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Development of a Genomic Microsatellite Library in Perennial Ryegrass (*Lolium perenne*) and its Use in Trait Mapping

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• *Background and Aims* Perennial ryegrass (*Lolium perenne*) is one of the key forage and amenity grasses throughout the world. In the UK it accounts for 70 % of all agricultural land use with an estimated farm gate value of £6 billion per annum. However, in terms of the genetic resources available, *L. perenne* has lagged behind other major crops in Poaceae. The aim of this project was therefore the construction of a microsatellite-enriched genomic library for *L. perenne* to increase the number of genetic markers available for both marker-assisted selection in breeding programmes and gene isolation.
• *Methods* Primers for 229 non-redundant microsatellite markers were designed and used to screen two *L. perenne* genotypes, one amenity and one forage. Of the 229 microsatellites, 95 were found to show polymorphism between amenity and forage genotypes. A selection of microsatellite primers was selected from these 95 and used to screen two mapping populations derived from intercrossing and backcrossing the two forage and amenity grass genotypes.
• *Key Results and Conclusions* The utility of the resulting genetic maps for analysis of the genetic control of target traits was demonstrated by the mapping of genes associated with heading date to linkage groups 4 and 7.

Key words: Microsatellites, *Lolium perenne*, perennial ryegrass, trait mapping.

INTRODUCTION

Genetic markers and linkage maps provide the basis for the development of superior varieties via marker-assisted selection in breeding programmes and for gene isolation via chromosome walking strategies (e.g. Armstead *et al.*, 2006, 2007). A wide range of genetic markers has been developed and used over the past two decades. The usefulness of a genetic marker is decided by a wide range of factors including cost of development, ease of use and transferability within varieties of the same species or between species. Microsatellites are highly polymorphic, co-dominant markers found throughout both the transcribed and non-transcribed regions of a genome (Varshney *et al.*, 2005). As PCR-based markers, their transferability both within and between species makes them a good choice for breeding programmes for, for example, tagging genes of interest.

Lolium perenne ($2n = 2x = 14$; DNA content = 2034 Mbp: Bennett and Smith, 1976) is one of the most economically and environmentally important grass species. For example, in the UK it accounts for 50 % of all land use and 70 % of all agricultural land use. Furthermore, this figure is likely to rise with increasing emphasis on sustainable agricultural practices, i.e. a move from concentrate to increasing grass consumption. The economic value of forage grass in the UK measured by its end products, meat and milk, is £6 billion per annum. This value compares well with £2·36 billion – the farm gate value of all cereals (wheat, barley and oats) (figures

taken from 'Agriculture in the UK'; DEFRA, 2002). In addition, the value of grass will continue to increase in line within an increase in the requirement for meat and milk at a rate of 7 % per annum.

In addition to conventional agricultural use, grass is of fundamental importance for the development of environmentally friendly biofuels and platform chemicals. Leisure activities, such as tourism, gardening and sport, and associated industries, e.g. garden machinery/equipment, sports equipment, sports media coverage, etc., are valued at hundreds of billions of euros per annum throughout the European Union.

The number of microsatellites for use in the *Lolium–Festuca* complex of grasses that are publicly available has really only started to increase in the last few years, e.g. Kubik *et al.* (1999, 2001), Jones *et al.* (2001, 2002), Warnke *et al.* (2004), Lauvergeat *et al.* (2005), Jensen *et al.* (2005) and Studer *et al.* (2006). This project was started with the aim of developing a genomic microsatellite library for *L. perenne*, which would then be combined with a set of EST-based microsatellites produced from a *L. perenne* GeneThresher database (Gill *et al.*, 2006). Genomic microsatellites can be produced from both transcribed and non-transcribed regions of the genome, but microsatellites derived from coding regions, e.g. ESTs and GeneThresher databases, tend to be concentrated in the gene-rich regions of the genome (Varshney *et al.*, 2005). Therefore a combination of the two sets of microsatellites would be expected to give as wide a genome coverage as possible.

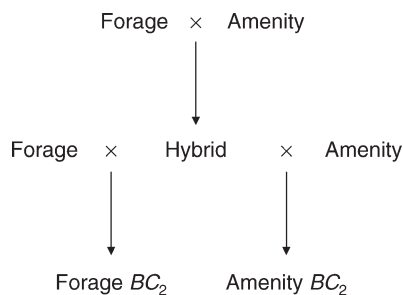* For correspondence. E-mail julie.king@bbsrc.ac.uk

Fig. 1. Development of forage and amenity mapping populations.

As already stated, genetic linkage mapping provides the basis for the development of superior varieties via marker-assisted selection in breeding programmes and for gene isolation via chromosome walking strategies. A key trait within crops principally grown for their vegetative organs, such as *L. perenne*, is the transition to flowering growth as this has impacts both on performance traits associated with the vegetative growth phase and seed yield. Therefore, as an initial step in assessing the utility of the microsatellite linkage maps for both marker-assisted selection and gene isolation, heading date was also studied in the *L. perenne* populations being screened with the microsatellites.

