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ORIGINAL PAPER

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An SSR- and AFLP-based genetic linkage map of tall fescue (*Festuca arundinacea* Schreb.)

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Abstract Tall fescue (*Festuca arundinacea* Schreb.) is commonly grown as forage and turf grass in the temperate regions of the world. Here, we report the first genetic map of tall fescue constructed with PCR-based markers. A combination of amplified fragment length polymorphisms (AFLPs) and expressed sequence tag-simple sequence repeats (EST-SSRs) of both tall fescue and those conserved in grass species was used for map construction. Genomic SSRs developed from *Festuca* × *Lolium* hybrids were also mapped. Two parental maps were initially constructed using a two-way pseudo-testcross mapping strategy. The female (HD28-56) map included 558 loci placed in 22 linkage groups (LGs) and covered 2,013 cM of the genome. In the male (R43-64) map, 579 loci were grouped in 22 LGs with a total map length of 1,722 cM. The marker density in the two maps varied from 3.61 cM (female parent) to 2.97 (male parent) cM per marker. These differences in map length indicated a reduced level of recombination in the male parent. Markers that revealed polymorphism within both parents and showed 3:1 segregation ratios were used as bridging loci to integrate the two parental maps as a bi-parental consensus. The integrated map covers 1,841 cM on 17 LGs, with an average of 54 loci per LG, and has an average marker density of 2.0 cM per marker. Homoeologous relationships among linkage groups of six of the seven predicted homeologous groups were identified. Three small groups from the HD28-56 map and four from the R43-64 map are yet to be integrated. Homoeologues of four of those groups were detected. Except for a few gaps, markers are well distributed throughout the genome. Clustering of those markers showing significant segregation distortion (23% of total) was observed in four of the LGs of the integrated map.

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Introduction

Tall fescue (*Festuca arundinacea* Schreb.) is a cool-season forage grass that is widely grown throughout the temperate regions of the world (Sleper 1985). It is the most important perennial forage species of the genus *Festuca*. In the United States, tall fescue is a major forage crop in Nebraska, Kansas and Oklahoma, and its cultivation extends through to the East Coast at approximately the same latitudes. The use of tall fescue in forage, soil conservation and turf increased dramatically during the early 1980s (Sleper and West 1996). It is an allohexaploid ($2n = 6x = 42$) with the genomic constitution $PPG_1G_1G_2G_2$(Sleper and West 1996). Meadow fescue (*F. pratensis* Huds.) is believed to be the donor of the P genome and tetraploid fescue (*Festuca arundinacea* var. *glaucescens* Boiss) the donor of the G_1G_2 genome (Xu et al. 1991).

Molecular linkage maps were initially constructed for a wide variety of plant species mainly through the application of restriction fragment length polymorphisms (RFLPs) (O'Brien 1993). The advent of PCR-based marker systems, along with high-throughput genotyping instruments, has enhanced the application of molecular markers in crop improvement with the result that the latter have been widely used for cultivar identification and parentage analysis (Sefc et al. 2000), genome mapping (Xu et al. 1995; Jones et al. 2002a; Alm et al. 2003) and the tagging of genetically important

traits (Cardinal et al. 2003). Quantitative trait loci (QTLs) can be identified in genetic linkage maps and associated markers can be used in marker-assisted selection to improve economically important traits of tall fescue (Xu et al. 1995).

The amplified fragment length polymorphism (AFLP) is an efficient marker system for molecular mapping, and a large number of markers can be generated within a short time (Vos et al. 1995). AFLP markers have been used to develop genetic linkage maps in various crop species in the grass family, including sorghum (Boivin et al. 1999), perennial ryegrass (Bert et al. 1999) and meadow fescue (Alm et al. 2003). Simple sequence repeat (SSR) markers are popular because they are highly informative, locus-specific and adaptable to high-throughput and because the primers are simple to maintain (Yu et al. 2002). In a number of earlier investigations SSR markers were developed from either non-enriched or enriched genomic libraries (Edwards et al. 1996), which entails a labor-intensive and costly process (Squirrell et al. 2003). However, more recently, expressed sequence tags (ESTs) have been identified as an attractive and inexpensive source of SSR markers (Temnykh et al. 2000; Thiel et al. 2003; Eujayl et al. 2004).

EST databases have grown exponentially because of their potential use in plant and animal genetic improvement programs, genomic approaches to drug discovery and the study of human genetic diseases (Messing and Llaca 1998). In addition, ESTs provide a valuable potential source of genetic markers that are informative for the construction of comparative maps of expressed genes in related species (Cato et al. 2001; Kantety et al. 2002). The frequency of SSRs in the EST databases of cereal crops (rice, wheat, maize, barley and sorghum) varies from 1.5% (maize) to 4.7% (rice), with an average of 3.2% across all five species (Kantety et al. 2002), while the frequency of SSRs in the first 20,000 of the tall fescue EST database was fairly low (1.3%) as compared to other grass species (Saha et al. 2004).

The development of SSRs from ESTs has been reported in various crop species, including rice (Cho et al. 2000), durum wheat (Eujayl et al. 2002), barley (Thiel et al. 2003), rye (Hackauf and Wehling 2002) and *Medicago truncatula* (Eujayl et al. 2004). Eujayl et al. (2002) observed that markers developed from the EST-SSRs amplified high-quality PCR products in wheat, however the rate of polymorphism of EST-SSRs was comparatively lower than those of genomic-SSRs (Cho et al. 2000; Thiel et al. 2003). The SSR markers developed from tall fescue ESTs (Saha et al. 2004) and the ESTs of the five cereal grass species (Kantety et al. 2002) and from the *Festuca* × *Lolium* genomic sequences (Marc Ghesquiere, Lusignan, INRA, France; personal communication) are useful for genetic mapping in tall fescue. Such markers may also be useful for comparative genomics of tall fescue with major cereal, forage and turf grass species—for example, rice, wheat, maize, barley, ryegrass and meadow fescue. Kantety et al. (2002) identified SSRs in many genes or transcripts with known function. The map location of such SSRs may provide functionally associated genetic markers for direct characterization of the QTLs for putatively correlated traits, which would be particularly advantageous for the genome mapping of crop species containing large genomes with relatively little genomic information, such as tall fescue.

The genetic improvement of tall fescue is impaired by its genome complexity, heterozygosity within clones, a high level of self-incompatibility and lack of morphological genetic markers (Xu et al. 1991). Its strong self-incompatibility system makes linkage mapping more complicated than for species that can readily be inbred. The two-way pseudo-testcross procedure (Ritter et al. 1990; Hemmat et al. 1994) is considered to be an efficient way to construct molecular marker-based genetic maps in plant species like tall fescue. In this procedure an F_1 progeny set developed by hybridizing two unrelated highly heterozygous parents constitutes a mapping family. Construction of linkage maps is complicated because one or both parents may be heterozygous at a certain locus, markers may be dominant or co-dominant and the linkage phase of marker alleles is usually unknown (Maliepaard et al. 1997). Thus, genomic information for many forage species is quite limited compared to major crop species. To date, efforts have relied mainly on the hybridization-based RFLP technique. The only available RFLP map of tall fescue covers 1,274 cM with an average of five loci per linkage group (LG) and a marker density of 17.9 cM per marker (Xu et al. 1995). The application of a PCR-based marker system is highly desirable because such a system is fast, simple and can be used for transferring genetic information across species. Consequently, this study was undertaken to construct the first PCR-based genetic map of tall fescue. It will be of value for studies designed to identify markers associated with traits of interest and for comparative analysis with other grass species.

