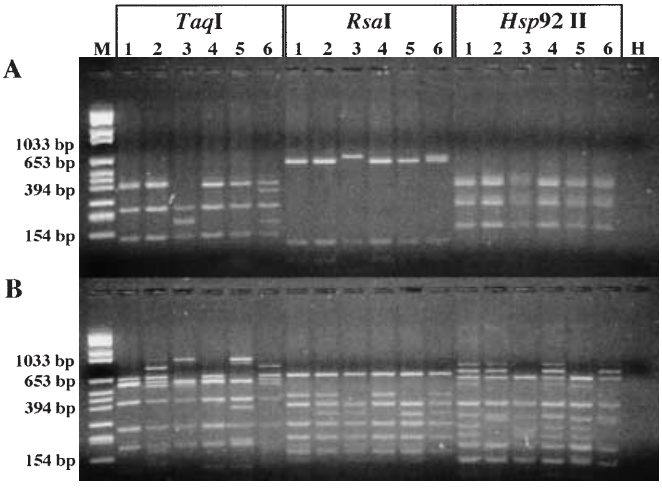

Fig. 1A, B Polymorphism detected in PCR amplification products generated in six *L. perenne* individuals using STS markers BCD450 (panel **A**) and Gln2 (panel **B**) after digestion from *Taq*I, *Rsa*I and *Hsp*92II. In both panels, *lanes 1 and 2* are individuals 1 and 2 from *L. perenne* ecotype NGB50, *lanes 3 and 4* are individuals 1 and 2 from *L. perenne* var. Veyo, and *lanes 5 and 6* are individuals 1 and 2 from *L. perenne* var. Borvi. *Lanes M and H* are DNA size markers (Boehringer Mannheim BMVI) and PCR water controls, respectively

Restriction enzyme digestion of *L. perenne* PCR products

Thirteen of the primer sets used in this study amplified sufficiently well in *L. perenne* to use as STS markers (Table 6). Consequently, these primer sets were ampli-

fied across individuals 1–6 from the ecotype "NGB50" and varieties "Veyo" and "Borvi", and the amplification products subjected to digestion using *Taq*I, *Rsa*I and *Hsp*92II.

Polymorphism was detected in at least one individual for 11 of the 13 markers. No polymorphism was detected in fragments generated using the markers MWG848 and F15. BCD450 amplified a 795-bp fragment in all individuals. Polymorphism was observed for two individuals with both *Taq*I and *Rsa*I (Fig. 1A).

The highest levels of polymorphism were observed for markers ASN and Gln2 (Table 6 and Fig. 1B). Both markers amplified fragments which contained large stretches of non-coding DNA as determined from sequence analysis. For both markers, polymorphisms differentiating between each individual were identified (Fig. 1B, *Taq*I).

Discussion

A total of 21 primer sets derived from barley, *T. tauchii* and *P. coerulescens* loci were assessed for their ability to amplify homologous sequences in *L. perenne*. Sequencing analysis of the PCR amplification products generated revealed that 11 primer sets (52%) successfully amplified homologous fragments from 10 of the 18 (55%) loci targetted. Analysis of homologous fragments generated in *L. perenne* reveals high levels of sequence identity and conservation in gene structure (i.e. presence, size and relative position of exons and introns) to barley, wheat and *P. coerulescens* homologues. The rate of success for primers designed from coding sequences was higher (58%) than for primers derived from genomic DNA sequences (44%). These results compare favourably to those of Erpelding et al. (1996) who showed that the transfer of STS markers between barley and wheat had a success rate of approximately 70%. Significantly, these authors observed that STS location in barley predicted STS location in wheat 75% of the time.

Four (40%) STS markers, MWG897, MWG913, MWG2264 and F15, amplified homologous fragments in *L. perenne*. In contrast, fragments derived from STS markers MWG532, cMWG694, MWG848, MWG900, MWG2033 and G49 were not homologous to the sequences from which the primers were derived. Similarly, PCR products generated using primers derived from cDNA clones BCD183 and CDO1508 were not homologous to their barley or oat counterparts. Other authors have observed the failure of STS primers to amplify, or to amplify homologous sequences, when transferred to other species (Erpelding et al. 1996; Talbert et al. 1996; Mano et al. 1999). The amplification of multiple products, most likely due to primers acting in a manner similar to RAPDs (Williams et al. 1990) has also been observed (Blake et al. 1996; Erpelding et al. 1996; Mano et al. 1999). Of the 22 STS markers selected, ten (45%) resulted in multiple amplification products. However, the amplification of homologous sequences cannot be ruled out due to the small number of clones analysed for each marker. It is possible that homologous fragments were amplified but not isolated. Where multiple products are generated, analysis via Southern hybridisation would seem a more robust and efficient method for assessing homology of the PCR products generated. This would also appear to be the most-efficient method for assembling a panel of markers for comparative mapping analyses in forage grasses.