MATERIALS AND METHODS

Plant material

Two *Lolium perenne* genotypes ($2n = 2x = 14$), one amenity (IGER accession Ba11773 3/33/8) and one forage (IGER accession Ba12142 3/34/8) were selected and intercrossed to produce an F_1 hybrid (Fig. 1). This was then cloned and backcrossed to the amenity and forage parental genotypes to produce two BC_2 mapping populations (Fig. 1).

Microsatellites

Six microsatellite-enriched libraries of a *L. perenne* genotype (Liprio) were constructed using the procedure of Edwards *et al.* (1996). DNA extraction was performed using a Nucleon Phytopure Kit (GE Healthcare, Little Chalfont, Bucks, UK). In order to minimize problems with restriction sites, the libraries were based on three enzymes (*Rsa*I, *Alu*I and *Hae*II). Two units of each enzyme were used to digest separately two replicates of 200 ng of genomic DNA in a volume of 50 μL for 2 h at 37 °C. At the end of 2 h, 1 μg of an *Mlu*I adaptor (consisting of a 21-mer oligonucleotide 5'-CTCTTGCTTAC GCGTGGACTA-3' and a phosphorylated 25-mer oligonucleotide: 5'-pTAGTCCACGCGTAAGCAAGAGCACA-3'), 3 μL 10 mM ATP and 1 unit of T4 ligase were added. Ligation was carried out overnight at room temperature in the dark. The ligated DNA was then denatured by boiling for 5 min. Enrichment for microsatellites was carried out using 100 ng of the ligated, denatured DNA in 500 μL hybridization buffer [50 % formamide, 3× standard saline citrate (SSC; 45 mM sodium citrate, pH 7·0, 450 mM

NaCl), 25 mM Na-phosphate, pH 7·0 and 0·5 % sodium dodecyl sulphate (SDS)] containing 1 μg of the 21-mer oligonucleotide (5'-CTCTTGCTTACGCGTGGACTA-3'). The DNA solution was incubated at 45 °C for ~24 h with a single Hybond N⁺ filter with bound oligonucleotides. [Hybond filters were prepared using two different oligonucleotides – $CA_{(15)}$ and $CT_{(15)}$. One hundred micrograms of one oligonucleotide brought to 1 mL with 2× SSC was spotted onto a $1·0 \times 15 \text{ cm}^2$ piece of Hybond® N⁺ (Amersham, Arlington Heights, IL, USA), air dried for 1 h and then baked for 1 h at 65 °C. The dry membrane was UV-treated for 30 s using a 260-nm transilluminator. Weakly bound oligonucleotides were washed off the membrane by washing twice in 10 mL of hybridization buffer at 45 °C for 2 d.] Thus six libraries were produced with DNA digested with three different enzymes undergoing filter selection for two dinucleotide repeats. Following hybridization, the filter was washed five times (5 min per wash) in 2× SSC, 1 % SDS at 65 °C and three times (5 min per wash) in 0·5× SSC, 1 % SDS at 65 °C. Bound DNA was eluted in 200 μL sterile distilled water by boiling for 5 min.

Three libraries were selected for characterization (based only on the criteria of two enzymes and two repeats). Three replicates of each library were amplified by PCR. The PCR conditions were (final volume of 50 μL): 1 μL of the eluted DNA, 0·4 μL Amplitaq Gold® *Taq* DNA polymerase with 5 μL of the manufacturer's buffer system, 1·25 mM of each dNTP and 200 ng of the 21-mer oligonucleotide. Thermal cycling was performed in an ABI9700 (Applied Biosystems, Warrington, UK) with an initial denaturation of 30 s at 94 °C, then 25 cycles of 20 s at 94 °C, 1 min at 60 °C and 3 min at 72 °C with a final extension of 10 min at 72 °C, after which like samples were pooled. Approximately 2·5 ng of the amplified DNA from the final amplification of the *Alu*I CA, *Alu*I CT and *Hae*II CA libraries was digested with 1 unit of *Mlu*I and ligated into 10 ng of a modified pUC19 vector (pJV1) containing a *Bss*HII site (obtained from K. J. Edwards, University of Bristol). Inclusion of *Mlu*I in the ligation ensured that each plasmid contained only a single insert. Plasmids were transformed into BRL DH5α competent cells (Invitrogen, Paisley, UK) and plated onto LB-agar containing 100 μg mL⁻¹ ampicillin and 40 mg mL⁻¹ X-gal. Colonies were transferred into micro-plates after overnight incubation at 37 °C. Microsatellite-containing clones were selected using an appropriate ³²P-labelled 30-mer oligonucleotide, i.e. $CA_{(15)}$ or $CT_{(15)}$. After sequencing with M13 forward and reverse primers, microsatellite containing sequences were entered into a local database to screen for redundancy. PCR primers were designed from the unique sequence flanking non-redundant microsatellites.