Materials and methods

Plant material

The mapping population of tall fescue (*Festuca arundinacea* Schreb.) used in this investigation was derived from a cross between two heterozygous highly polymorphic genotypes, ♀HD28-56 and ♂R43-64. The female parent, HD28-56, was originally provided by Dr. David Sleper, University of Missouri and was characterized as having high forage digestibility. The male parent, R43-64, originates from a population collected in northwestern Oklahoma in 1997 (Mian et al. 2002). An AFLP diversity analysis revealed substantial variation between the two genotypes (Mian et al. 2002). Ninety-one F_1 plants germinated from the seeds obtained from the HD28-56 parent constituted the pseudo-testcross mapping population. All genotypes were

maintained as clonally propagated tillers. All F_1 plants were tested by SSR/AFLP markers to confirm their hybrid status.

DNA extraction

From each genotype, approximately 200 mg of young leaf tissue from greenhouse-grown plants was collected in a 2-ml microtube and immediately frozen in liquid nitrogen. Each sample was ground to fine powder using a Mixer Mill Type MM 300 (Retsch, Hann, Germany). The DNA was extracted following a slightly modified protocol of Qiagen (Valencia, Calif.), which included the addition of 500 µl AP1 buffer, 5 µl RNase A, 165 µl AP2 buffer and 90 µl AE buffer to each tube of ground tissue. DNA concentrations were quantified using a Hoefer Dyna Quant 200 (Amersham Biosciences, Piscataway, N.J.) DNA fluorometer. DNA quality and integrity were assessed by electrophoresis on 1.0% (w/v) agarose gels.

Amplified fragment length polymorphism

The AFLP procedure was conducted according to the protocol supplied with the AFLP Plant Mapping kit of Perkin-Elmer Applied Biosystems (Foster City, Calif.) with minor modifications. The pre-amplification products were diluted 20-fold for template DNA for selective amplifications. Selective amplification was performed with the fluorescently labeled *Eco*RI or *Pst*I selective primers (Table 1). The samples were electrophoresed on an ABI 3100 capillary genetic analyzer (Perkin-Elmer Applied Biosystems) with an injection time of 8 s and a run time of 28 min. Raw data were analyzed with GENESCAN (ver. 2.1, Perkin-Elmer Applied Biosystems), and the resulting GENESCAN trace files were imported into GENOGRAPHER ver. 1.2 (Benham et al. 1999; http://hordeum.oscs.montana.edu/genographer). The AFLP fragments 50–500 bp in length were scored in GENOGRAPHER as present (A) or absent (B). Scores were recorded and formatted for analyses using the CONVERT GENOGRAPHER software (Noble Foundation, Ardmore, Okla; the

Table 1 AFLP and SSRs primer combinations used for mapping the tall fescue population

AFLPs	SSRs		
	Tall fescue EST-SSRs[a]	Conserved grass EST-SSRs[b]	*F*×*L* genomic SSRs[c]
*Pst*I + *Mse*I	NFFA002, NFFA005	CNL035	B1A11
PCC_CGC	NFFA011, NFFA013	CNL039	B1A2
PCC_GCT	NFFA014, NFFA015	CNL044	B1A9
PCC_GGA	NFFA016, NFFA017	CNL047	B1B6
PCC_TCG	NFFA018, NFFA020	CNL050	B1B12
PCC_TG	NFFA021, NFFA029	CNL051	B1C1
PGG_AG	NFFA030, NFFA031	CNL052	B1C9
PGG_GCG	NFFA032, NFFA033	CNL053	B2A1
PGG_TCG	NFFA034, NFFA037	CNL055	B2G9
PGG_TG	NFFA039, NFFA040	CNL056	B3A1
PGT_AGC	NFFA043, NFFA045	CNL058	B3A3
PGT_CGC	NFFA046, NFFA047	CNL061	B3B1
PGT_CTA	NFFA048, NFFA057	CNL063	B3B7
PGT_GCG	NFFA059, NFFA060	CNL064	B3B8
PGT_GCT	NFFA062, NFFA065	CNL068	B3C11
PGT_GGA	NFFA067, NFFA068	CNL081	B3C4
PGT_TCG	NFFA071, NFFA073	CNL083	B3C5
PGT_TG	NFFA074, NFFA075	CNL085	B3D1
PGT_TGC	NFFA082, NFFA087	CNL100	B3D3
	NFFA088, NFFA090	CNL101	B3D12
*Eco*RI + *Mse*I	NFFA096, NFFA098	CNL102	B3D3
EAG_TG	NFFA101, NFFA103	CNL103	B3D4
ECC_AG	NFFA104, NFFA108	CNL112	B3F2
EGG_GCG	NFFA109, NFFA110	CNL114	B3F3
EGG_TCG	NFFA112, NFFA113	CNL119	B4C4
	NFFA116, NFFA117	CNL124	B4C10
	NFFA120, NFFA129	CNL128	B4D7
	NFFA130, NFFA131	CNL134	B4D9
	NFFA134, NFFA135	CNL139	B5E1
	NFFA138, NFFA140	CNL140	B5F9
	NFFA142, NFFA146	CNL141	B5G4
	NFFA152, NFFA154	CNL142	
	NFFA155, NFFA157	CNL143	
		CNL145	
		CNL147	
		CNL151	
		CNL158	

[a]The tall fescue EST-SSRs were developed at the Noble Foundation, and sequences of these primer pairs were published in Saha et al. (2004)

[b]The conserved grass EST-SSRs were developed at the Cornell University, and these primer sequences are available from Dr. Mark Sorrells, Cornell University, USA

[c]*Festuca* (F) × *Lolium* (L) hybrid genomic SSRs are available from Dr. Marc Ghesquiere, Lusignan, INRA, France

software can be obtained by e-mail correspondence with the authors). Scores thus obtained were verified on the gel images. A total of 56 *Eco*RI/ *Pst*I + *Mse*I primer combinations were first screened with two parents and six progenies. On the basis of high polymorphism and clean products, 22 primer combinations were selected for assaying the whole population (Table 1).

Simple sequence repeats

A set of 157 tall fescue (TF)-EST-SSRs (NFFA), 101 conserved grass EST-SSRs (CNL) and 60 *Festuca* × *Lolium* F_1 genomic SSRs (*F*× *L*) were used for genetic mapping in this tall fescue pseudo-testcross population. TF-EST-SSRs were developed at the Noble Foundation. Information on the CNL was kindly provided by Dr. Mark E. Sorrells, Cornell University, Ithaca, N.Y. The primer sequences for *F*× *L* genomic SSRs were kindly provided by Dr. Marc Ghesquiere, Lusignan, INRA, France (the sequence information of these primers can be obtained by contacting Dr. Ghesquiere). All of the primer combinations were first screened with the parents to find the polymorphic primer pairs (PPs). The selected PPs were then screened on a subset of six F_1 progeny lines of the population to select markers for mapping in the whole population. Some of the polymorphic PPs with non-specific amplifications and/or too faint products were discarded from the final population assay.

