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S. E. Warnke · R. E. Barker · Geunhwa Jung ·
Sung-Chur Sim · M. A. Rouf Mian · M. C. Saha ·
L. A. Brilman · M. P. Dupal · J. W. Forster

Genetic linkage mapping of an annual × perennial ryegrass population

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Abstract Annual (*Lolium multiflorum* Lam.) and perennial (*L. perenne* L.) ryegrass are two common forage and turfgrass species grown throughout the world. Perennial ryegrass is most commonly used for turfgrass purposes, and contamination by annual ryegrass, through physical seed mixing or gene flow, can result in a significant reduction in turfgrass quality. Seed certifying agencies in the United States currently use a test called seedling root fluorescence (SRF) to detect contamination between these species. The SRF test, however, can be inaccurate and therefore, the development of additional markers for species separation is needed. Male and female molecular-marker linkage maps of an interspecific annual × perennial ryegrass mapping population were developed to determine the map location of the SRF character and to identify additional genomic regions useful for species separation. A total of 235 AFLP markers, 81 RAPD markers, 16 comparative grass RFLPs, 106 SSR markers, 2 isozyme loci and 2 morphological characteristics, 8-h flowering, and SRF were used to construct the maps. RFLP markers from oat and barley and SSR markers from tall fescue and other grasses allowed the linkage groups to be numbered, relative to the Triticeae and the International *Lolium* Genome Initative reference population P150/112. The three-generation population structure allowed both male and female maps to be constructed. The male and female maps each have seven linkage groups, but differ in map length with the male map being 537 cm long and the female map 712 cm long. Regions of skewed segregation were identified in both maps with linkage groups 1, 3, and 6 of the male map showing the highest percentage of skewed markers. The (SRF) character mapped to linkage group 1 in both the male and female maps, and the 8-h flowering character was also localized to this linkage group on the female map. In addition, the *Sod-1* isozyme marker, which can separate annual and perennial ryegrasses, mapped to linkage group 7. These results indicate that *Lolium* linkage groups 1 and 7 may provide additional markers and candidate genes for use in ryegrass species separation.

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S. E. Warnke (✉)
National Arboretum, USDA-ARS,
3501 New York Ave. NE,
Washington, DC 20002, USA
e-mail: warnkes@ars.usda.gov
Fax: +1-202-2454579

R. E. Barker
National Forage Seed Production Research Center, USDA-ARS,
3450 SW Campus Way,
Corvallis, OR 97331-7102, USA

G. Jung · S.-C. Sim
Department of Plant Pathology, University of Wisconsin,
Madison, WI 53706, USA

M. A. Rouf Mian · M. C. Saha
The Noble Foundation,
Ardmore, OK 73402, USA

L. A. Brilman
Seed Research of Oregon,
Corvallis, OR 97333, USA

M. P. Dupal
Amersham Biosciences Pty. Ltd.,
Unit 1, 22 Hudson Avenue,
NSW 2154 Castle Hill, Australia

J. W. Forster
Department of Primary Industries, Primary Industries Research Victoria, Plant Biotechnology Centre, La Trobe University,
3086 Bundoora, Victoria, Australia

Introduction

The two primary cultivated members of the *Lolium* genus are Italian or annual (*Lolium multiflorum* Lam.) and perennial (*L. perenne* L.) ryegrass. These two species have very similar genomes and are fully interfertile. However, they are morphologically distinct, with annual ryegrass primarily used for forage production and perennial ryegrass types utilized for either turf or forage purposes.

Gene flow between these species or physical mixing of seed can result in a low quality turfgrass, and as a consequence, seed certification agencies utilize the seedling root fluorescence (SRF) test to identify seed lots contaminated with annual ryegrass. SRF is a genetic trait that is caused by the exudation of a fluorescent compound called annuloline. The gene or genes that condition this trait are only weakly linked to genes that result in an annual growth habit and therefore, it is possible to develop fluorescent perennial ryegrasses (Nyquist 1963).

Inaccuracy of the SRF test (Floyd and Barker 2002) has led to a search for an alternative or supplemental test to more accurately detect annual ryegrass contamination of perennial ryegrass (Warnke et al. 2002). Two leaf isozymes have been identified that can be used as species indicators. A phosphoglucose isomerase locus (*Pgi-2*) and a superoxide dismutase locus (*Sod-1*) show allele frequency differences between *L. perenne* and *L. multiflorum* (Charmet and Balfourier 1994). The *Pgi-2* locus is well studied in ryegrass and was mapped to linkage group 1 (Armstead et al. 2002). Warnke et al. (2002) reported on the segregation of the *Sod-1* locus, and the present paper is the first to assign this locus to a defined linkage group.

One of the primary differences between annual and perennial ryegrass is the control of flowering in these species. Annual ryegrass has an annual to weakly perennial growth habit and, in most cases, no vernalization requirement. In addition, annual ryegrass has a photoperiod-induction period that is less than 12 h, with most plants being photoperiod insensitive. In contrast, perennial ryegrass varieties used as turfgrass in the United States have a strong vernalization requirement and generally require a photoperiod over 12 h before flowering will be initiated (Jung et al. 1996). Therefore, genetic markers in chromosomal regions that control flowering may provide good candidates for ryegrass species separation.

A genetic map-based approach is one method that could be used to expedite the identification of markers for species separation. Several genetic maps of ryegrass have been reported (Jones et al. 2002a). However, the majority of these maps have been based on the P150/112 reference mapping population that was derived by crossing a highly heterozygous *L. perenne* parent of complex descent, as the pollinator, with a doubled-haploid *L. perenne* female parent (Bert et al. 1999; Jones et al. 2002a, 2002b). Hayward et al. (1994) utilized an annual × perennial ryegrass cross that was then crossed with a doubled-haploid perennial parent, and the map identified the location of several genomic regions with influence on developmental characteristics.