Primers were used to amplify DNA from the amenity and forage parents. The PCR amplification conditions were (final volume of 10 μL): 10 ng of DNA sample, 1 U of Faststart *Taq* polymerase with the manufacturer's buffer system (Roche, Lewes, Sussex, UK) 1·25 mM of each dNTP and 0·2 μM of each primer. Thermal cycling was performed with an initial denaturation of 5 min at 96 °C, then 40 cycles of 15 s at 96 °C, 30 s at 60 °C (the annealing

TABLE 1. *Microsatellite primer sequences*

Microsatellite	Primer (forward and reverse)	Microsatellite motif
LpACT13H2	f – TAACATTGATGCATGGGTTTC r – ACTATTTAACTAGGATCCAAC	$CT_{(35)}$
LpACT14A4	f – CATGCTTGGACACTGTTAGCC r – CTCTGTGCATATTCACGAGGG	$CT_{(21)}$
LpACT14C9	f – AATGATGGCACGGAGCAATCG r – CTGTAATTCCAGGTCACTACC	$CT_{(22)}$
LpACT15H3	f – GACATCCATGCAAAATGTAAG r – TCGTCACTTGCAAATATGAAC	$CT_{(33)}$
LpACT43C6	f – AGCGGGAAAGATACAGCGAAG r – GAGCAAATTGGTGATCGCTAC	$CT_{(29)}$
LpACT44A7	f – CACGTAGAAGCCACACTTTAC r – GTCACATTCCATTCACTTCCG	$CT_{(60)}$
LpACT44B9	f – TATGCAGCTTATGGGATCTTG r – CGCATGCTGCACAATATCTGG	$CT_{(38)}$
LpACA31C9	f – CTGAATGCGCAAGCCGCATTC r – CGATTTGCAAAACCGATGGTG	$CA_{(40)}$
LpHCA16B2	f – TGACAGTGTAGGCTAGTGATG r – GAGTACAACATAGCAGATACC	$CA_{(16)}$
LpHCA17C6	f – AACAGCATCATGGATGCTAGG r – ACGGTATGGGTATGCTGATCC	$CA_{(20)}$
LpHCA17C11	f – ACCGGGACAAAGGGCTAGTAC r – CGCTCGATCGATCTGATCGTG	$CA_{(23)}$
LpHCA18B12*	f – TCACGCTTGAAAGATAAACCC r – AACTCATGCAACACATGCATG	$CA_{(5)}CA_{(9)}$
LpHCA18F11	f – TTCACATGGCATGCACAAACC r – AGGTATGACGTGCCGACATGC	$CA_{(15)}$
LpHCA20F6	f – GGGTCATCAGAGATCTTGCTC r – TGCAGCTGGATGGATCAGCTC	$CA_{(5)}$

* The microsatellite motif detected by this pair of primers is an interrupted one; the two parts are separated by a 7-base insertion.

temperature was adjusted for a small number of the microsatellite primers) and 30 s at 72 °C, with a final extension of 4 min at 72 °C. Products were run on 6 % denaturing polyacrylamide gels. Microsatellites were visualized using the Silver Sequence DNA Staining Reagents (Promega, Southampton, UK) with bands scored as present or absent.

Genetic mapping

Primers which generated polymorphisms between the two parental genotypes were identified. Fourteen of these primer pair sequences are given in Table 1 but academic research licenses are available for the primer sequences for all microsatellite markers developed from the genomic library (Institute of Grassland and Environmental Research, Plas Gogerddan, Aberystwyth, UK). A selection of these primers was then used to screen the two BC_2 mapping populations (Fig. 1). JOINMAP, version 2·0 (Stam, 1993; Stam and van Ooijen; 1995), was used to place the genomic microsatellite markers into linkage groups in both the forage and amenity populations. Analyses were performed using a LOD threshold of 3 and Kosambi's mapping function (Kosambi, 1944). Selected microsatellite primers from the Vialactia GeneThresher database were also used (Gill *et al.*, 2006). Primers selected were those already mapped to the Vialactia genetic linkage map of *L. perenne* and/or to a linkage map for water-soluble carbohydrate (WSC) produced at IGER (Turner *et al.*, 2006). GeneThresher microsatellites derived from each of the seven linkage groups of *L. perenne* were used in order to allow the newly derived genomic microsatellites to be assigned to specific linkage groups.

The forage mapping population was also screened for heading date in order to assess the utility of the microsatellites generated. The 100 genotypes making up the forage population were grown as spaced plants and induced to flower under field conditions. Heading date was scored as number of days from 1 April until there was a minimum of three inflorescences emerging from the flag leaf sheath. QTL analysis was carried out using MapQTL 4·0 (van Ooijen *et al.*, 2002) using interval and MQM mapping. Co-factors for MQM mapping were identified using the automatic co-factor selection option within MapQTL 4·0.

In addition to screening the amenity forage parents, primers were also used to screen seven *Lolium/Festuca* substitution lines (King *et al.*, 2002*a*, *b*). In each of these lines one of the seven *L. perenne* chromosomes has been replaced by its homoeologous equivalent from *Festuca pratensis* ($2n = 2x = 14$; DNA content = 2181 Mbp: Bennett *et al.*, 1982) (King *et al.*, 2008). Screening of the substitution lines was carried out on an ABI3100 (Applied Biosystems, Warrington, Lancs, UK).

