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ORIGINAL PAPER

Development of intron-flanking EST markers for the *Lolium/Festuca* complex using rice genomic information

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Abstract DNA markers able to distinguish species or genera with high specificity are valuable in the identification of introgressed regions in interspecific or intergeneric hybrids. Intergeneric hybridization between the genera of *Lolium* and *Festuca*, leading to the reciprocal introgression of chromosomal segments, can produce novel forage grasses with unique combinations of characteristics. To characterize *Lolium/Festuca* introgressions, novel PCR-based expression sequence tag (EST) markers were developed. These markers were designed around intronic regions which show higher polymorphism than exonic regions. Intronic regions of the grass genes were predicted from the sequenced rice genome. Two hundred and nine primer sets were designed from *Lolium/Festuca* ESTs that showed high similarity to unique rice genes dispersed uniformly throughout the rice genome. We selected 61 of these primer sets as insertion-deletion (indel)-type markers and 82 primer sets as cleaved amplified polymorphic sequence (CAPS) markers to distinguish between *Lolium perenne* and *Festuca pratensis*. Specificity of these markers to each species was evaluated by the genotyping of four cultivars and accessions (32 individuals) of *L. perenne* and *F. pratensis*, respectively. Evaluation using specificity indices proposed in this study suggested that many indel-type markers had high species specificity to *L. perenne* and *F. pratensis*, including 15 markers completely specific to both species. Forty-nine of the CAPS markers completely distinguish between the two species at bulk level. Chromosome mapping of these markers using a *Lolium/Festuca* substitution line revealed syntenic relationships between *Lolium/Festuca* and rice largely consistent with previous reports. This intron-based marker system that shows a high level of polymorphisms between species in combination with high species specificity will consequently be a valuable tool in *Festulolium* breeding.

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Introduction

The genera *Lolium* and *Festuca* are widely used as a major source of forage in temperate grasslands. Forage grass breeders have combined favorable traits of *Lolium* and *Festuca* in a single genotype, i.e. *Festulolium*, by

producing amphidiploids of the two genera or by introgression of the appropriate traits in one species to the other (reviewed by Yamada et al. 2005). Recently, a few *Festuca* chromosome segments able to increase tolerance to abiotic stresses such as freezing and drought were identified in a *Lolium* background by genomic in situ hybridization (GISH) (reviewed by Kopecký et al. 2008). However, GISH is labor intensive and time consuming and thus could not be used as a tool in conventional breeding programmes comprising large numbers of plants. Furthermore, difficulties can arise in the identification of small introgressed chromosomal segments (Humphreys et al. 1998).

DNA markers able to distinguish the genomes of two species (or genera) provide an important tool for the identification and characterization of introgressed chromosomal segments in interspecific or intergeneric hybrids (introgression mapping). Several DNA marker systems that enable distinction between *Lolium* and *Festuca* genomes have been developed (Stammers et al. 1995; Pašakinskiene et al. 2000; Momotaz et al. 2004). Simple sequence repeat (SSR) markers, in particular, are efficient tools for introgression mapping due to their abundance, ubiquitous distribution in plant genomes, ease of polymerase chain reaction (PCR)-based analysis and detection of co-dominant multi-allelic loci (Momotaz et al. 2004; Lauvergeat et al. 2005). Currently, however, only a limited number of SSR markers are publicly available for *Lolium/Festuca*, and the development of SSR makers is both costly and labor intensive.

More recently, marker systems developed using rice genomic information (International Rice Genome Sequencing Project 2005) have been proposed for related Poaceae crops (Lem and Lallemand 2003; Bertin et al. 2005; Fredslund et al. 2006; Ishikawa et al. 2007). These systems involved the development of PCR-based markers from cDNA sequences, including expression sequence tags (ESTs), of the related species that showed high similarity to single-copy rice genes (with no paralogous genes). Thus, exploitation of the sequenced rice genome in combination with marker technology and comparative genomics enables the identification of orthologous loci in related species (Gale and Devos 1998). In this marker system, primer design is based on the sequences of exonic regions flanking introns of rice genes. Because intronic regions generally include more polymorphisms than exonic regions (Lyons et al. 1997; Choi et al. 2004; Wei et al. 2005), a higher frequency of polymorphic markers is expected using this strategy.