Comparison of *L. perenne* sequences to the nucleotide, EST and protein databases maintained at GenBank and EMBL revealed significant similarity between a number of sequences with previously characterised genes in other organisms. In the study of Michalek et al. (1999), MWG913 was found to have homology to ferric leghemoglobin reductase from *Glycine max*. These authors apparently did not compare genomic DNA fragments against protein sequence databases. In contrast, comparison of these genomic fragments to protein sequence databases revealed strong similarity to dihydrolipoamide dehydrogenase from *T. aestivum*.

Thirteen primer sets were optimised sufficiently to be employed as STS markers in *L. perenne*. Eleven primer sets amplified fragments of the same size equally efficiently in all individuals tested. Significantly, seven (70%) of the barley and *T. tauchii* STS markers tested, reliably amplified fragments suitable for the development of STS markers in *L. perenne*. Given the hundreds of STS markers now available in barley and wheat, and the likelihood that many will amplify homologous sequences in *L. perenne*, their application in *L. perenne* would seem a rapid, cost effective and rewarding means of generating useful, new markers in this species. A preliminary analysis of polymorphism was carried out by digesting PCR products with *Taq*I, *Rsa*I and *Hsp*92II, and showed that mappable restriction fragment length polymorphisms could be identified in at least one individual for 11 of the 13 markers. Levels of polymorphism increased with increasing PCR product length and were highest in fragments containing large stretches of non-coding DNA (i.e. ASN and Gln2).

In order for any marker to be used as an "anchor locus" in linkage mapping studies, it must first be able to detect orthologous loci outside of the species of origin and, second, its map position within the species of origin must be known. Ten of the STS markers developed in this study have been shown to amplify homologous sequences in *L. perenne*. In each case, the map position in barley and rye is known. The chromosome location in barley of STS markers developed in this study were in complete agreement with their RFLP chromosome location as determined using the wheat-barley addition lines. Homology between the *L. perenne* fragment with corresponding fragments from the barley AS1 and AS2 loci is 94% and 87%, respectively. This would suggest that the fragment amplified in *L. perenne* is homologous to the AS1 locus located on barley chromosome 5HL. STS primers derived from LpGln2 amplified a product in barley which was mapped to the long arm of barley chromo-

some 2H. Since Gln2 maps to barley chromosome 2HL (GrainGenes 2000), it would appear that this primer set directs amplification from the Gln2 locus in both barley and *L. perenne*.

The current linkage maps available for *Lolium* are based upon RFLP, RAPD, AFLP and a small number of isozyme and EST markers (Hayward et al. 1998; Bert et al. 1999). Few of the RFLP markers mapped in *Lolium* have been mapped in other members of the *Poaceae*. While RAPD and AFLP markers are highly polymorphic and provide a ready source of polymorphic markers in forage grasses (Charmet et al. 1997; Bert et al. 1999; Kolliker et al. 1999), their transfer between populations is likely to be problematic. Moreover, both RAPDs and AFLPs are generally dominant, and are expected to provide little or no information regarding synteny. Consequently, there is no possibility of aligning current *Lolium* linkage maps with those of rice, barley or wheat, although ongoing mapping of heterologous anchor RFLP probes in *L. perenne* by a number of research groups will ultimately allow the alignment of maps to those of other *Poaceae* species. Data presented in this paper suggest that the probability of amplifying homologous loci in *L. perenne* using primers derived from wheat and barley (and perhaps other grass species) is approximately 50%. Given that the ability to predict STS location in wheat based upon STS location in barley is 75% (Erpelding et al. 1996), it would seem reasonable to assume that STS location in barley will be an accurate predictor of STS location in *L. perenne*. Significantly, this work indicates that it should be possible to assemble a panel of "anchor" STS loci for comparative mapping in forage grasses.

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