RESULTS

The microsatellites developed from the genomic libraries were screened on the forage parent, the amenity parent and the forage/amenity hybrid to test for polymorphism

TABLE 2. *Microsatellites from the three genomic libraries screened on the amenity/forage parents (comparison of amplification products produced and levels of polymorphism)*

	*Alu*I CT	*Alu*I CA	*Hae*II CA
Polymorphic	43	30	22
Non-polymorphic	31	41	25
No amplification	8	6	6

(Table 2). A chi-square test, carried out on the number of polymorphic versus non-polymorphic microsatellites in the three libraries, proved slightly significant ($\chi^2 = 3.867$, 1 d.f.) at the 5 % level of probability. This therefore suggests that the level of polymorphism found in the microsatellites may vary depending both on the enzyme used to make the library and the repeat selected for. The *Alu*I CT library proved the best source of polymorphic microsatellites for *L. perenne* with a ratio of polymorphic to non-polymorphic of 49 % : 35 %. In the *Alu*I CA library the ratio was reversed, i.e. 36 % : 49 %, whereas the *Hae*II CA library provided an almost even split of polymorphic to non-polymorphic, i.e. 38 % : 43 %. Genomic microsatellites

TABLE 3. *Number of polymorphic bands per microsatellite primer pair in the forage and amenity populations*

No. of polymorphic bands	Forage population	Amenity population
0	11	9
1	16	14
2	15	18
3	13	12
4	6	5
5	4	1
6	1	2
7	0	0
8	0	1
NS	11	15

NS shows number of microsatellites not scored due to scoring problems.

which produced products in *L. perenne* were also used to screen a set of seven *Lolium perenne/Festuca pratensis* monosomic substitution lines (data not shown), each with 13 *L. perenne* chromosomes and one *F. pratensis* chromosome. The microsatellite primers did not all produce an amplification product in the *F. pratensis* genotype used with the *Hae*II CA library giving the lowest percentage
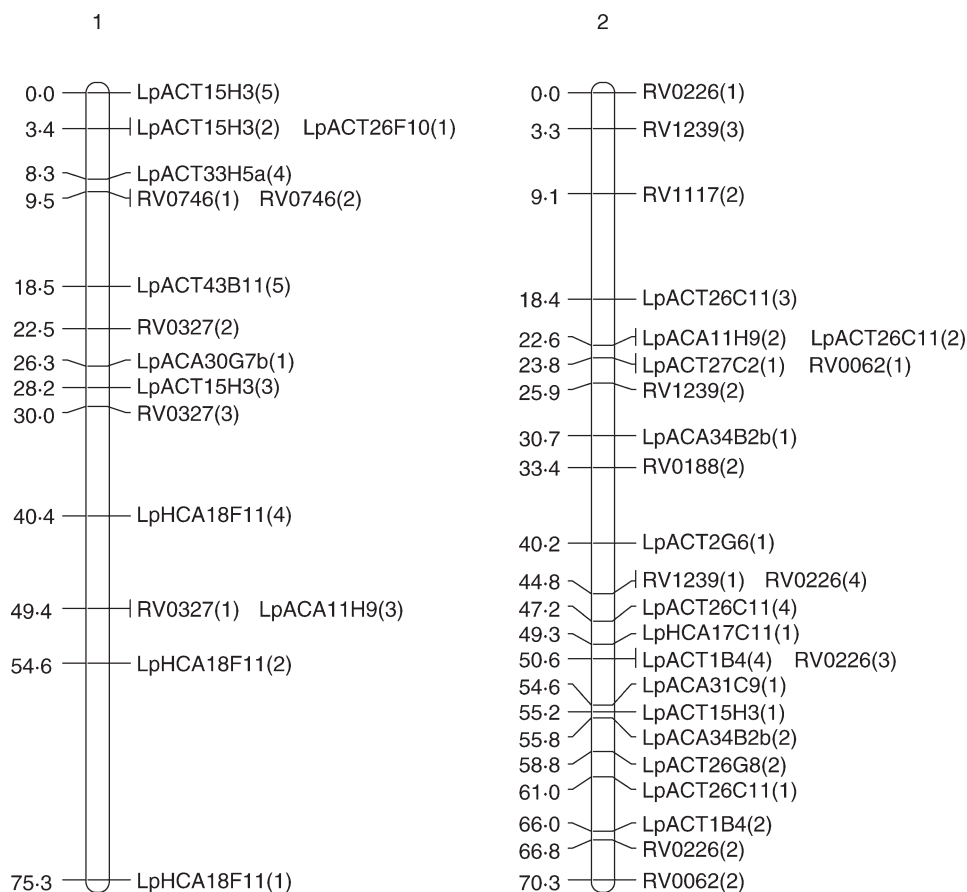