In this report, we have applied intron-flanking EST markers, developed using rice genomic information, to characterize introgressions in the *Lolium/Festuca* complex, i.e. hybrids and derivatives. We developed 143 PCR-based EST markers that distinguished between the *L. perenne* and *F. pratensis* genomes. The markers were evaluated for their specificity to the *Lolium* and *Festuca* genomes, and were assigned to linkage groups using *Lolium/Festuca* monosomic substitution genotypes. Finally, we discuss the potential of the intron-flanking EST markers for the analysis of *Lolium/Festuca* complexes.

Materials and methods

Plant materials

Twelve cultivars and accessions of *Lolium* and *Festuca* were used in this study for the development of markers and the evaluation of their species specificities: *L. perenne* cv 'Reveille' ($2n = 4x$) bred in the Netherlands, cv 'Riikka' ($2n = 2x$) bred in Finland, cv 'Pokoro' ($2n = 4x$) bred in Japan and accession 'Yatsugatake D-13' ($2n = 2x$) bred in Japan; *F. pratensis* cv 'Harusakae' ($2n = 2x$) bred in Japan, cv 'Pradel' ($2n = 2x$) bred in Switzerland, cv 'Tammisto' ($2n = 2x$) bred in Finland and accession 'Severodvinskaya' ($2n = 2x$) which originated from the Arkhangelsk region of Russia provided by N. I. Vavilov All-Russian Research Institute of Plant Industry; *L. multiflorum* cv 'Waseaoba' ($2n = 2x$) bred in Japan and cv 'Billion' ($2n = 4x$) bred in the Netherlands; *F. arundinacea* cv 'Hokuryo' ($2n = 6x$) bred in Japan and cv 'Falcon' ($2n = 6x$) bred in the USA. Seven monosomic substitution plants (each containing 13 *L. perenne* chromosomes and a single *F. pratensis* chromosome from each of the seven linkage groups) and the three parents (two *L. perenne* and one *F. pratensis*) (King et al. 1998, 2007, pers. commun.) were used to assign the markers to individual chromosomes.

Development of *L. perenne* ESTs

A cDNA library constructed from cold-acclimated *L. perenne* plants (Shinozuka et al. 2005) was used to develop ESTs. *E. coli* transformants from the cDNA library were selected at random and plasmid DNA extracted. Nucleotide sequences of 5′ and 3′ ends of the cDNA clones were determined using the CEQ 8000 Genetic Analysis System (Beckman Coulter, CA, USA). Sequence data of ESTs used for primer designs were deposited in DDBJ and Genbank data libraries under accession numbers AB373997 to AB374042.

Screening of *Lolium/Festuca* ESTs homologous to unique rice genes

A total of 21,276 ESTs originating from *L. perenne*, *L. multiflorum* and *F. arundinacea* (Table 1) were searched by Blastn against the rice pseudomolecule release 4 downloaded from the ftp sites (ftp://ftp.tigr.org/pub/data/Eukaryotic_Projects/o_sativa/annotation_dbs/

Table 1 Screening of *Lolium/Festuca* ESTs homologous to unique rice genes

Origin	Reference	Screened ESTs	Selected ESTs[a]	Corresponding unique rice genes
L. perenne	In this study	2,327	765	494
L. multiflorum	Ikeda et al. (2004)	5,818	1,874	1,125
F. arundinacea	Plant GDB[b]	13,131	7,411	4,899
Total		21,276	10,050	5,830

[a] ESTs homologous to unique rice genes were selected using Blastn as described in "Materials and methods"