L. perenne and *L. multiflorum* are both highly outcrossing species. Self-fertility is limited by a gametophytic, two-locus incompatibility system with the *S* and *Z* loci acting at the stigmatic surface (Cornish et al. 1980). The *S* and *Z* loci have recently been mapped to *Lolium* linkage groups 1 and 3 (Thorogood et al. 2002). The locations of self-incompatibility loci have been predicted based on the clustering of loci with distorted segregation ratios (Wricke and Wehling 1985).

Most plant species of agronomic importance are self-fertile and, therefore, mapping pedigrees involving back-crosses or F_2s are constructed. These population structures simplify mapping because genetic segregation is the result of the meiotic recombination from a single F_1 genotype. Therefore, only two alleles segregate in the mapping populations and, because two homozygous inbred lines are used to produce the F_1 parent, linkage phase among alleles in the population is known (Sewell et al. 1998).

In self-incompatible outcrossing species, high genetic load typically prevents the development of inbred lines. Therefore, both parents of a mapping population are highly heterozygous, with as many as four alleles segregating at a given locus, and any given marker can segregate in two (1:1), three (1:2:1), or four (1:1:1:1) genotypic classes. Additionally, phase relationships among alleles are not known, but must be determined from the inheritance within a three-generation population structure or from progeny segregation data. Crosses between highly heterozygous parents allow progeny data from dominant markers such as AFLPs and RAPDs to be subdivided into two independent data sets that contain the meiotic segregation data from each parent, allowing independent linkage maps to be constructed. Codominant markers segregating from both parents can then be used to align linkage groups and produce a sex-averaged map (Devey et al. 1994).

This paper reports on the development of genetic maps in an interspecific three-generation mapping population between annual ryegrass and perennial ryegrass and provides the first evidence of the likely genome location of the SRF and 8-h flowering traits in ryegrass.

Materials and methods

Plant material

Two annual ryegrass plants from the cultivar Floregon were crossed with two perennial ryegrass plants from the cultivar Manhattan. From the resultant F_1 populations, two random plants were chosen (one from each) and crossed to develop a three-generation population (*n*=91, Fig. 1). All of the seed used to develop the progeny population was obtained from MFA-4, making it the female parent and MFB-2 the male parent for the mapping population. Dominant AFLP and RAPD markers segregating from each of the parents were collected and maintained as separate data sets. Therefore, markers coming from the female parent represent female recombination events, while those from the male parent represent male recombination.

DNA extraction

Genomic DNA was extracted using a modified CTAB (cetyltrimethylammonium bromide) extraction (Saghai-Maroof et al. 1984) from 100 mg lyophilized plant leaf tissue.

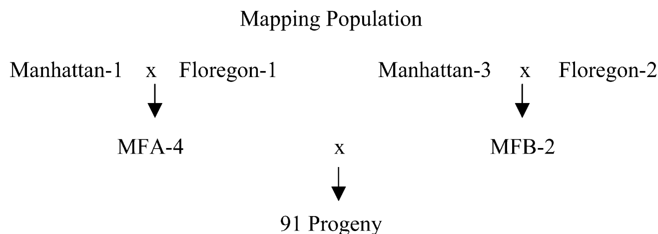

Fig. 1 Three-generation population structure used to develop annual × perennial ryegrass genetic maps. All seed of the progeny was harvested from the MFA-4 F_1 parent, making this the female parent and MFB-2 the male parent. Manhattan grandparents were from the perennial ryegrass cultivar Manhattan, and annual ryegrass grandparents were from the annual ryegrass cultivar Floregon

AFLP analysis

DNA digestions, adaptor ligations, and preselective and selective amplifications were performed according to instructions provided with an AFLP analysis kit purchased from GIBCO BRL (Gaithersburg, Md., USA) and standard AFLP procedures (Vos et al. 1995). Fluorescent 6-FAM (6-carboxy fluorescein) primers labeled on the 5′ nucleotide were used to carry out the selective amplifications with three selective bases per primer (Table 1). The amplified fragments were detected with an ABI373XL instrument (PE Applied Biosystems, Foster City, Calif., USA) using a 34-cm well to read polyacrylamide gels formulated with 5.75% (w/v), 7 M urea, and 1× TBE running buffer. Each sample lane included the GS500-ROX internal lane standard (Larson et al. 2001). The gel electrophoresis procedures were performed by the Oregon State University Gene Center, Corvallis, Ore. Fluorescent fragments between 50 nucleotides (nt) and 500 nt were identified by GeneScan 3.1 software (PE Applied Biosystems). Genescan trace files were then analyzed for the presence and absence of AFLP products, in 1-nt intervals, using the computer program Genographer (Benham et al. 1999).

RAPD analysis

Reactions for RAPD analysis were performed in 10-µl volumes in 96-well plates in an MJ PTC-100 incubator (MJ Research, Watertown, Mass.) following the methods of Johns et al. (1997). All RAPD reaction products were electrophoresed on agarose gels as described by Johns et al. (1997). Gels were run for 2 h at 300 V, stained with ethidium bromide, illuminated by UV light, photographed, and manually scored for presence/absence of clear bands. All 10mer primers were obtained from Operon Technologies (Table 2).

RFLP analysis

For Southern blotting, 10 µg genomic DNA from each plant was digested with four different restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, and *Hin*dIII). Restriction endonuclease-digested DNAs were electrophoretically fractionated in 1% agarose gels and transferred to Hybond-N+ nylon membranes (Amersham, Piscataway, N.J.) by capillary transfer. Prehybridization and hybridization were performed in a rotary hybridization chamber (Techne, Burlington, N.J.) at 65°C. Probes were labeled with ^{32}P by the random hexamer primer method (Feinberg and Vogelstein 1984). The membranes were washed in 0.5× SSC and 0.1% SDS for 50 min at 65°C.

The cDNA probes used to develop the genetic linkage map of ryegrass were derived from a common set of 152 anchor probes provided by S. McCouch of Cornell University, Ithaca, N.Y. (Van Deynze et al. 1998). Additional oat and barley (BCD) cDNA clones were obtained from the United States Department of Agriculture, Albany, Calif. (Table 3). The probes were screened to detect polymorphism using the heterozygous parents. Polymorphic probes with a simple segregating pattern were selected and tested on a progeny set of up to 89 randomly selected individuals.