FIG. 2. Genetic maps of the seven linkage groups based on the forage mapping population incorporating both the genomic microsatellites (prefixed Lp) and selected EST microsatellites from Vialactia (prefixed RV or 13). In the genomic microsatellites the letter following the Lp prefix donates the enzyme used to produce the library (A = *Alu*I; H = *Hae*II) and the second and third letters show the dinucleotide repeat enriched for (CA or CT). Figures within parentheses show which band was being scored as polymorphic for a particular primer pair.
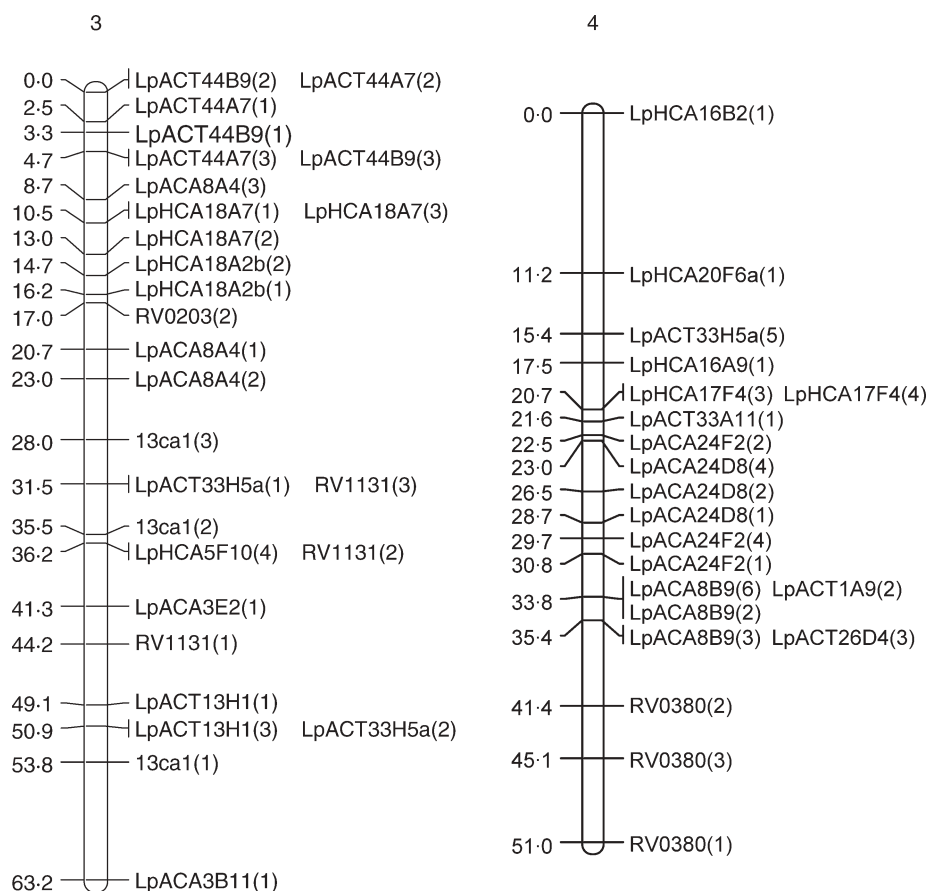
3

0·0	LpACT44B9(2) LpACT44A7(2)
2·5	LpACT44A7(1)
3·3	LpACT44B9(1)
4·7	LpACT44A7(3) LpACT44B9(3)
8·7	LpACA8A4(3)
10·5	LpHCA18A7(1) LpHCA18A7(3)
13·0	LpHCA18A7(2)
14·7	LpHCA18A2b(2)
16·2	LpHCA18A2b(1)
17·0	RV0203(2)
20·7	LpACA8A4(1)
23·0	LpACA8A4(2)
28·0	13ca1(3)
31·5	LpACT33H5a(1) RV1131(3)
35·5	13ca1(2)
36·2	LpHCA5F10(4) RV1131(2)
41·3	LpACA3E2(1)
44·2	RV1131(1)
49·1	LpACT13H1(1)
50·9	LpACT13H1(3) LpACT33H5a(2)
53·8	13ca1(1)
63·2	LpACA3B11(1)

4

0·0	LpHCA16B2(1)
11·2	LpHCA20F6a(1)
15·4	LpACT33H5a(5)
17·5	LpHCA16A9(1)
20·7	LpHCA17F4(3) LpHCA17F4(4)
21·6	LpACT33A11(1)
22·5	LpACA24F2(2)
23·0	LpACA24D8(4)
26·5	LpACA24D8(2)
28·7	LpACA24D8(1)
29·7	LpACA24F2(4)
30·8	LpACA24F2(1)
33·8	LpACA8B9(6) LpACT1A9(2) / LpACA8B9(2)
35·4	LpACA8B9(3) LpACT26D4(3)
41·4	RV0380(2)
45·1	RV0380(3)
51·0	RV0380(1)

Fɪɢ. 2. Continued.

(38 % of *Alu*I CT primers and 39 % of *Alu*I CA primers produced amplification products in both the *Lolium* and *Festuca* genotypes compared with just 21 % of *Hae*II primers). Conversely, of those primers that did produce amplification products in both the *Lolium* and *Festuca* genotypes, the highest frequency of polymorphism was with those primers from the *Hae*II CA library (55 % of the *Hae*II CA primers giving amplification products in both *Lolium* and *Festuca* were polymorphic compared with 43 % of the *Alu*I CT primers and 40 % of the *Alu*I CA primers).

A total of 77 microsatellites was screened on 100 forage backcross plants and 79 amenity backcross plants (Fig. 1). Bands were screened as present or absent. The number of bands per microsatellite varied considerably (Table 3). In a number of the microsatellites, bands could only be scored for one of the populations as the bands in the other population were non-polymorphic (either absent from all BC_2 plants or present in all).