[b] http://www.plantgdb.org/download/download.php?dir=/Sequence/ESTcontig/Festuca_arundinacea/previous_version/151a

pseudomolecules/version_4.0) in the TIGR rice genome annotation database (Yuan et al. 2005). *Lolium/Festuca* gene sequences matched against rice cDNA with a first-hit similarity score of more than 150 and a second-hit similarity score of less than 150 were selected for marker development. As a result, 10,050 redundant ESTs, with high similarity to unique rice genes, were selected, corresponding to 5,830 rice gene loci (Table 1). To develop markers dispersed throughout the whole *Lolium/Festuca* genome, we selected 209 clusters of *Lolium/Festuca* ESTs corresponding to rice unique genes dispersed throughout the rice genome that contained at least one intron. In addition to the TIGR rice database, the rice pseudomolecule build 4 of the International Rice Genome Sequencing Project (IRGSP) and the functional annotation database in the Rice Annotation Project (RAP) build 2 (Ohyanagi et al. 2006) were used for the description of gene-loci number and gene annotation data.

Design of primer sets

Multiple alignments of the genomic sequence of the unique rice genes and corresponding *Lolium/Festuca* ESTs were performed using Clustal W program in the DNASIS®Pro ver. 2.0 software (Hitachisoft, Japan). Primers were designed from regions of *Lolium/Festuca* ESTs that were expected to flank at least one intronic region based on the rice genome sequence. In each case, primers were designed from regions of exonic DNA that showed conservation between *Lolium/Festuca* and rice. Mean PCR fragment size was estimated as 603 bp (minimum: 154, maximum: 1,180) on the basis of rice gene structure. In some indel-type markers, to clarify the fragment-size polymorphisms among *Lolium/Festuca* species, nested primers were designed near the indel regions based on the *L. perenne* and *F. pratensis* genomic sequences obtained by the direct sequencing of PCR amplicons described later.

PCR, restriction enzyme reaction and electrophoresis

Genomic DNA was extracted from plants using a modified CTAB method (Murray and Thompson 1980). The PCR amplification reactions were performed in a 10 µl reaction volume containing 50–80 ng of genomic DNA, 1 µl of 10× PCR buffer (50 mM KCl, 10 mM Tris–HCl, pH 8.3) (Applied Biosystems), 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 0.5 mM each of the forward and reverse primers and 0.25 units of Ampli Taq polymerase (Applied Biosystems, CA, USA). The cycling regime for the PCR amplification consisted of an initial denaturation step of 4 min at 94°C, 10 cycles of touch down: 30 s at 94°C, 30 s at 65 to 56°C (decreasing 1°C each cycle) and 1.5 min at 72°C, 25 cycles of 30 s at 94°C, 30 s at 55°C and 1.5 min at 72°C, and a final extension of 7 min at 72°C. For both the PCR-restriction fragment length polymorphism (RFLP) analysis and genotyping using cleaved amplified polymorphic sequence (CAPS) markers, PCR products were treated with 0.1 unit/µl of each restriction enzyme overnight. Amplification products were loaded onto 4% agarose gels in TAE buffer for electrophoresis. When the fragment size was <200–300 bp, 6% polyacrylamide gels were used for electrophoresis with TBE buffer. The banding patterns were visualized following staining with ethidium bromide and were photographed under ultraviolet light.

Direct sequencing of PCR amplicons

Direct sequencing was performed on PCR products amplified from the bulked genomic DNA derived from 8 individuals of *L. perenne* cv 'Reveille' or *F. pratensis* accession 'S215' ($2n = 4x$). After treatment with ExoSAP-IT (GE Healthcare, UK) to eliminate primers and short nucleotides, the fragments were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit and ABI3730xl capillary sequencer (Applied Biosystems) with the primers used for the amplification.