The PCR reactions were run under standard conditions for all primers using 1 U AmpliTaq Gold with GeneAmp PCR buffer II (Applied Biosystems/Roche, Branchburg, N.J.), 3 m *M* $MgCl_2$, 200 μM dNTPs, 0.2 m *M* of each primer and 20 ng of template DNA in a 10-µl reaction. PCR reaction conditions consisted of 10 min at 95°C, followed by 40 cycles of 50 s at 95°C, 50 s at a temperature between 58°C and 64°C (optimum annealing temperature for each PP varied), 90 s at 72°C, and a final extension step of 10 min at 72°C. The PCR products were resolved by analyzing samples on 6% polyacrylamide denaturing gels run under standard conditions. The amplified products were visualized by silver staining. Polymorphism was determined by the presence or absence of a SSR locus according to the single-dose restriction fragment approach described by Wu et al. (1992).

Linkage analysis and map integration

Markers with alleles segregating in one or both parents were analyzed in the entire population of 91 F_1 progenies. Markers segregating from one parent or both parents were analyzed separately. Both of the parents were used to define their respective coupling LGs using JOINMAP 3.0 (Van Ooijen and Voorrips 2001). Construction of the linkage map was accomplished by treating the segregating data as a cross pollinator (CP). After constructing the LOD grouping trees, we selected LGs with a LOD grouping threshold of 5.0. The calculation of the linkage maps utilized all pair-wise recombination estimates of less than 0.499 and a LOD score greater than 0.01 (ripple value = 1, jump threshold = 5 and a triple threshold = 7). Map distances were calculated using the Kosambi mapping function (Kosambi 1944).

Two parental maps were first constructed with markers segregating in a 1:1 ratio. Those markers present in both parents that segregated in a 3:1 ratio were later included and used to find the homologous groups in the two parental maps. The homologous group nodes of two parental maps were selected, and the integrated LGs were constructed using the map integration command under the MAP tab. The SSR PPs that detected multiple alleles, diagnostic of duplicated loci, were used to define the homoeologous groups for each set of chromosomes. LGs with putative homoeoalleles detected by a single PP were considered to be homoeologous in nature. Following construction of the LGs, the graphics were done using MAPCHART 2.1 (Voorrips 2002).

Results

Molecular markers

Fifty-six selective AFLP primer pairs were tested on a subset of the tall fescue population; of these, 22 (39%) were scored on the whole population. The selected primer combinations were highly polymorphic in the tall fescue population. On average, 35 polymorphic loci were scored from each primer combination. Both the highest (53) and the lowest (24) numbers of polymorphic markers were scored using the *Eco*RI + *Mse*I combinations EACC + MCAG and EAGG + MCTCG, respectively. The average number of polymorphic loci obtained from the *Eco*RI + *Mse*I and *Pst*I + *Mse*I combinations was similar (data not shown). In total, 773 polymorphic bands were generated. The four *Eco*RI + *Mse*I combinations generated 136 segregating loci and the 18 *Pst*I + *Mse*I combinations had 637 polymorphic loci. Of these 773 AFLP loci, 343 were polymorphic in R43-64 only, 279 were polymorphic in HD28-56 only, and the remaining 151 segregated from both parental genotypes. A total of 430 and 494 AFLP loci were used to construct maps of HD28-56 and R43-64, respectively (Table 2).

In total, 66 TF-EST-SSR (42% of the total PPs screened; designated NFFA_), 37 conserved cereal EST-SSR (37% of the total PPs screened; designated CNL_) and 31 *Festuca* × *Lolium* hybrid genomic SSR (52% of the total PPs; designated B_N_) PPs were ultimately used to construct the linkage map (Table 1). The TF-EST-SSR PPs generated 197 polymorphic loci, with an average of three loci per PP; the CNL EST-SSR PPs generated 87 polymorphic loci, with an average of 2.4 loci per PP; the *F*× *L* PPs generated 59 polymorphic loci, with an average of 1.9 loci per PP. A total of 343 SSR

Table 2 Summary of the female (HD28-56) and male (R43-64) parental maps

Parameters	HD28-56	R43-64
AFLP		
Scored	430	494
Mapped	370 (86)[a]	426 (86)
Unlinked	60 (14)	68 (14)
SSRs		
Scored	212	182
Mapped	188 (89)	153 (84)
Unlinked	24 (11)	29 (16)
Total		
Scored	642	676
Mapped	558 (87)	579 (86)
Unlinked	84 (13)	97 (14)
Skewed markers		
AFLP	86 (20)	120 (24)
SSR	46 (22)	49 (27)
Total	132 (21)	169 (25)
Linkage analysis		
Linkage groups	22	22
Map length (cM)	2013	1722
Marker density (cM/marker)	3.61	2.97

[a] The number in parenthesis indicates the percentage of the total for that specific category

loci were scored from 134 selected PPs, with an average of 2.6 loci per PP. Of these, 131 were polymorphic in R43-64 only, 161 were polymorphic in HD28-56 only and the remaining 51 were segregated from both parental genotypes. We used 212 SSR loci to construct the HD28-56 parental map and 182 loci to construct the R43-64 map (Table 2).

Marker segregation

Tall fescue is an allohexaploid with disomic inheritance. On the basis of Mendelian inheritance, segregation ratios of 1:1 (testcross) or 3:1 (dominant) were expected. Marker loci segregating from one parent or both parents were scored. From a total of 1,116 loci scored for mapping, 82% segregated from one parent (Aa×aa or aa×Aa) and the remaining18% segregated from both parents (Aa×Aa). Segregation of each marker locus was verified using a chi-square test. Of the total number of markers scored, 77% followed a normal Mendelian segregation, with approximately 88% segregating 1:1, while the remaining 12% showed a 3:1 segregation ratio (data not shown). Among the 23% of the markers showing a skewed segregation, 14% showed segregation from one parent while 9% segregated from both parents. Twenty-five percent of the markers segregating from the R43-64 parent were skewed compared to 21% of the markers segregating from HD28-56 (Table 2). The skewed markers were included in linkage map construction. Clusters of skewed markers were evident on four of the HD28-56 LGs and five of the R43-64 LGs. Apart from this observation, relatively few skewed markers were present on the remaining LGs of each parental maps.

Parental map construction

The AFLP and SSR markers were used initially to construct the parental maps on the basis of segregation data obtained from 91 F_1 progeny. The HD28-56 parental map was constructed with 370 AFLP and 188 SSR loci, leaving 60 AFLP and 24 SSR loci unlinked (Table 2). A total of 558 marker loci were distributed in 22 LGs, with the AFLP loci constituting the major part of the LGs. However, SSR loci are predominant in LGs 8, 17 and 21 (Table 3 and ESM). The TF-EST-SSRs were present in all LGs and constituted most of the SSR loci. The CNL and *F*×*L* SSRs were present in 15 and 13 of the LGs, respectively. Eleven of the LGs were covered by markers from all four classes (Table 3 and ESM). Markers in the 22 LGs covered a length of 2,013 cM, with an average of 25 loci per LG (Table 2). LG 3 was the longest group with 43 loci spanning 147.3 cM, and LG 21 was the shortest with seven loci spanning 27.5 cM (Table 3 and ESM). The average size of the LGs was 91.5 cM. The average marker density in this parental map was 3.61 cM per marker.