In both populations, JOINMAP produced seven distinct linkage groups although not all markers were included (Fig. 2). In the forage population, 52 primer pairs produced 118 segregating loci, whereas 47 primer pairs produced 94 segregating loci in the amenity population. With the exception of eight markers, those scored as polymorphic in both populations were placed in the same linkage groups. The

eight exceptions to this were LpACA3B7a(1), LpACA3B7b(1), LpACT15H3(1), LpACT26C11(2), LpACT26C11(3), LpHCA18F11(2), LpHCA18F11(3) and LpHCA18F11(4). These eight markers were placed in linkage groups in the forage population but were left unlinked to any other markers by JOINMAP in the amenity population.

Selected Vialactia *L. perenne* GeneThresher microsatellite primers were also run on the 100 plants of the forage backcross population. The GeneThresher microsatellites were developed from a *L. perenne* GeneThresher library (Gill *et al.*, 2006) and were therefore mainly developed from coding sequences, i.e. EST microsatellites. The mapping information for the GeneThresher microsatellites was combined with that obtained for the genomic microsatellites and JOINMAP run on the total data set. The GeneThresher microsatellites remained in the same linkage groups respective to each other in the forage population as they had previously been mapped to in the Vialactia genetic linkage map of *L. perenne* (Gill *et al.*, 2006). The linkage groups in the GeneThresher-derived microsatellite map of *L. perenne* have previously been aligned to the orthologous Triticeae groups by comparison to a RFLP based map (Armstead *et al.*, 2002; Gill *et al.*, 2006). The seven linkage groups produced here were therefore designated LG1 to LG7 in accordance with the
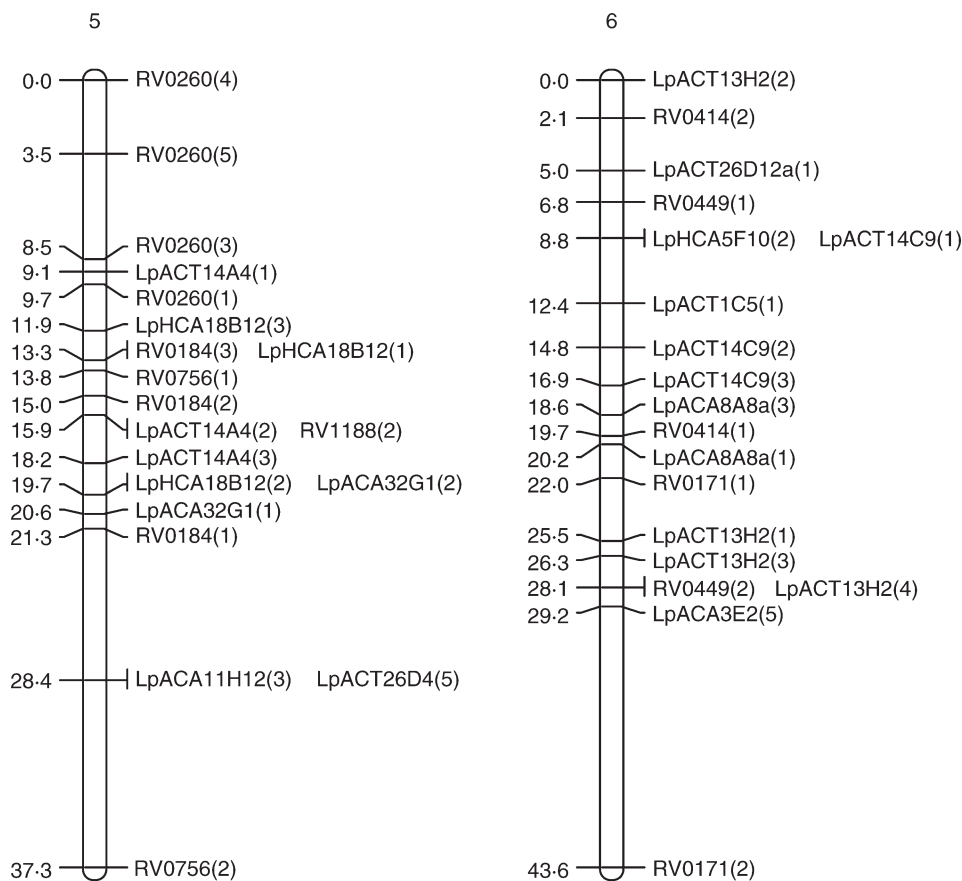
Fig. 2. Continued.

orthologous Triticeae group by reference to the Vialactia microsatellites (Fig. 2).

JOINMAP was then used to estimate the genetic distances between markers in each of the seven linkage groups of the forage population. The map derived from the forage mapping population covered a total genetic distance of 433·8 cM, with the smallest linkage group of 37·3 cM and the largest of 93·1 cM. JOINMAP was also used to estimate the genetic distances between markers in each of the seven linkage groups of the amenity population (although with considerably fewer data points available due to lower plant numbers and fewer markers these data can only be viewed as preliminary). The map derived from the amenity population covered a total genetic distance of 438·5 cM, with the smallest linkage group of 45·9 cM and the largest of 87·2 cM.

The genomic microsatellites generated were tested to confirm their potential for mapping genes which control target phenotypic traits. Heading date was the trait initially used for this work. Heading date in the forage population varied from a minimum of 55 d after 1 April to a maximum of 73·8 d after 1 April (mean = 64·85; $n = 94$). The plants of the forage backcross population were therefore screened for heading date (Fig. 3) and the results analysed with MapQTL 4·0 (van Ooijen *et al.*, 2002). Association for heading date was shown by a number of markers from linkage group 4 and a strong association for heading date with nearly all the markers from linkage group 7 (Fig. 4).