Evaluation of the specificity of markers to *Lolium* and *Festuca*

A total of 96 individuals consisting of 8 plants from each of the *Lolium* and *Festuca* cultivars and accessions described in "Plant materials" were used for the evaluation of the species specificity of indel-type markers. Species specificity

of indel-type markers was evaluated by the difference of fragment frequencies between the two species as follows. Frequencies (0–1) of a fragment (e.g. fragment A) with the same size in two populations belonging to different species (e.g. species X and Y) were defined as $F({}_{\mathrm{A}}F_{\mathrm{X}}$ and ${}_{\mathrm{A}}F_{\mathrm{Y}})$. The specificity of fragment A was then defined as ${}_{\mathrm{A}}S_{\mathrm{X}} = |{}_{\mathrm{A}}F_{\mathrm{X}} - {}_{\mathrm{A}}F_{\mathrm{Y}}|$ if ${}_{\mathrm{A}}F_{\mathrm{X}} > {}_{\mathrm{A}}F_{\mathrm{Y}}$, or ${}_{\mathrm{A}}S_{\mathrm{Y}} = |{}_{\mathrm{A}}F_{\mathrm{X}} - {}_{\mathrm{A}}F_{\mathrm{Y}}|$ if ${}_{\mathrm{A}}F_{\mathrm{X}} < {}_{\mathrm{A}}F_{\mathrm{Y}}$. After S values were scored for all fragments of different sizes, the maximum S values for a marker in both species X and Y were defined as S_{X} and S_{Y}, respectively, and used as specificity indices to evaluate the degree of specificity of the marker to the two species.

Four sets of bulked genomic DNA of 8 individuals from each cultivar or accession of *L. perenne* and *F. pratensis* were used to estimate the species specificity of the CAPS markers. Each marker was classified following the species specificity of the banding pattern: class A, specific fragments from both species; class B, specific fragments from only one species; class C, no specific fragment.

Chromosome mapping

Assignment of the markers to individual chromosomes was determined by screening the seven *Lolium/Festuca* substitution plants for the presence of fragments specifically derived from the *F. pratensis* parent plant.

Results

PCR and PCR-RFLP analyses with intron-flanking EST primer sets

All (209) primer sets were tested to detect polymorphisms between *L. perenne* and *F. pratensis* by PCR and PCR-RFLP analyses. To do this, we used two bulks of genomic DNA of eight individuals from *L. perenne* cv 'Reveille' and *F. pratensis* cv 'Harusakae', respectively, because both synthetic cultivars contain multiple genotypes. One hundred and ninety-five (93.3%) of the primer pairs successfully amplified clear fragments in both species, four (1.9%) primer pairs amplified fragments in only one species, and ten (4.8%) primer pairs did not amplify clear DNA fragments in either species. Electrophoresis on 4% agarose gels revealed that PCR fragments amplified by 61 primer pairs showed fragment-length polymorphisms between the two species. For the 134 primer pairs of PCR amplicons with no fragment-length polymorphism between the two species, PCR-RFLP analysis was performed using four restriction enzymes (4 bp recognition sites), *Afa*I, *Dde*I, *Mse*I and *Taq*I. Mean detection frequency of primer pairs of class A (specific fragments from both species) and class B (specific fragments from only one species) banding patterns following each restriction enzyme reaction were 18.1 and 18.7%, respectively. No significant differences were found between the detection frequency of class A and that of class B with any restriction enzymes. Ninety-nine (73.9%) showed class A or B banding pattern, and sixty-four (47.8%) primer sets showed class A banding patterns, respectively, using at least one of the four restriction enzymes.

Sequencing analysis of PCR amplicons

In order to confirm the polymorphisms at the sequence level, direct sequencing was carried out on 87 pairs of PCR fragments randomly selected from the 195 pairs amplified from *L. perenne* and *F. pratensis*. Sixty-one pairs of the PCR fragments were successfully sequenced. A total of 29,748 bp of sequence, including 22,479 bp of putative intron sequence (based on *F. pratensis* sequences), were obtained from *L. perenne* and *F. pratensis* allowing comparisons to be made between the two species. These included 25 pairs of PCR fragments showing indel polymorphisms by electrophoresis on 4% agarose gels. A total of 2,191 bp of indel mutations between *L. perenne* and *F. pratensis* were found at 126 sites in 45 genes including 20 genes which did not show clear indel polymorphisms using 4% agarose gel electrophoresis. All indels were found in putative intronic regions. Fifty-eight (46.0%) indels consisted of more than 5 bp (Fig. 1). SSR motifs with mono-, di- or trinucleotide repeats were found around 35 (27.8%) of the indels (Fig. 1). All SSR indels were composed of