The R43-64 map was constructed with 426 AFLP and 153 SSR loci, leaving 68 AFLP and 29 SSR loci unlinked (Table 2). All of the 579 loci were arranged in 22 LGs. AFLP loci constituted the major part of all LGs except LGs 3, 7 and 17. The TF-EST-SSRs were missing in LG 4 (Table 3). The CNL and *F*×*L* SSRs covered 15 and 12 of the LGs, respectively. Eight of the LGs had loci from all four marker classes (Table 3). Map length covered by all LGs was 1,722 cM. LG 11 was the longest group, spanning 187 cM with 61 loci, and LG 22 was the smallest group, with five loci spanning 29.6 cM. The average distance between markers was 2.97 cM (Table 2).

Map integration

The AFLP and SSR loci that were heterozygous in both parents provided the bridging loci between homologous LGs of the two parental maps and allowed us to construct 17 integrated LGs. Four LGs of HD28-56 correspond to two groups of R43-64 (HD13 and HD15 with R11, and HD19 and HD22 with R18). Two LGs of the R43-64 map (R8 and R16) correspond to a HD28-46 LG 16 (Table 4 and ESM). All of the other integrated LGs have one LG from each parent. In total 17 integrated LGs were constructed using 37 parental LGs. Homologous groups for three HD28-56 LGs (4, 7 and 20) and four R43-64 LGs (3, 4, 19 and 22) could not be established due to the lack of common markers.

The 17 integrated LGs contained 922 markers, of which 653 were AFLP and 269 were SSR loci (Table 4). AFLP and NFFA loci were present in all combined groups. The CNL and *F*×*L* SSRs were represented on 16 and 12 of the LGs, respectively. Eleven of the LGs had loci from all four marker classes (Table 4). Map length covered by the LGs was 1840.9 cM. LG 1-A was the

Table 3 Distribution of AFLP, tall fescue (NFFA) and conserved grass (CNL) EST-SSR and *Festuca* × *Lolium* hybrid (*F*×*L*) genomic SSR marker loci in different linkage groups of the two parental maps

Linkage group	Parental map									
	HD28-56					R43-64				
	AFLP	NFFA	CNL	*F*×*L*	Total	AFLP	NFFA	CNL	*F*×*L*	Total
1	25	6	1	3	35	42	6	3	2	53
2	29	11	6	3	49	23	3	0	0	26
3	28	8	3	4	43	12	5	4	4	25
4	18	6	4	1	29	23	0	1	0	24
5	20	3	3	1	27	12	2	2	0	16
6	10	6	0	0	16	23	7	2	3	35
7	8	2	1	0	11	12	12	0	3	27
8	20	25	0	5	50	32	1	0	2	35
9	34	6	3	1	44	15	5	3	0	23
10	24	11	1	1	37	11	4	0	3	18
11	21	5	2	2	30	49	6	2	4	61
12	16	2	1	0	19	20	4	2	1	27
13	19	2	0	3	24	14	4	1	0	19
14	15	3	3	2	23	35	2	3	4	44
15	7	2	0	0	9	23	7	2	3	35
16	23	2	1	5	31	12	1	0	1	14
17	10	7	3	0	20	8	6	5	0	19
18	16	4	2	1	23	28	2	2	0	32
19	13	3	0	0	16	13	5	1	0	19
20	7	2	0	0	9	8	1	3	1	13
21	3	2	2	0	7	7	2	0	0	9
22	4	2	0	0	6	4	1	0	0	5
Total	370	120	36	32	558	426	86	36	31	579

longest group, with 88 loci covering a distance of 181.7 cM. LG 1-C had the same number of loci and spanned 113.2 cM, with a marker every 1.3 cM. LG 4-B was the smallest group with 15 loci spanning 45.9 cM. Integration of the two parental maps significantly improved marker density in the combined map. On average, one marker was present in every 2.0 cM. Each marker locus was present in less than 2 cM in LGs 1-C, 2-A, 2-B, 6-A and 7-A (Table 4). The marker density varied from 1.1 cM to 3.7 cM per locus in the different linkage groups.

Forty-two SSR primers with multiple alleles were used to find the homoeologous groups within the integrated LGs. Three homoeologous groups of each of six

Table 4 Distribution of different types of marker loci, map length and marker density in LGs of the integrated map. Integrated LGs along with their parental origin are presented

Integrated linkage groups	Parental groups		Origin of marker loci					Map length (cM)	Marker density (cM/marker)
	HD map	R map	AFLP	NFFA[a]	CNL[b]	*F*×*L*[c]	Total		
1-A	13,15	11	71	10	2	5	88	181.7	2.1
1-B	12	9	30	6	3	0	39	82.9	2.1
1-C	10	1	65	16	4	3	88	113.2	1.3
2-A	3	14	59	9	6	8	82	93.6	1.1
2-B	16	8,16	56	4	1	7	68	107.2	1.6
2-C	8	7	32	34	0	8	74	145.1	2.0
3-A	5	12	35	7	4	1	47	149.3	3.2
3-B	19,22	18	41	6	2	0	49	110.9	2.3
3-C	6	13	22	9	1	0	32	117.3	3.7
4-A	18	10	23	7	2	3	35	70.9	2.0
4-B	21	21	9	4	2	0	15	45.9	3.1
4-C	9	2	51	8	3	1	63	126.8	2.0
5-A	17	17	14	12	7	0	33	77.4	2.3
5-B	1	5	32	6	3	3	44	91.0	2.1
6-A	14	6	36	9	4	4	53	91.1	1.7
6-B	2	20	38	10	6	4	56	133.0	2.4
7-A	11	15	39	8	4	3	54	103.6	1.9
Total/Aver.	19	18	653	165	54	50	922	1840.9	2.0

[a]Tall fescue (TF)-EST-SSRs; Noble Foundation, USA
[b]Conserved grass EST-SSRs; Cornell University, USA
[c]*F*×*L* = *Festuca* × *Lolium* hybrid genomic SSRs; INRA, France

sets of chromosomes were identified. All three were identified for groups 1, 2, 3 and 4, while two were identified for groups 5 and 6 (Fig. 1). Homoeologues for group 7 could not be detected due to lack of homoeologous loci. Four of the parental LGs had their respective homoeologues in the integrated LGs 5 and 6. One LG from HD28-56 and two from the R43-64 parental maps had no homoeologue and were marked as "X", "Y" and "Z." Some of the SSR PPs detected duplicate non-homologous loci, both within individual chromosomes and also between non-homologous linkage groups.

In general, marker loci were distributed evenly throughout the genome except for a large gap of 20.3 cM in LG 3-C and a few gaps of 15–20 cM in LGs 3-B and 4-C (Fig. 1). The AFLP loci were more evenly dispersed throughout the LGs than the SSR loci. Clustering of SSR loci was evident in several LGs (LGs 1-C, 2-C and 4-A), while a large portion of LGs 1-A, 3-B, 4-A and 6-B, did not have any SSR loci at all (Fig. 1). While the marker order in the parental maps and the integrated maps usually remained the same, rearrangements were evident in some of the linkage groups. Skewed markers were distributed throughout the genome, although clustering of those markers was evident in LGs 1-A, 1-B, 2-C and 6-B (Fig. 1).