DISCUSSION

Genomic microsatellite markers, with only eight exceptions, formed the same linkage groups and same order in both the amenity and forage mapping populations. This demonstrates the reliability of the markers despite the relatively small number of genotypes from each population used to produce the genetic linkage groups. The relatively small size of the two populations probably explains some of the discrepancies found in the microsatellite markers between the two populations, e.g. the eight common markers placed in linkage groups in the forage population but left unlinked by JOINMAP in the amenity population.

The two sets of microsatellites used in this project, the genomic microsatellites produced at IGER and the GeneThresher microsatellites produced at Vialactia, complement each other. The genomic microsatellites have been shown to be highly polymorphic both in the two populations outlined in this paper and also in a set of *L. perenne/F. pratensis* introgression lines (data not shown). The banding patterns on the polyacrylamide gels and also data obtained from an ABI sequencer have often been very complex. Genomic microsatellites have generally been reported as being more polymorphic than EST
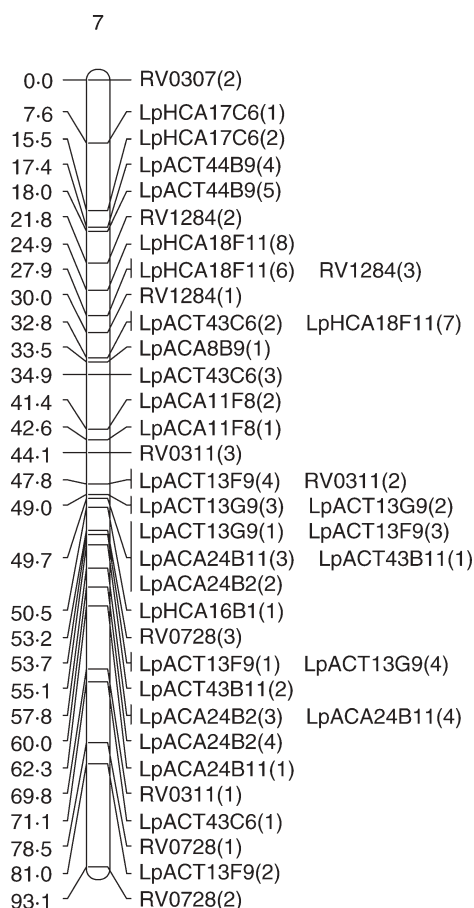
7

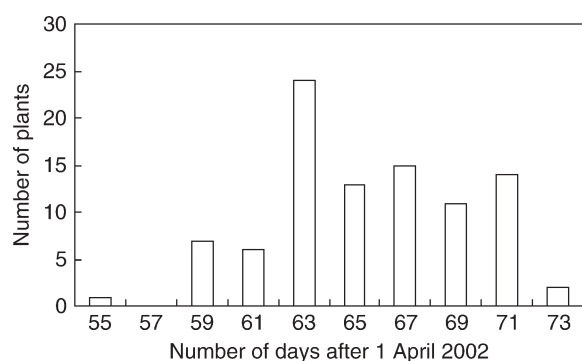| cM	Marker	
0·0	RV0307(2)	
7·6	LpHCA17C6(1)	
15·5	LpHCA17C6(2)	
17·4	LpACT44B9(4)	
18·0	LpACT44B9(5)	
21·8	RV1284(2)	
24·9	LpHCA18F11(8)	
27·9	LpHCA18F11(6)	RV1284(3)
30·0	RV1284(1)	
32·8	LpACT43C6(2)	LpHCA18F11(7)
33·5	LpACA8B9(1)	
34·9	LpACT43C6(3)	
41·4	LpACA11F8(2)	
42·6	LpACA11F8(1)	
44·1	RV0311(3)	
47·8	LpACT13F9(4)	RV0311(2)
49·0	LpACT13G9(3)	LpACT13G9(2)
	LpACT13G9(1)	LpACT13F9(3)
49·7	LpACA24B11(3)	LpACT43B11(1)
	LpACA24B2(2)	
50·5	LpHCA16B1(1)	
53·2	RV0728(3)	
53·7	LpACT13F9(1)	LpACT13G9(4)
55·1	LpACT43B11(2)	
57·8	LpACA24B2(3)	LpACA24B11(4)
60·0	LpACA24B2(4)	
62·3	LpACA24B11(1)	
69·8	RV0311(1)	
71·1	LpACT43C6(1)	
78·5	RV0728(1)	
81·0	LpACT13F9(2)	
93·1	RV0728(2)	

FIG. 2. Continued.

FIG. 3. Heading dates in 200 plants of forage backcross mapping population.

FIG. 4. Significance of *t*-tests for marker/heading date association in the forage backcross mapping family for microsatellite markers across seven linkage groups (LG).