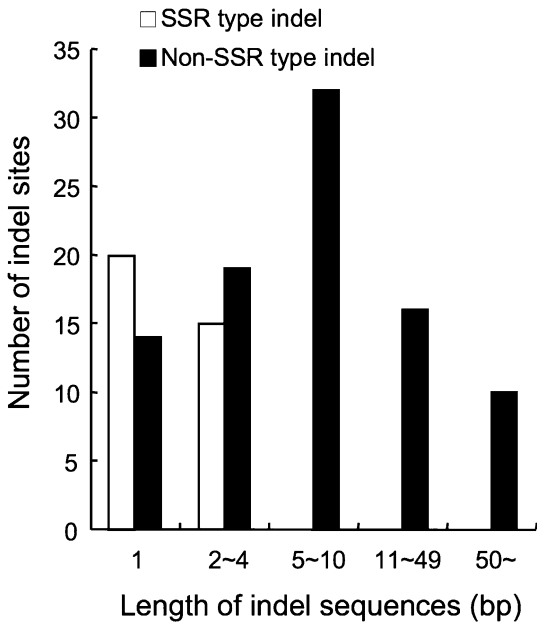

Fig. 1 Distribution of length, in bp, of 126 indel mutations between *L. perenne* and *F. pratensis* in intron regions of 45 genes. SSR type indels included mono-, di- and trinucleotide repeats

less than 4 bp and 57.1% of them were composed of a single nucleotide indel (Fig. 1). However, we could not accurately determine the frequency of nucleotide substitutions between the two bulks of DNA (i.e., it was difficult to discriminate nucleotide substitutions between species from those within species).

Intron-flanking EST markers that distinguish between the *L. perenne* and *F. pratensis* genomes

On the basis of the electrophoresis, PCR-RFLP and sequence data, 61 primer pairs for indel-type markers and 82 primer pairs for CAPS markers using 11 restriction enzymes (4 used in PCR-RFLP screening and 7 selected based on polymorphic sites obtained from sequence data) showing clear species specific fragments from both *L. perenne* cv 'Reveille' and *F. pratensis cv* 'Harusakae' in bulk assays were selected as the intron-flanking EST markers to distinguish between the *L. perenne* and *F. pratensis* genomes (Tables 2, 3). For some indel-type markers, primers were redesigned closer to the indel to detect clear polymorphisms. Detailed data of markers including primer sequences are described in S1 and S2.

Specificities of intron-flanking EST markers that distinguish between the *Lolium* and *Festuca* genomes

To estimate the utility of the intron-flanking EST markers as tools to identify *Lolium* and *Festuca* genomes in the *Lolium/Festuca* complex, we confirmed the specificity of the markers to *Lolium* and *Festuca* species, respectively. For indel markers, species specificity was evaluated as the degree of difference of the fragment frequency between two species, defined as *S* values in this study as described in the "Materials and methods". We investigated the S_{Lp} (specificity to *L. perenne*) and S_{Fp} (specificity to *F. pratensis*) values by genotyping 32 individuals originating from four cultivars and accessions of *L. perenne* and *F. pratensis*, respectively (Fig. 2). The mean number of fragments per primer set was 1.82 in the *L. perenne* population, 1.52 in the *F. pratensis* population and 2.82 in total. Twenty-six (42.6%) of the sixty-one indel-type markers were estimated to be completely specific to *L. perenne* with a S_{Lp} value of 1.0 (i.e. fragments common to all *L. perenne* individuals but which did not appear in *F. pratensis*) (Fig. 3). Similarly, 24 (39.3%) indel-type markers were estimated to be completely specific to *F. pratensis* with a S_{Fp} value of 1.0 (Fig. 3). In total, 15 (24.6%) markers showed complete specificity to both species with S_{Lp} and S_{Fp} both equal to 1.0 (Table 2). Genotyping data for 16 plants of *L. multiflorum* and *F. arundinacea* were then included and mean *S* values calculated for all possible combinations among the four species (Table 4). Both S_{Lp} and S_{Fp} in the combination between *L. perenne* and *F. pratensis* were higher than the *S* values in other *Lolium/Festuca* combinations. *S* values for combinations within the same genus were also lower than *S* values for combinations between different genera. The only exception was S_{Fa} (specificity to *F. arundinacea*), which was nearly equal against *F. pratensis* and *L. multiflorum* (Table 4).