Table 1 Summary of AFLP markers segregating in the MFA-4 × MFB-2 population

Primer combination	Marker code	Total polymorphic bands	Polymorphic bands mapped
*Eco*RI-AAG/*Mse*I-CAT	(P, A)[a]-EaagMcat-(x)[b]	6	6
*Eco*RI-AAC/*Mse*I-CAC	(P, A)-EaacMcac-(x)	8	8
*Eco*RI-AAC/*Mse*I-CTG	(P, A)-EaacMctg-(x)	18	0
*Eco*RI-AAC/*Mse*I-CTT	(P, A)-EaacMctt-(x)	20	16
*Eco*RI-AAG/*Mse*I-CTT	(P, A)-EaagMctt-(x)	8	8
*Eco*RI-ACA/*Mse*I-CAC	(P, A)-EacaMcac-(x)	20	19
*Eco*RI-ACA/*Mse*I-CAT	(P, A)-EacaMcat-(x)	18	16
*Eco*RI-ACA/*Mse*I-CTA	(P, A)-EacaMcta-(x)	14	13
*Eco*RI-ACA/*Mse*I-CTG	(P, A)-EacaMctg-(x)	6	5
*Eco*RI-ACA/*Mse*I-CTT	(P, A)-EacaMctt-(x)	6	6
*Eco*RI-ACC/*Mse*I-CAA	(P, A)-EaccMcaa-(x)	17	16
*Eco*RI-ACC/*Mse*I-CAC	(P, A)-EaccMcac-(x)	22	19
*Eco*RI-ACC/*Mse*I-CAT	(P, A)-EaccMcat-(x)	16	0
*Eco*RI-ACC/*Mse*I-CTA	(P, A)-EaccMcta-(x)	22	19
*Eco*RI-ACG/*Mse*I-CAA	(P, A)-EacgMcaa-(x)	23	19
*Eco*RI-ACT/*Mse*I-CTG	(P, A)-EactMctg-(x)	7	7
*Eco*RI-AGC/*Mse*I-CAT	(P, A)-EagcMcat-(x)	27	22
*Eco*RI-AGC/*Mse*I-CTG	(P, A)-EagcMctg-(x)	21	20
*Eco*RI-AGG/*Mse*I-CTT	(P, A)-EaggMctt-(x)	18	16
Total		297	235

[a]Designation of the fragment by *P* indicates that the fragment was present in the perennial grandparent and its presence in the annual grandparent is unknown. *A* indicates that the fragment was not present in the perennial grandparent; therefore, the fragment originated from the annual grandparent
[b]Identifies the fragment size of the segregating band scored in the MFA-4 × MFB-2 population

Table 2 Naming of RAPD primers and fragments used to construct the linkage maps of MFA-4 and MFB-2

Primer name on map	Fragments scored	Fragments mapped
B11_xxxx[a]	3	3
B17	3	2
C11	2	2
C19	6	5
D16	4	2
D2	2	1
E14	4	3
E3	4	4
E6	6	5
E8	5	4
F13	6	5
F14	5	5
F9	5	4
G7	2	2
G10	6	6
G11	4	3
H13	4	3
H4	4	3
K12	4	4
K2	5	4
K8	3	5
K9	6	6
Total	94	81

[a] *_xxxx* Approximate size in base pairs of polymorphic fragment scored

Table 3 Origin of cDNA probes use to construct the linkage maps of MFA-4 and MFB-2

Acronym on map	Probe source	Number of loci mapped
CDO	Oat leaf cDNA	13
BCD	Barley leaf cDNA	1
RZ	Rice leaf cDNA	2
Total		16

SSR analysis (tall fescue and conserved grass EST-SSRs)

A total of 30 tall fescue EST-SSR and 25 conserved grass EST-SSR primer pairs were found to be polymorphic in this population and used for mapping (Tables 4, 5, 6). Twenty nanograms DNA was used as template for each PCR reaction. The PCR reactions consisted of one unit of Ampli*Taq* Gold with GeneAmp PCR Buffer II (Applied Biosysytems/Roche, Branchburg, N.J.), 3 mM $MgCl_2$, 200 µM of dNTPs, and 0.2 mM each primer in a 10 µl reaction. The PCR amplification conditions were: denaturation at 95°C for 10 min; followed by 40 cycles at 95°C for 50 s, 50 s at a temperature between 58°C to 64°C (the optimum annealing temperature for the respective primer pair), 72°C for 90 s; and a final extension step of 10 min at 72°C. The PCR products were resolved on 6% polyacrylamide denaturing gels (Gel Mix 6, Invitrogen Life Technologies, Carlsbad, Calif.). The gels were silver stained using Silver Sequence Kit (Promega, Madison, Wis.) for SSR-band detection. The band size is reported for the most intensely amplified band for each SSR or the average of the stutter if the intensity were the same using a 10-bp DNA ladder (Invitrogen Life Technologies) as the reference point. The loci were scored as dominant markers for the presence or absence of the SSR band. The genomic DNA-derived SSR (LPSSR) markers developed by Primary Industries Research, Victoria, Australia are proprietary in nature; therefore, primer sequences are not presented, but information for research purposes may be obtained on request.

Linkage analysis

JoinMap 3.0 software (van Ooijen and Voorrips 2001) was used to carry out the linkage analysis using the CP-type data classification. Separate maternal (MFA-4) and paternal (MFB-2) maps were calculated. The determination of linkage groups of markers originating from MFA-4 and MFB-2 was performed with log-of-odds (LOD) ratio thresholds of 7.0, with the exception of linkage group 1 of the MFA-4 map; an LOD ratio of 5.0 was used. The calculations of the linkage maps were done using all pairwise recombination estimates <0.499 and an LOD score >0.001 (ripple value = 1, jump threshold = 5, and a triplet threshold = 7). The Kosambi mapping function (Kosambi 1944) was used to convert recombination units into genetic distances.