TABLE 4. *Comparison of frequency of polymorphism of EST microsatellites and genomic microsatellites in the forage mapping population*

	EST microsatellites	Genomic microsatellites
Total number tested	37	66
No. of polymorphic in forage	22	55
% polymorphic in forage	59·5	83
No. of non-polymorphic in forage	15	11
% non-polymorphic in forage	40·5	17

microsatellites in crop plants because of less DNA sequence conservation in non-transcribed regions (Cho *et al.*, 2000; Eujayl *et al.*, 2001; Russell *et al.*, 2004). The GeneThresher microsatellites were shown to produce strong bands and distinct allelic peaks. However, the level of polymorphism shown by the GeneThresher microsatellites was lower than those produced from the genomic library when tested on the forage and amenity populations (Table 4). The EST microsatellites were selected from those already shown to be polymorphic in the population
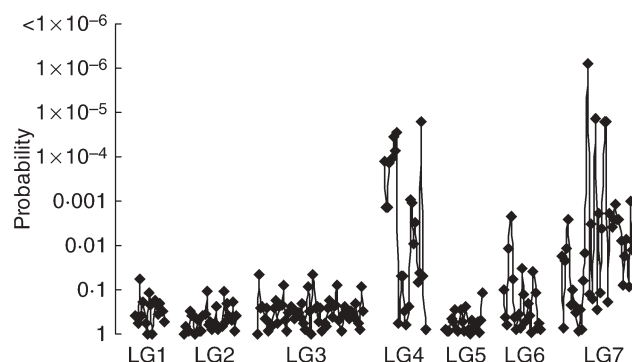
used to produce a genetic linkage map of *L. perenne* (Gill *et al.*, 2006) and therefore the figures shown in Table 3 may inflate the frequency of polymorphism of GeneThresher-derived microsatellites. The lower level of polymorphism shown by the GeneThresher-derived microsatellites was also found in *Lolium/Festuca* introgression lines. One hundred and sixty-one GeneThresher-derived microsatellite primer pairs were tried on the substitution lines, of which 36 % did not amplify a product in *Festuca* and 27 % were non-polymorphic between *Lolium* and *Festuca*. In comparison, of 58 genomic microsatellite primer pairs tried, 52 % did not amplify a band in *Festuca* but only 19 % were non-polymorphic between *Lolium* and *Festuca*.

The estimated total map length of the forage grass genetic map was 433·8 cM. This is considerably shorter than most of the recent genetic maps published in *L. perenne* (all examples of which below are forage populations). For example, Vialactia published a distance of 675·6 cM for their framework map (Gill *et al.*, 2006) made up of 376 SSR markers and nine RFLP markers. Armstead *et al.* (2002) reported 515 cM in an RFLP-based map and 565 cM in a backcross pedigree. Bert *et al.* (1999) reported a map distance of 930 cM (based on 463 AFLP markers), Jones *et al.* (2002) reported a map distance of 811 cM (based on 96 SSR loci) and Faville *et al.* (2004) reported map distances of 963 cM in one parent and 757 cM in the other (based on a combination of SSR and RFLP markers). Since the map reported here

contains only genomic and a few EST microsatellite markers it would suggest that the microsatellites are not evenly distributed across the genome and indeed microsatellite loci isolated from genomic libraries have previously been shown to cluster on a number of genetic maps, e.g. barley (Ramsey *et al.*, 2000). It is therefore possible that the inclusion of different types of markers, e.g. AFLP and RFLP, would give greater genome coverage.

QTL analysis revealed a major QTL for heading date on linkage group 7 in the forage population with a large number of the markers assigned to this group showing significant association to this QTL (Fig. 3). Analysis also revealed a second, considerably smaller QTL for heading date on linkage group 4. QTLs for heading date have been identified in Triticeae species associated with linkage groups 2, 4 and 7 (Laurie *et al.*, 1995; Lundqvist *et al.*, 1997; Börner *et al.*, 2002). In *L. perenne*, Armstead *et al.* (2004) located a major QTL for heading date to linkage group 7 and have shown that candidate orthologues of rice *Hd1* and barley *HvCO1* also map to linkage group 7 (Armstead *et al.*, 2005). Further smaller QTLs were also shown to be located on linkage groups 2, 4 and 7. QTL mapping with only 100 individuals is obviously of limited value. However, the main objective of this work was the construction of the microsatellite library and an initial assessment of its utility as regards its main objectives of marker-assisted selection within breeding programmes and gene isolation. The association of the microsatellite markers on linkage groups 4 and 7 with known QTLs for heading date provides further verification for the accuracy of the linkage map produced in this study and raises the confidence levels in the ability of the microsatellites to look for less well-understood QTL.

The genomic microsatellites described in this work, and the GeneThresher-derived microsatellites are presently being exploited for marker-assisted selection in breeding programmes. Key targets are the development of new varieties adapted to sustainable agricultural practices (e.g. low nitrogen input), climate change traits (e.g. heading date) and biorenewables (e.g. fermentability). In addition, the microsatellites are being exploited for the development of introgression maps of all seven *Lolium/Festuca* chromosomes. Introgression mapping is enabling the establishment of the syntenic relationship between the monocots and the determination of the genetic control of target traits (King *et al.*, 2002*a*, *b*, 2007). These microsatellites in combination with introgression maps will form the basis for the development of a physical map of grass and thus provide a springboard for gene isolation via map-based cloning (Armstead *et al.*, 2007).

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