For the 82 selected CAPS markers, we estimated the species specificity of markers using 4 genomic DNA bulks of *L. perenne* and *F. pratensis*, respectively, and classified the polymorphic patterns into three specificity classes—A, B and C. Fragment patterns of CAPS markers with specificities evaluated as class A and B are shown in Fig. 4. Forty-nine (59.8%) markers were categorized as class A, i.e. markers able to apparently distinguish between the two species at bulk level with co-dominant-like polymorphisms, while only four (4.9%) markers were ranked as C showing no specific fragments to either of the two species (Table 3).

Chromosome mapping of intron-flanking EST markers

To identify the chromosomal location of the intron-flanking EST markers, chromosome mapping was performed using *Lolium/Festuca* monosomic substitution plants (King et al. 1998, 2007, pers. commun.). Out of 143 intron-flanking EST markers, 107 (74.8%) markers were assigned to one of the 7 *Festuca* chromosomes (Tables 2, 3). The remaining 36 markers (15 indel-type markers and 21 CAPS markers) could not clearly discriminate alleles originating from the *F. pratensis* genome in the monosomic substitution plants. The comparative relationship of unique gene loci between *Lolium/Festuca* and rice revealed several syntenic segments largely in agreement with previous reports (Jones et al. 2002; Alm et al. 2003; Sim et al. 2005; King et al. 2007).

Discussion

In general, intronic regions have higher frequencies of polymorphism with indels and base substitutions than exonic regions (Lyons et al. 1997; Choi et al. 2004; Bertin et al. 2005; Wei et al. 2005), i.e. they are less well conserved. In the study of hexaploid wheat, sequencing of 24 genes revealed that intron regions had indel polymorphisms among homoeologous genes circa every 100 bp, while length of exonic regions were generally equal among them (Ishikawa et al. 2007). Similarly, all indel sites found here during the sequencing comparisons between *L. perenne* and *F. pratensis* were located in intronic regions, even though about a quarter of the compared sequences were exonic. Although we could not estimate accurately the

Table 2 List of 61 indel-type markers showing co-dominant polymorphism between *L. perenne* cv 'Reveille' and *F. pratensis* cv 'Harusakae'