Phenotypic analysis

SRF was scored on each plant in the mapping population by germinating the seed on white blotter paper and examining the seedling roots for the presence of fluorescence for 18 days following germination. When a seedling root exhibited fluorescence, it was removed from the filter paper and planted in a greenhouse. SRF was scored as a dominant trait with plants exhibiting fluorescence scored “ab” and those not fluorescing scored “aa.”

Data for the 8-h flowering character was evaluated by growing two replications of each clone of the mapping population in a growth chamber under vernalization conditions of a 8-h photoperiod (425 µmol m^{-2} s^{-1} PAR) at 5°C for 116 days. The plants were then moved to a different growth chamber, maintained at 8-h photoperiod (425 µmol m^{-2} s^{-1} PAR) at 25°C for 116 days, and the days to flowering was recorded for each clone. A limited number of the clones in the mapping population were capable of flowering under these conditions, and the trait was scored as a recessive trait with plants flowering scored “aa” and plants not flowering scored “ab.”

Table 4 Origin of SSR primers used to construct the linkage maps of MFA-4 and MFB-2

Acronym on map	Source	SSR origin	Number of SSR primer pairs used	Number of SSR loci mapped
TFxx[a]-xxx[b]	Noble Foundation	Tall fescue	30	44
K or H	Agriculture Victoria	Ryegrass	54	25
CG-xx-xxx	Cornell University	Gramineae	25	37
Total			109	106

[a] *xx* Identification number given to primer pair
[b] *xxx* Size of fragment amplified using primer pair

Table 5 List of tall fescue EST-SSR primers used in mapping the ryegrass population

Marker name on map	Noble Foundation marker name	Forward primer sequence 5′ to 3″	Reverse primer sequence 5′ to 3′	Temp. (°C)	Mapped loci	Mapped to linkage group
TF2	NFFa002	GCTCCAGCTTCTCCATCATC	ACCAAGTCGTCCAAGTCAGC	62	0	-
TF4	NFFa004	AAGCGGGAGGAGAGATGG	CACCACGACGTCGCTCTC	64	4	A1, B3, A4–B4[a]
TF9	NFFa009	TCCAACCCTAGATCCACACC	GGTGAACTCGTCGGTGATCT	62	1	A3
TF15	NFFa015	GCGTCCACTAACAACACCAA	AGCAAGGCCAGCAAAAATTA	60	2	A6–B6
TF19	NFFa019	TGGATTTGCAATTAGCCTCA	GCTCGTGTATGGCCTTCAAT	60	**1**	**A7–B7**[b]
TF21	NFFa021	CACAGCTCGTATAGGCGTCA	CTTGTCGAAGAGCGGGAAC	62	3	A2, A7–B7
TF23	NFFa023	AGTCGGTGGTGAAGCTGAAG	TACAACTAGGGGGCTGGTCA	62	1	B2
TF24	NFFa024	TGCCCACGAGGTCTATCTTC	AGCTTCCCCTTCATTCCACT	62	1	B7
TF27	NFFa027	CGAGGTCTCAATCCTCCATT	GACAGAGACGACGACGACAT	62	2	A5–B5
TF30	NFFa030	AGTCGGTGGTGAAGCTGAAG	ACAACTAGGGGGCTGGTCA	62	1	B2
TF31	NFFa031	ACGGTCTGTACCGTGGATGT	GCTGTAGACTCAGCCGAACC	64	2	A1–B1
TF34	NFFa034	GCTGGGTGTAGGGCTGTAAA	CTCCTTTCCATCACCTCTGG	62	2	A7, B3
TF36	NFFa036	AGAGGAAGAGCGAAAGAGCA	CCCTGGTACTCGTGGATGTT	60	2	A6–B6
TF39	NFFa039	GTCTGCACCCCTCTCCTCTC	CTCCTTATCTTGGCGATGGA	64	2	A2–B2
TF41	NFFa041	TCCTGAGAGACATCGAGCAG	TCAAAAGCCCAAACACTTCC	60	1	A6
TF45	NFFa045	ACGAGGGAAAGGTAGGGTTT	GATGAAGCCAATTTCCTTGG	60	2	A4–B4
TF47	NFFa047	TTCCTTCCTCTTTCCCAACA	ATGGTCTCCCTCTGCTCGTA	60	1	A1
TF48	NFFa048	CAGGCTGTTAACGGTGTCCT	CCTTCTTCTTGGGAGGGAAA	60	2	A6–B6
TF49	NFFa049	CTACTACGGGGGAGGTGGAG	CGCAACAGTTGTACCGACAG	64	1	A6
TF52	NFFa052	GTGGATCCAAACGAATCGAA	GTGCTCTTCCTTCCAGTTCC	60	0	-
TF58	NFFa058	CAATCTACCGTCGCTTCACC	CAAAACCAGGTGGCAGATTT	60	1	A7
TF59	NFFa059	GTCGCCGGAGAAGAGAAGAG	AACGCTAGCCGTGATGACTT	62	2	A5–B5
TF61	NFFa061	TGGATTTGCAATTAGCCTCA	GCTCGTGTATGGCCTTCAAT	60	1	B7
TF64	NFFa064	TCATTTGACGCCACTTGAAC	GTCTTAGCGCCTTCCTTGGT	60	1	A5
TF66	NFFa066	CTCCCCGTCCTTCCATCT	CAACCTCCTCCACCATCTTG	62	**1**	**A1–B1**
TF68	NFFa068	GAGGTTTTGCTCCCTCCTGT	AGGGTCCTTCTGCAAGTCCT	60	1	B2
TF69	NFFa069	CCCAAGAAGAAGACGACCAA	ACGACCGAATGGACAGAGAC	62	1	A7–B7
TF73	NFFa073	TTCCTTCCTCTTTCCCGAAC	ATGGTCTCCCTCTGCTCGTA	60	1	B1
TF74	NFFa074	TTCCTTCCTCTTTCCCAACA	ATGGTCTCCCTCTGCTCGTA	60	1	A1
TF75	NFFa075	CTCTGCCCTTCCTTCCTCTT	ATGGTCTCCCTCTGCTCGTA	60	2	A1–B1