Discussion

We have constructed the first PCR marker-based genetic linkage map of tall fescue that contains substantial numbers of AFLP and SSR loci. The advantage of using AFLP markers is that a large number of markers can be readily generated to give substantial genome coverage. These markers facilitate linkage between framework markers, for example SSRs. The AFLP markers significantly improved the construction of the present map with respect to both genome coverage and in providing links to associate distantly located SSR loci. However, due to their reduced power in genetic mapping and QTL detection, AFLPs are not commonly used in framework mapping (Jones et al. 2002b). Also, AFLPs are not suitable for comparative mapping.

Microsatellite or SSR markers have become the marker class of choice for the molecular mapping of many crop species. These markers have high rates of transferability across species within a genus (Gaitán-Solís et al. 2002) but can also be extended across genera and beyond (Peakall et al. 1998; Roa et al. 2000). SSR markers are also considered to be a useful tool for aligning trait-specific maps with reference maps (Jones et al. 2002a). Many of the tall fescue EST-SSR markers (NFFA) developed at the Noble Foundation that were used to construct this map have been shown to amplify loci in different forage and cereal grass species (Saha et al. 2004), while conserved grass EST-SSR markers (CNL) developed at Cornell University have been mapped in hexaploid wheat (Yu et al. 2004). Some of these NFFA and CNL markers have also been mapped in a ryegrass population (Warnke et al. 2004). *F*×*L* genomic SSRs used to construct this map were found to be usable in cross-species transportability within *Festuca-Lolium* complex species and other related grasses (Marc Ghesquiere, personal communication).

The vast majority of the markers showed a 1:1 Mendelian segregation ratio and were used to construct the framework map. This indicates that most of the loci were in a heterozygous state in one parent and in a homozygous state in the other parent. Only 12% of the markers showed a 3:1 segregation pattern, indicating a heterozygous state in both parents. Maliepaard et al. (1998) found that markers present in both parents and showing a 3:1 segregation ratio were useful for identifying homologous groups between maps. Of the 202 AFLP and SSR loci segregating from both parents, 99 were found associated with 37 LGs of both parental maps. These markers allowed us to construct 17 integrated LGs. Some of these markers mapped in only one parent, perhaps due to a lack of linked loci. The reason for the lack of map integration for many of these loci may be due to the low confidence of linkage estimation between marker pairs segregating 1:1 and 3:1 (Scalfi et al. 2004). Map construction at an LOD threshold value of 5.0 also caused several markers to remain unlinked.

High levels of segregation distortion were observed in the cross between two distantly related heterozygous tall fescue genotypes in this study. Various processes can cause segregation distortion, including gametic selection and/or faulty chromosome pairing, an association between heterozygosity and plant vigor and the selection of one parental type (Xu et al. 1995). High levels of segregation distortion have frequently been reported in tall fescue (Xu et al 1995), ryegrass (Warnke et al. 2004) and other species (Brummer et al. 1993; Wang et al. 1994). In tall fescue, genetic self-incompatibility has been well established (Xu et al. 1995), and genes linked to self-incompatibility could cause segregation distortion. However, as this population was developed by crosses between two heterozygous unrelated parents, the involvement of self-incompatible genes in segregation distortion is doubtful. The distribution of skewed markers in both parental maps indicated that both the male and female gametophytes and/or sporophytes are involved in the segregation distortion.

The length of the genetic map for the female parent was estimated to be 2,013 cM and that of the male parent to be 1,722 cM. Similar variations in parental maps were reported by Warnke et al. (2004). Differences in map length can result from a variation in the number of recombination events in the two parents as well as variations in the numbers and locations of mapped loci. In this investigation we observed a distinctly reduced level of recombination in the male parent compared to the female parent (1.6 and 1.8 chiasmata per LG, respectively). Differences in recombination frequency between parents have also been reported for other species (Sewell et al. 1998). However, as Alm et al. (2003)