Marker name/ corresponding rice locus number in TIGR pseudomolecule 4	Original EST clones derived from			1st hit score of Blastn versus rice gene[d]	Specificity values (S)[e] to		Number of alleles in			Locating chromosome[f]
	F. arundinacea[a]	*L. perenne*[b]	*L. multiflorum*[c]		*L. perenne* (S_{Lp})	*F. pratensis* (S_{Fp})	*L. perenne* ($n = 32$)	*F. pratensis* ($n = 32$)	Total ($n = 64$)	
Os01g01080			AU249702	184	1.00	1.00	1	1	2	6
Os01g10840		AB373998		244	1.00	0.72	2	1	2	3
Os01g18320	151a-24922783		AU247466	680	0.56	0.81	1	2	2	3
Os01g34480		AB374005		519	1.00	1.00	1	1	2	3
Os01g36930	151a-6580			402	0.78	0.97	2	2	4	3
Os01g43070	151a-19929		AU246653	381	1.00	1.00	1	1	2	3
Os01g53250	151a-11460		AU248952	434	0.38	0.88	2	2	3	
Os01g68710	151a-18904		AU245738	462	0.75	1.00	1	2	2	3
Os02g22140	151a-19874			640	0.91	0.84	3	1	3	
Os03g01200	151a-14112			396	1.00	0.81	2	2	3	4
Os03g06220		AB374000		295	0.91	1.00	2	1	3	4
Os03g23950	151a-10338		AU248514	392	1.00	0.88	3	1	3	4
Os03g38980		AB374025		472	0.75	1.00	3	1	4	4
Os03g50480	151a-15822777	AB374002		289	1.00	1.00	1	1	2	4
Os03g64210	151a-3415			615	1.00	1.00	1	1	2	4
Os04g01230			AU246534	389	0.69	0.84	3	1	4	
Os04g02000		AB374034		208	0.91	0.91	2	1	2	2
Os04g06790	151a-21548			369	1.00	1.00	1	1	2	4
Os04g11880	151a-4722779			337	1.00	0.81	1	2	3	2
Os04g16680	151a-13096			1,205	1.00	0.81	1	2	3	3
Os04g30420	151a-176			222	1.00	1.00	1	2	3	2
Os04g46620		AB374022		575[g]	1.00	0.66	2	1	2	
Os04g54410	151a-6211			575	1.00	1.00	1	2	3	2
Os05g01270	151a-15286		AU246867	553	0.41	0.97	1	1	2	1
Os05g11850	151a-2073			561	0.81	0.03	2	1	2	
Os05g19630	151a-1411			216	1.00	1.00	1	1	2	1
Os05g24550	151a-10922784		AU248117	650	1.00	1.00	1	1	2	4
Os05g32140	151a-12022780			1,061	0.44	0.94	3	1	3	1
Os05g48510	151a-10422783	AB374040		809	0.84	1.00	3	2	4	5
Os05g51700		AB374010		345	0.66	0.88	2	3	3	1
Os06g01390	151a-11093			1,285	0.06	0.81	3	1	4	2
Os06g06090	151a-3122781		AU247261	807	0.78	0.81	1	2	2	
Os06g11040	151a-18122780		AU245606	646	1.00	1.00	1	1	2	7
Os06g27760	151a-995		AU249087	281	1.00	0.93	3	1	3	7
Os06g36700	151a-3522781	AB374036		961	0.34	1.00	1	1	2	7
Os06g41390	151a-1521		AU246331	281	1.00	0.81	4	2	6	7
Os06g41790	151a-21954		AU248531	440	1.00	1.00	1	1	2	7
Os06g47220	151a-12896			575	1.00	0.94	1	2	3	
Os06g50110		AB374023		224	0.94	1.00	1	1	2	5
Os06g51029	151a-19197		AU248818	983	0.88	1.00	1	2	2	7
Os07g04840	151a-20822783	AB374027		579	0.97	1.00	1	1	2	
Os07g12730	151a-2722782		AU248089	222	0.47	0.19	2	2	3	
Os07g22350	151a-14279			385	0.78	0.38	2	1	2	
Os07g25430		AB374006		234	1.00	1.00	2	1	3	2

Table 2 continued

Marker name/ corresponding rice locus number in TIGR pseudomolecule 4	Original EST clones derived from			1st hit score of Blastn versus rice gene[d]	Specificity values (S)[e] to		Number of alleles in			Locating chromosome[f]
	F. arundinacea[a]	*L. perenne*[b]	*L. multiflorum*[c]		*L. perenne* (S_{Lp})	*F. pratensis* (S_{Fp})	*L. perenne* ($n = 32$)	*F. pratensis* ($n = 32$)	Total ($n = 64$)	
Os07g49320	151a-21251			948	0.66	0.50	3	4	5	
Os08g07830	151a-12944		AU250953	492	0.31	0.31	3	2	4	7
Os08g36774		AB374004		317	0.66	0.53	2	3	3	
Os08g45210	151a-487			406	0.53	1.00	3	1	4	7
Os09g03610	151a-8722			321	1.00	0.84	2	1	2	5
Os09g08660	151a-21930			488	0.66	0.56	3	2	4	
Os09g26730	151a-9181		AU247962	504	0.69	0.44	2	2	3	5
Os09g30466	151a-9622779			563	1.00	0.91	1	2	3	5
Os09g34910		AB374003		309	0.88	0.97	2	3	5	5
Os09g34970	151a-19414		AU248518	989	0.56	0.81	1	2	2	5
Os09g35670	151a-7695			509	0.88	0.34	3	1	3	
Os09g39740	151a-1677			238	0.91	0.91	2	2	3	4
Os10g25360		AB374012		359	1.00	1.00	1	1	2	7
Os10g31000	151a-4222782		AU247977	498	0.91	0.91	2	1	2	4
Os11g02580			AU247482	458	0.91	0.88	2	2	4	4
Os11g09280	151a-18322784			739	1.00	1.00	1	1	2	4
Os11g20689	151a-13004			559	0.91	0.53	3	2	4	