[a]A (–) connecting two linkage groups indicates that two loci amplified by the same primer pair were mapped on the same linkage group on both female and male maps
[b]The marker in *boldface* was common to both parents and segregated in the progeny and was mapped on both female and male maps

Results

Genetic linkage map

Linkage groups were numbered according to the order proposed by Jones et al. (2002a). Congruence of linkage groups was established based on common RFLP loci. The largest gap on the MFA-4 map was 22 cM on linkage group 1, and the average distance between markers was 3.00 cM/marker (Fig. 2). The largest gap on the MFB-2 map was 17 cM on linkage group 2, and the average distance between markers is 2.39 cM/marker (Fig. 3). The map length of MFA-4 (female parent) was 712 cM, and the length of MFB-2 (male parent) was 537 cM.

Dominant markers (AFLP, RAPD)

A total of 297 AFLP bands were scored using 19 *Eco*R1 +3/*Mse*1+3 selective primer combinations (Table 1), and 235 were mapped. The primer combination *Eco*R1-AGC/ *Mse*1-CAT produced the highest number of polymorphic and mapped fragments. On average, 16 polymorphic fragments were scored per primer pair, and 11 of them were mapped.

Eighty-one polymorphic fragments were mapped using 22 RAPD primers (Table 2).

RFLPs

A total of 16 codominant RFLP markers were polymorphic and placed on the male and female linkage maps. The

Table 6 List of conserved grass EST-SSR primers used in mapping the ryegrass population

Marker name on map	Cornell Name	Forward primer sequence 5′ to 3′	Reverse primer sequence 5′ to 3′	Optimum primer temperature for annealing (°C)
CG-002.1	CNL51	CTAGGGTTTCCCACCTCTCA	AATGTCCTTGGCGTTGCT	58
CG-002.2	CNL50	AAAGGTAGGGTTTCCAGTTGC	AATGTCCTTGGCGTTGCT	58
CG-004	CNL53	CGCAGCAAGTAGGGTTAGGA	CCTCGTGGTGGATCTGCAT	62
CG-009.2	CNL58	AAAGTGACAGCAACAATGACC	CAGATGGCGTCGTAGTCGAG	60
CG-019	CNL141	ATTAACATGCGGTGTTGCAT	GAGTGGAGGTGGAGGATGAG	58
CG-025	CNL142	AATTCGGCACCAGCTCCT	CTGGAAGTCGAGGTTGAGGT	60
CG-026(1)	CNL77	GCAAGTGGGCGCTCTCCT	GTCCATGAGCCTGGACACCTC	64
CG-026(2)	CNL78	GCAAGTGGGCGCTCTCCT	GGCATTGACTTGGAGCTTCTTAG	64
CG-028	CNL83	ATCGACGGCACGATCAAG	GGTGGCAGTGGAAGTGCTAT	60
CG-030	CNL144	AGAAGGCGGCTCAGAAGAAG	GCTCCAACTCAGAATCAACAA	60
CG-032	CNL145	ATCTCCTCTCCTCCGTCCTC	TCACCGACATAGGCATCCTT	60
CG-033	CNL86	CAACAACGTCAACGCCTTC	GCGTCTTGAACCTCTTGTCC	60
CG-035	CNL147	GGCTAGGGTTTCGACTCCTC	AGATGGCGAACTCGACCTG	62
CG-041.1	CNL151	AGAAATCCATCCATCCCATT	AGGCGTTGTCGAGGCTGT	58
CG-042	CNL152	ACAAAGGCTCACCGTGGAA	GTCGGAGGCGATGAACTCT	60
CG-044	CNL153	CCTTCTTCCGCGACTACCTC	TCTGACATTGCTTCCATTGC	60
CG-045	CNL155	GTATTCTCCCATGGCGACTG	GCCTCCTTGATCATCTCGAA	60
CG-053	CNL108	GAGGAGGAGAGCGGATTCT	CTCCATCGTTTCCCAAGC	60
CG-065	CNL124	TCGTTGTTCTTGGTTGGTTG	GGTTGATGTTGGCCTTCG	58
CG-066.2	CNL126	CTACGGCATCAGGAAGCTC	GTCTCTGTGCTTCACCACCA	62
CG-077.2	CNL43	GCCAGCTAGTGAGTGTGTGC	CCGACGATGTTGAAGGAGAG	62
CG-082	CNL48	AGAGTACACCAACGCCATCA	CCGTCAACATTCCCTTCATT	58
CG-085	CNL132	GCAGCAACAACAACCAGTTC	TTGCAGGAACACCTTGCAT	58
CG-086	CNL133	CAGGCAACAACCACCATTT	GAACATGCCCTTCATCTGCT	58
CG-088	CNL135	TTCACAGCAACAACAACCAG	TGCGACCTAGCAAGACGTT	58

codominance of these markers allowed the male and female maps to be combined. These markers are primarily part of the Cornell grass anchor set and allow linkage groups in this population to be numbered according to the International *Lolium* Genome Initiative (ILGI) reference map (Table 3).

SSRs

SSR primer pairs from three sources were used in map development (Table 4). The perennial ryegrass genomic SSR markers were only mapped in the MFB-2 parent because missing data complicated map construction in the MFA-4 parent. A total of 109 SSRs were evaluated, and 106 were mapped with some of the EST-SSR loci mapping to more than one location.