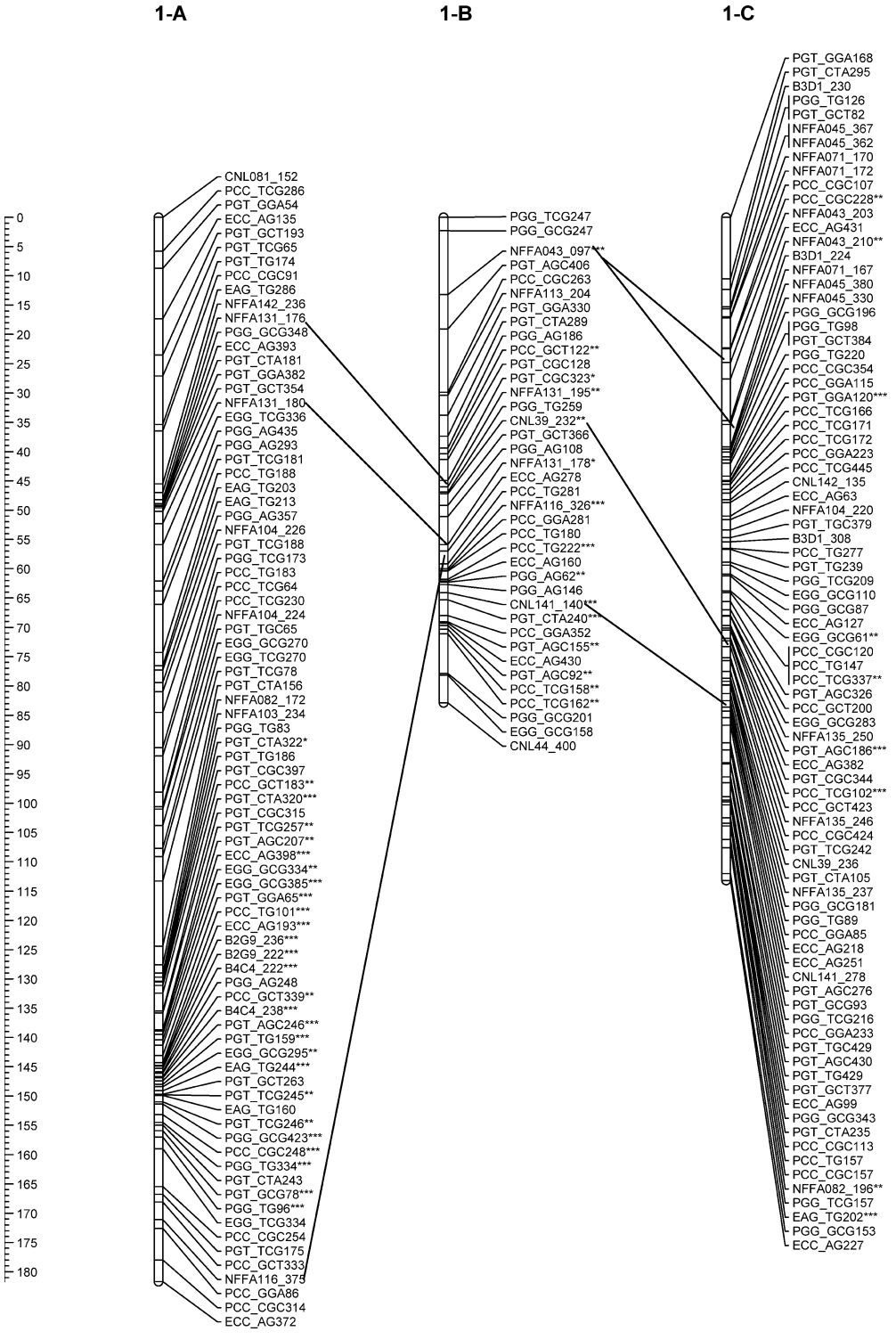
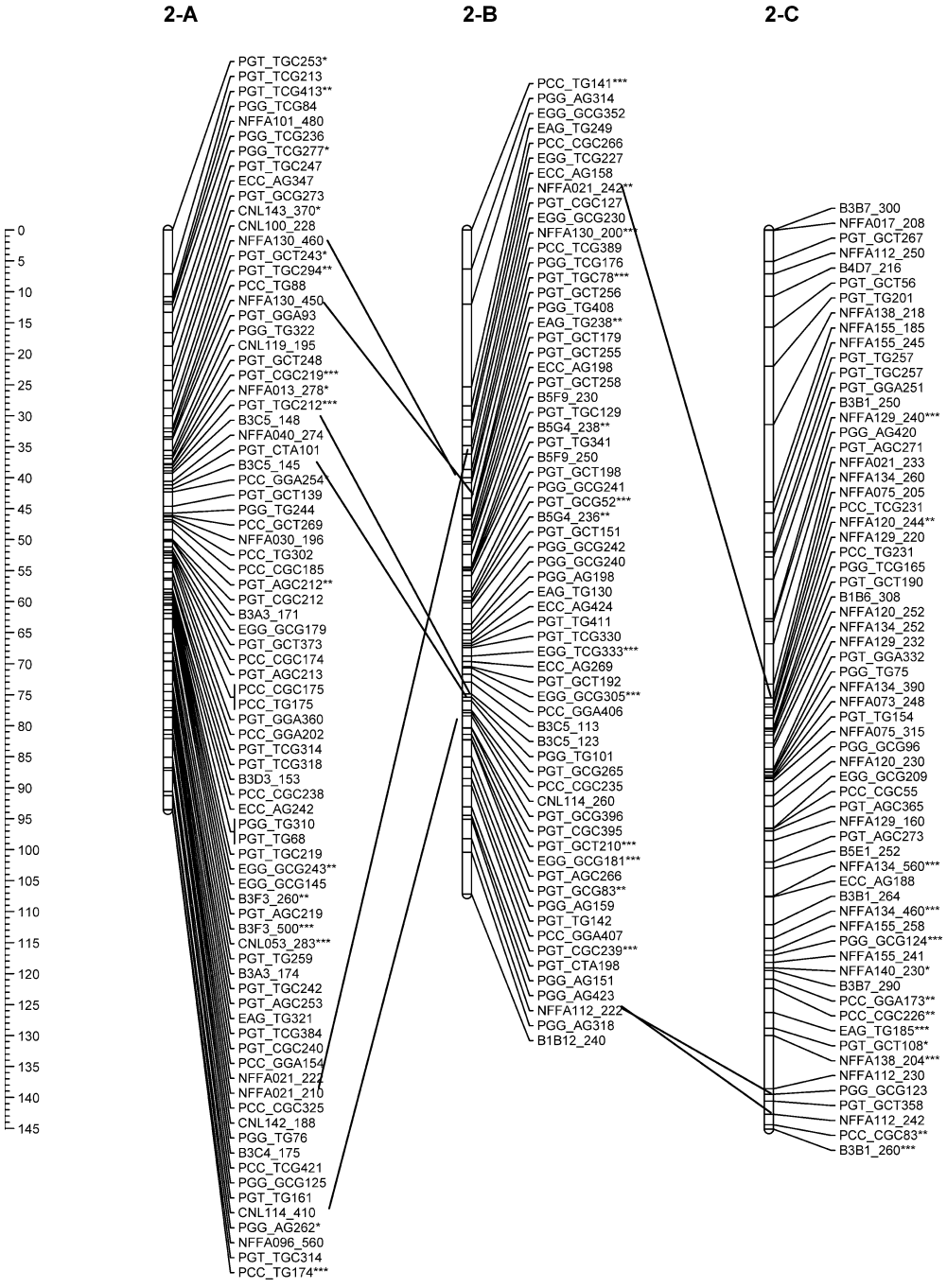


Fig. 1 Tall fescue integrated genetic linkage maps. The seven homoeologous linkage groups are arbitrarily numbered from *1* to *7*, and homoeologues within each group are designated *A*, *B* and *C*. Three of the parental linkage groups without homoeologues are denoted *X*, *Y* and *Z*. The skewed markers are marked with *asterisks*; (*, ** and *** indicate significance levels of 0.10, 0.05 and 0.01, respectively). The centiMorgan distance scale is found at the *extreme left* of the page. The *prefixes* of AFLP markers begin with a *P* or an *E*, the tall fescue EST-SSR markers begin with an *N*, the conserved grass EST-SSR markers begin with a *C* and the *Festuca* × *Lolium* genomic SSR markers begin with a *B*. The *three-digit number at the end* of an AFLP marker name indicates the size of the AFLP band in basepairs. The *three-digit extension after the underscore* (_) in the name of an SSR marker indicates the size of the SSR band in basepairs

reported that in meadow fescue the variation in recombination frequencies in female and male parents was not pronounced, the parental variation in recombination frequencies that we observed might be a species-specific characteristic. King et al. (2002) reported a 1:1 correspondence between chiasma frequency and recombination in a *Festuca-Lolium* hybrid.

The average length of the female and male parental linkage groups was 91.5 cM and 78.3 cM, respectively, and that of the combined groups was 102.2 cM. The average length of the meadow fescue linkage groups was 86.0, 83.0 and 94.1 cM for the maternal, paternal and combined groups, respectively (Alm et al. 2003). The *Lolium* map had an average length of 116 cM per link-