Forty-six tall fescue EST-SSR loci were mapped from the 30 primer pairs (Table 5). The markers from two primer pairs (NFFa002 and NFFa052) remained unlinked. Between two to four loci were mapped from each of 15 primer pairs, while one SSR locus was mapped from each of the remaining 13 primer pairs. Two markers (NFFa019-170 and NFFa066-294) segregated from both parents and were therefore included on both parental maps. The markers detected by primer pair NFFa019-170 mapped to linkage group 7 on both parental maps, while the markers detected by primer pair NFFa066-294 mapped to linkage group 1 on both maps. This type of segregating marker is common to both parents and can be used to merge the two parental maps. The multiple loci amplified by the same primer pair in some instances mapped to different locations on the genome.

Thirty-eight conserved grass EST-SSR loci were mapped from 21 primer pairs, and the markers from 4 primer pairs remained unlinked. One segregating marker [CG026.(1)] that was common to both parents was mapped to linkage group 7 in both parents. Again, between two to four loci were mapped from each of nine primer pairs, and one locus was mapped from each of the remaining 12 primer pairs. A number of loci originating from the same primer pair mapped to different places on the genome. One to six conserved grass SSR loci were mapped to each linkage group in both maps.

A total of 43 perennial ryegrass genomic SSR loci (LPSSR) primer pairs detected polymorphic loci segregating from the MFB-2 genetic map, of which ten detected multiple loci. One primer pair (designed to clone LPSSRH09E12) detected three separate genetic loci. A total of 54 polymorphic loci were used for the genetic map analysis and a total of 23 loci were assigned to the MFB-2 parental genetic map. The map locations of LPSSR loci on

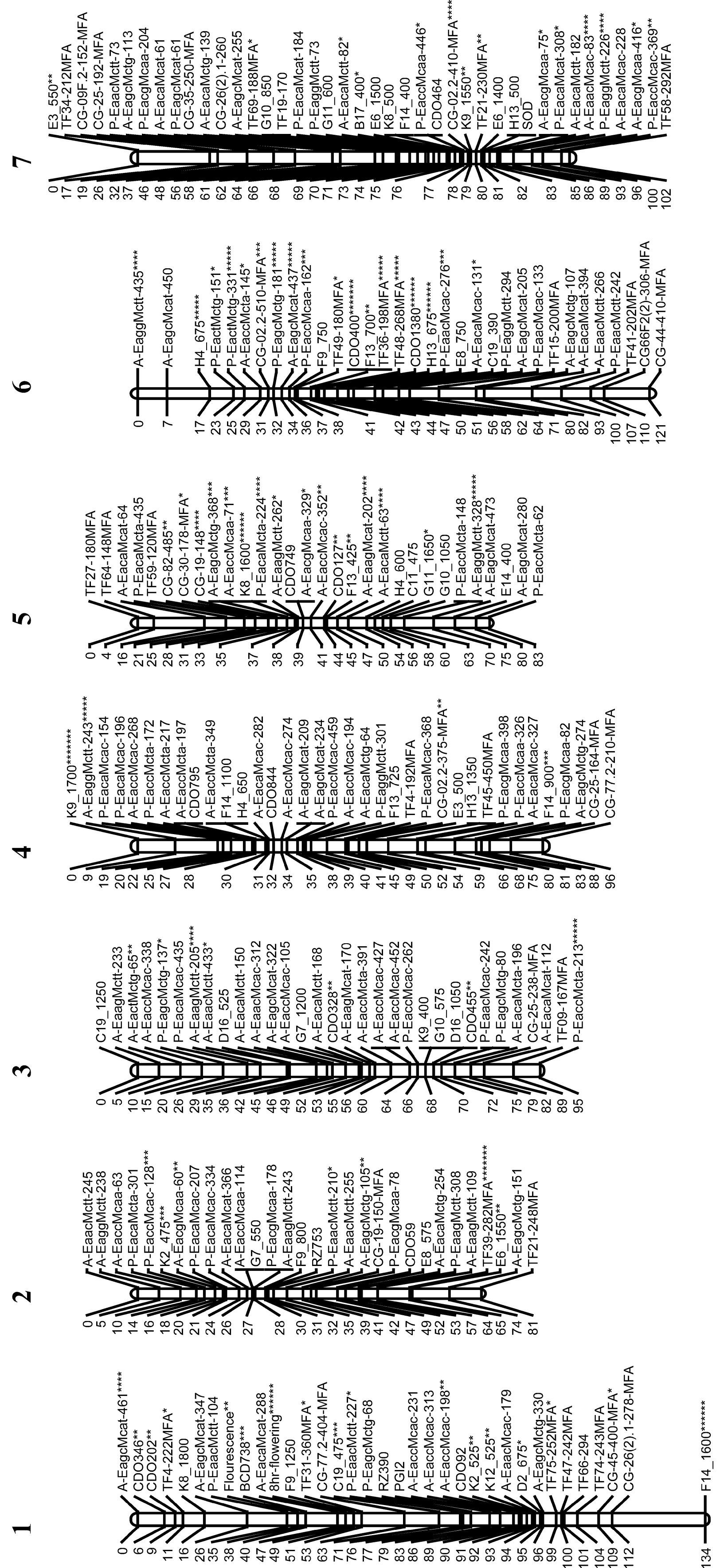

Fig. 2 Molecular-marker linkage map of the MFA-4 female parent from the MFA-4 × MFB-2 cross. Group numbering is according to Jones et al. (2002), a based on common RFLP markers. Loci labeled with *asterisks* showed distorted segregations (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001, *****P<0.00001). Marker types and names are presented in Tables 1, 2, 3, 4, 5, and 6. Two isozyme loci (*Pgi-2* and *Sod-1*) and two morphological markers, Fluorescence and 8-h flowering, were also mapped

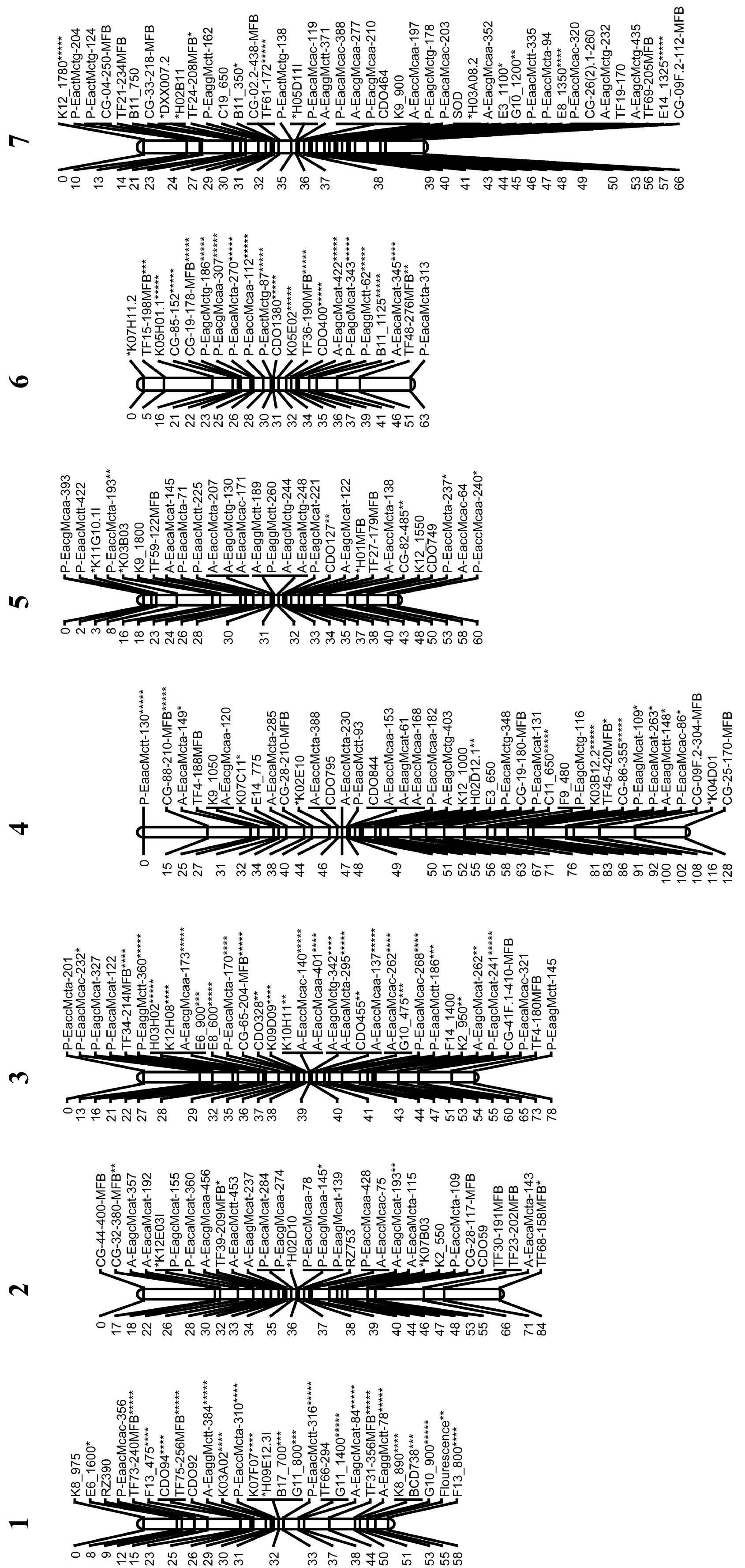

Fig. 3 Molecular-marker linkage map of the MFB-2 male parent from the MFA-4 × MFB-2 cross. Group numbering is according to Jones et al. (2002a), based on common RFLP and genomic DNA-derived SSR markers. Loci labeled with *asterisks* showed distorted segregations (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001, ******P*<0.00001). Marker types and names are presented in Tables 1, 2, 3, 4, 5, and 6. Two isozyme loci (*Pgi-2* and *Sod-1*) and one morphological marker, Fluorescence, were also mapped

the MFB-2 map provide further confirmation of the assignment of linkage groups to the reference maps of Jones et al. (2002a, 2002b). Loci detected by primer pairs LPSSRK03A02 and LPSSRH09E12 have been assigned to linkage group1 in both the P150/112 and the MFB-2 maps. This also applies to loci detected by primer pairs LPSSRK12E03 and LPSSRH02D10 on linkage group 2, loci detected by primer pairs LPSSRH03H02 and LPSSRK12H08 on linkage group 3, loci detected by primer pairs LPSSRK07C11, LPSSRK03B12, and LPSSRK04D01 on linkage group 4, the locus detected by primer pair LPSSRK03B03 on linkage group 5, and the locus detected by primer pair LPSSRK05H01 on linkage group 6. One anomalous location was identified (e.g., the locus detected by primer pair LPSSRH02D12 on linkage group 4, attributed to linkage group 3 in the reference map), and the remaining loci were unmapped in the reference population. These latter markers provide an expansion of the coverage of this marker set.

Morphological markers

The SRF character mapped to linkage group 1 in both the MFA-4 and MFB-2 linkage maps. The SRF trait was placed on linkage group 1 at an LOD of 7.0 in the MFB-2 map; however, LOD scores had to be lowered to 5.0 to show significant linkages on group 1 in the MFA-4 map. At an LOD score of 5.0, the 8-h flowering character could be placed on linkage group 1 of the MFA-4 map. Both traits showed significant distortion from the expected 3:1 segregation ratios. The morphological markers are 11 cM apart on this linkage group near the RFLP marker BCD738.

Segregation distortion

Markers with distorted segregation ratios were present in both the MFA-4 and MFB-2 maps. Thirty-four percent of the markers mapped from the MFA-4 female parent showed distorted segregation ratios. Clusters of skewed markers were evident on linkage groups 5 and 6, with over 50% of the markers showing distorted ratios. Forty-two percent of the markers from the MFB-2 map show distorted ratios, with linkage groups 1, 3, and 6 having over 70% of the markers showing segregation distortion.