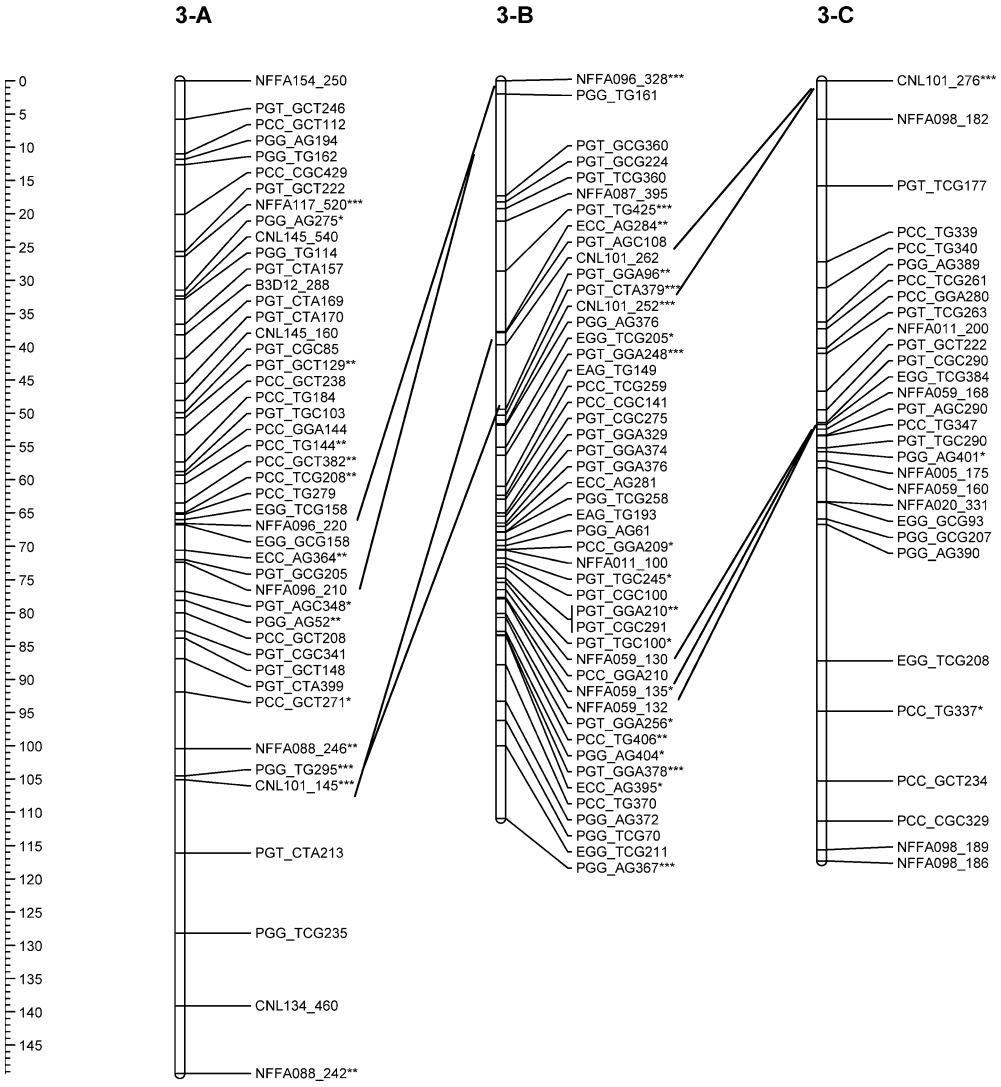
Fig. 1 (Contd.)



age group (Jones et al. 2002a). An average length of 102.2 cM corresponds to approximately 2.0 chiasmata per chromosome in the tall fescue genotypes of this study. An earlier report by Simonsen (1975) indicated that the mean number of chiasmata per chromosome ranged from 1.5 to 1.96 in 50 genotypes of a Norwegian cultivar of meadow fescue. The meadow fescue map— with an average length of 94.1 cM per chromosome—had approximately 1.9 chiasmata per chromosome (Alm et al. 2003). These results suggest that tall fescue, ryegrass and meadow fescue have similar frequencies of chiasma formation during metaphase I.

The marker density in the parental maps varied from 3.61 cM (HD28-56) to 2.97 (R43-64) cM per marker, and the average density in the integrated map was 1.9 cM per marker. Markers were highly clustered in some regions, but there were gaps of 10–21 cM in some LGs, indicating that either recombination events or mapped loci were not evenly distributed throughout the genome. Recombination can be drastically reduced in chromosomes with inversions and translocations, and a high frequency of chromosomal aberrations, such as translocations (22%) and paracentric inversions (42%), have been reported for meadow fescue (Simonsen 1975).

Fig. 1 (Contd.)

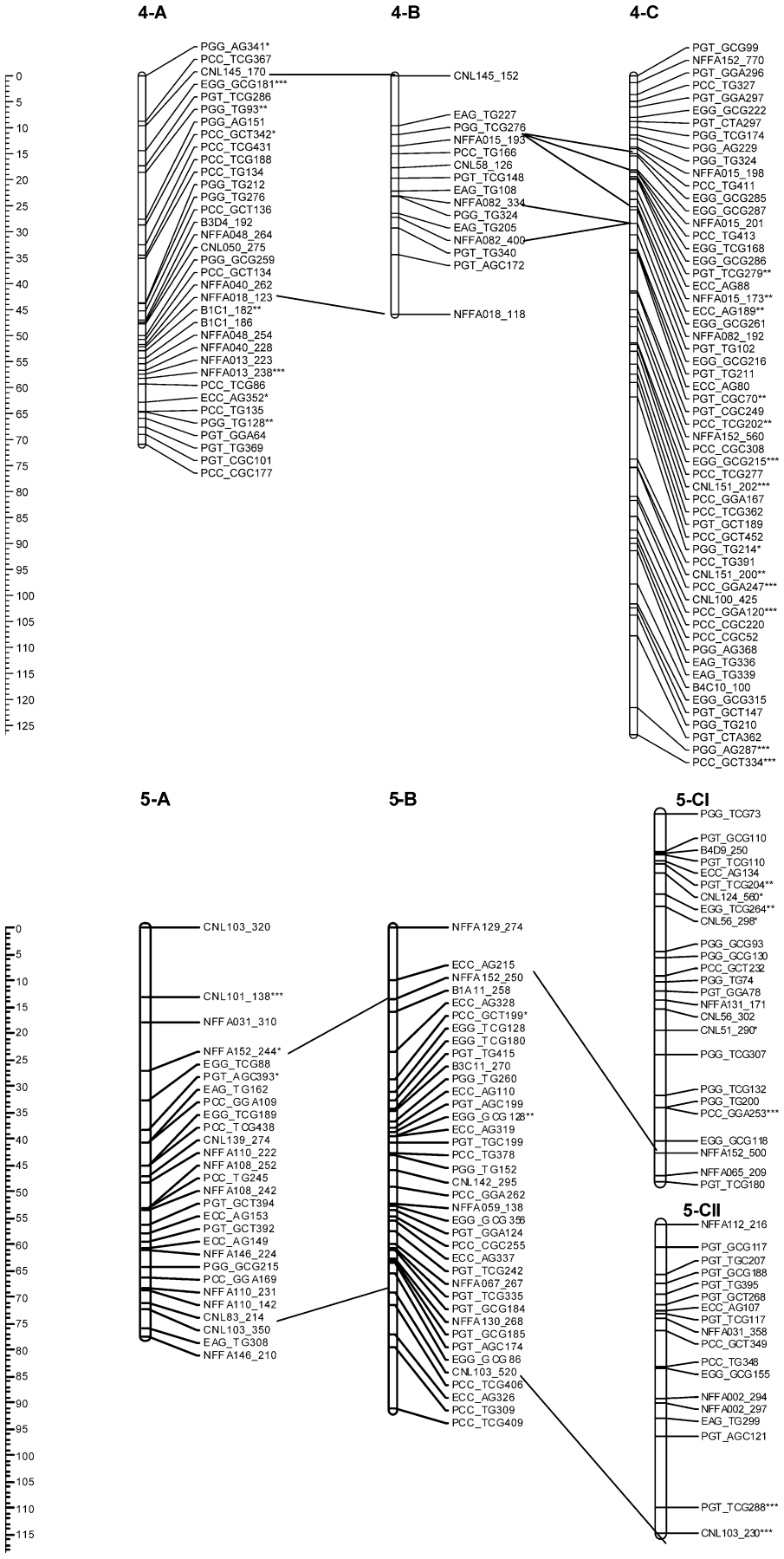


It is therefore possible that chromosomal aberrations were also present in the tall fescue population used in this study. Characterization of the meiotic behavior of the parental genotypes during homologous chromosome pairing could provide useful information about the type and number of chromosomal rearrangements in this population.