Discussion

The mapping population structure allowed development of male and female linkage maps for this population because all of the seed for the establishment of the population was taken from MFA-4, making this the female parent and MFB-2 the male parent. The use of dominant AFLP markers allowed separate maps to be developed for each parent (Knapp et. al. 1995). Difference in map length was shown and may indicate a reduced level of recombination in the male parent. Recombination frequency differences between male and female parents have been reported in other species (Sewell et al. 1998); however, to determine if this is a species-wide characteristic or a cross-specific phenomenon will require analysis in additional populations. The map length of the MFA-4 female parent (712 cm) is comparable to the previously published P150/112 map (811 cm), which is a male map with respect to the dominant markers used in map construction (Jones et al. 2002a).

Clusters of markers with distorted segregation ratios were visible on linkage groups 5 and 6 in the MFA-4 map and groups 1, 3, and 6 in the MFB-2 map. The MFB-2 clusters were more significant, with over 70% of the markers on groups 1 and 3, and 90% of the markers on group 6 showing distortion. Group 1 is known to contain the *S* self-incompatibility locus and this is the likely cause of distorted ratios on this linkage group. The *Z* self-incompatibility locus maps to linkage group 2 (Thorogood et al. 2002), but allelic variability in this cross may not lead to any distorted segregation in this region. Linkage group 5 is the location of a self-compatibility locus in rye (Fuong et. al. 1993) and may be the cause of the distorted segregation on this group in the MFA-4 map. Both the MFB-2 and P150/112 maps identify a region of skewed markers on linkage group 3 (Jones et al. 2002a).

AFLP markers were the first markers used in the development of this map followed by RAPD, RFLP, and SSR markers. The dominant AFLP and RAPD markers are useful for increasing map coverage, while the RFLP markers were very valuable in alignment of these maps with the P150/112 reference population developed through ILGI (Jones et al. 2002a). Based on the 16 RFLP markers used in this study and the map-assigned LPSSR markers on the MFB-2 map, chromosome numbering matched that of the p150/112; however, the identification of the level of synteny with the Triticeae and the location of major chromosome rearrangements will require a more complete RFLP analysis.

Codominant SSR markers are a useful tool for alignment of trait-specific maps with reference maps (Jones et al. 2002a) because these markers are portable between crosses and are highly polymorphic. A number of tall fescue EST-SSR markers developed at the Noble Foundation (Mian et al. 2003) were added to this map, as well as conserved grass EST-SSR markers developed at Cornell University (Kantety et al. 2002; Ju-Kyung et al. 2003). The EST-derived markers offer the opportunity for gene discovery and increase the value of genetic markers by surveying variation in transcribed gene-rich regions of the genome. EST-derived markers are likely to be more conserved and thus more transferable across species than genomic DNA-derived markers (Decroocq et al. 2003). Nearly 50% of the EST-SSR primer pairs produced multiple loci that were mapped to different positions in the genome. Similarly, 39% of the conserved grass EST-SSR primer pairs produced multiple loci in wheat (Mark E. Sorrells, 2003, personal communication). The portion of multiple loci-detecting EST-SSR primer pairs was

double that of primer pairs derived from genomic DNA (20%). This is due to higher conservation of gene-rich regions of the genome compared to the intergenic regions. The map locations of such multiple loci amplified by a single primer pair indicate the position of conserved or duplicated genomic regions in *Lolium*.

The tall fescue and the conserved grass SSR markers are being mapped in a tall fescue genetic map at the Noble Foundation. The map location of common SSR loci between the tall fescue and the ryegrass map will allow the alignment of the maps of the two species. The further development of freely available SSR markers should allow for rapid development of chromosome-aligned maps in trait-specific populations from the *Festuca*-*Lolium* complex.

The population structure used to develop these genetic maps was established to help identify the *Lolium* chromosomal regions that are important in separating the two major *Lolium* species, *L. multiflorum* and *L. perenne*. The *Sod-1* locus maps to linkage group 7; however, it is not possible to determine if the utility of this isozyme marker for species separation is due to linkage with a locus that causes morphological variation or if the common perennial ryegrass allele at the *Sod-1* locus improves long-term survival of this species. The *Pgi-2* locus, SRF, and the 8-h flowering characteristics on linkage group 1 can be used to separate annual from perennial ryegrass and provide support for developing markers from this chromosomal region for species-separation tests. The SRF character was first reported by Gentner (1929), and since then, it has been studied by several scientists with the most current report on genetics by Nyquist (1963). However, this is the first research to localize the trait to a specific chromosomal region. The 8-h flowering character was identified following vernalization of the mapping population. Certain genotypes flowered under short 8-h day lengths, indicating that they were essentially photoperiod insensitive following vernalization. This locus may be homologous to the *ppd-H2* locus in barley that is located on linkage group 1H and controls flowering under short day lengths; however, there are not enough common markers between maps to establish synteny in this region (Laurie et al. 1995).

The SRF and 8-h flowering traits map in a region that has been shown to contain the *S* self-incompatibility locus (Thorogood et al. 2002). Both the SRF character and the 8-h flowering trait have skewed segregation relative to the expected 3:1 ratios. It is interesting that the 8-h flowering trait only maps on the MFA-4 map; this may be due to the fact that there are far fewer skewed markers in this region of the female map. It can also be hypothesized that the 8-h flowering trait is the character being selected against when SRF is used as a species-separation tool. Recombination between these two loci might have led to the reduction in utility of SRF as a species-separation tool. Future map base cloning efforts in *Lolium* may make it possible to identify the gene responsible for the 8-h flowering trait. The cloning of a gene responsible for true phenotypic differences between annual and perennial ryegrass will allow for the development of a fast accurate DNA based species-separation tool.

Marker nomenclature

The EST-SSR markers were designated according to their source of development. The tall fescue EST-SSR markers developed in the Noble Foundation have the prefix "NFFa," and conserved grass EST-SSRs markers developed at Cornell University have the prefix "CNL." More information on the conserved grass SSRs can be obtained from GrainGene Web site (http://www.wheat.pw.usa.gov) under the section of "Triticeae EST-SSR coordination."

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