Tall fescue is an allohexaploid ($2n = 6x = 42$) with three sets of chromosomes (Sleper and West 1996). Three homoeologues of each of seven chromosomes are expected. SSR PPs with multiple loci mapped in different LGs were used to identify the homoeologous groups. Loci from 42 PPs identified all three homoeologous groups of six chromosomes; the seventh group was not detected due to a lack of homoeologous loci. Barrett et al. (2004) used SSR loci to find the homoeologous groups in white clover and were able to distinguish all eight homoeologous groups . In our study, non-homoeologous amplification of SSR loci was also observed, but as non-homoeologous loci were present within homologues and between linkage groups, identification of the homoeologous chromosome in this study is tentative and requires additional verification. Barrett et al. (2004) reported the presence of non-homoeologous loci in white clover. The presence of non-homoeologous loci within and between LGs might be due to duplicated genomic regions and translocations. In wheat, Anderson et al. (1992) reported 25–30% gene duplication. Saha et al. (2004) observed that a significant number of TF-EST-SSRs amplified more bands than expected in six different grass species. Thirty-nine percent of the EST-SSRs detected multiple loci in wheat (Yu et al. 2004).

The PCR-based tall fescue map developed in this study covered 1,737 cM of the genome, with 54 markers per linkage group and a map density of 1.9 cM per marker, which is a substantial improvement over the existing RFLP-based map that covers 1,274 cM, with five loci per linkage group and 17.9 cM between loci (Xu et al. 1995). Tall fescue can be clonally propagated, and all parental and F_1lines are maintained clonally. This will facilitate further improvement in the mapping effort

Fig. 1 (Contd.)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PCC_TCG188
PCC_TG134
PGG_TG212
PGG_TG276
PCC_GCT136
B3D4_192
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CNL050_275
PGG_GCG259
PCC_GCT134
NFFA040_262
NFFA018_123
B1C1_182**
B1C1_186
NFFA048_254
NFFA040_228
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NFFA013_238***
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PGG_TG128**
PGT_GGA64
PGT_TG369
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PCC_CGC177
CNL145_152
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PGG_TCG276
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PCC_TG166
CNL58_126
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EAG_TG108
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NFFA018_118
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5-B
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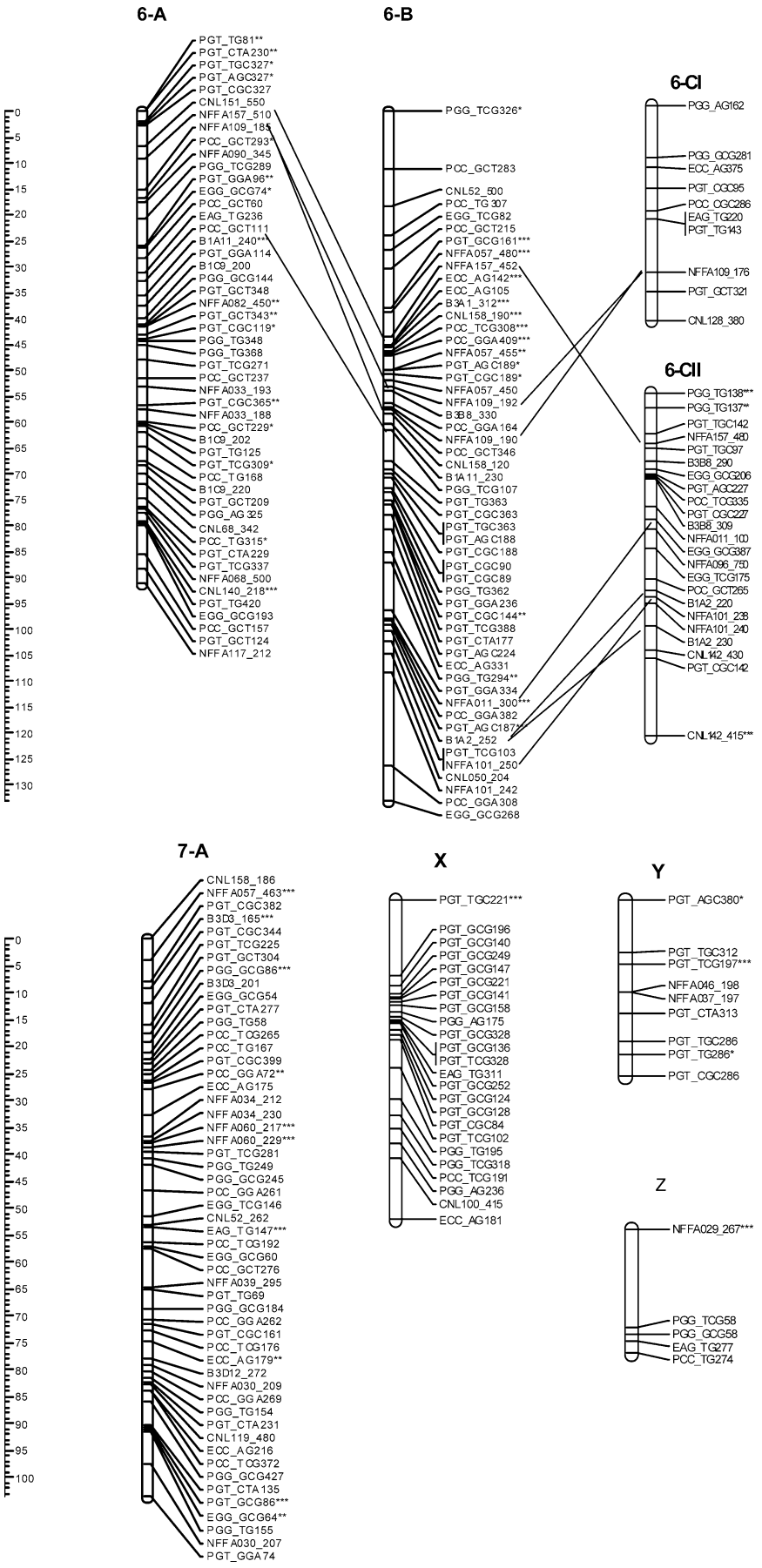
Fig. 1 (Contd.)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7-A
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Y
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10
15
20
25
30
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40
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50
55
60
65
70
75
80
85
90
95
100
105
110
115
120
125
130

and facilitate QTLs mapping studies over multiple environments. The addition of a substantial number of SSR loci, especially the EST-SSRs, opens the door for comparative mapping with other forage, turf and cereal grass species.

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