[a] Abbreviated from the EST names in Plant GDB. For example, PUT-151a-Festuca_arundinacea-3 was abbreviated to 151a-3 in this study

[b] Described as accession numbers. All ESTs were obtained in this study

[c] Described as accession numbers. All ESTs were obtained by Ikeda et al. (2004)

[d] The highest score obtained by Blastn between *Lolium*/*Festuca* EST cluster and rice cDNA in pseudomolecule release 4 in TIGR rice genome annotation database

[e] Species-specificity (S) value was evaluated from the genotyping of 32 individuals of *L. perenne* and *F. pratensis* respectively as described in "Materials and methods." S value of 1 means that indel marker has at least one PCR fragment which is completely specific to the species

[f] Estimated by genotyping of *Lolium*/*Festuca* monosomic substitution genotypes (King et al. 1998)

[g] Scores obtained between the EST of the other end of the same cDNA (AB374035) and the rice cDNA

frequency of nucleotide substitutions between *L. perenne* and *F. pratensis*, we were able to confirm a tendency for single nucleotide substitutions to occur more frequently in intronic regions than in exonic ones (data not shown). Meanwhile, primers designed within highly conserved exonic regions provide the means for amplification across related species or genera (King et al. 2007). In this study, for example, 93.3% of primer sets successfully amplified fragments from both *Lolium* and *Festuca* genomes.

Indel-type markers can be used more easily than CAPS markers as they do not need to be treated with restriction enzymes. In this study, we have developed 61 indel markers from 209 primer sets (29.2%), which show clear differences in fragment size between *L. perenne* and *F. pratensis* by short electrophoresis on agarose or acrylamide gels. In the future, the number of indel markers would be expected to increase if all the PCR amplicons are sequenced.

Species specificity of markers is an important factor for the exploitation and characterization of interspecific and intergeneric hybrids and their derivatives. In this study, markers were analyzed to select those showing specific fragments from two bulked PCR amplicons derived from *L. perenne* cv 'Reveille' and *F. pratensis* cv 'Harusakae', respectively. Although some of the markers did not show specificity to other cultivars and accessions, many showed high specificities to both species. Specificities of markers to *L. perenne* and *F. pratensis* were evaluated by calculating the maximum difference of fragment frequencies between the two species for indel markers, and by the fragment patterns of the CAPS markers, using populations thought to contain wide genetic diversity. In chromosome mapping

Table 3 List of 82 CAPS markers showing co-dominant polymorphisms between *L. perenne* cv 'Reveille' and *F. pratensis* cv 'Harusakae'

Marker name/corresponding rice locus number in TIGR pseudomolecule 4	Original EST clones derived from			1st hit score of Blastn vs. rice gene	Restriction enzymes	Species-specificity class[a]	Locating chromosome
	F. arundinacea	*L. perenne*	*L. multiflorum*				
Os01g01010	151a-3254			176	*Xsp*I	A	6
Os01g02880	151a-20012		AU249900	676	*Afa*I	A	3
Os01g25370	151a-61			262	*Afa*I	A	3
Os01g43250		AB374021		377	*Dde*I	B	3
Os01g49330		AB374020		260	*Taq*I	A	3
Os01g55540	151a-12786		AU245817	660	*Dde*I	A	
Os01g60650	151a-15290		AU247348	309	*Mse*I	A	3
Os01g63270	151a-19122777	AB374031		1,267	*Afa*I	C	3
Os01g64720		AB374001		268	*Dde*I	A	3
Os01g66240		AB374014		343	*Sac*II	B	
Os02g01170	151a-14707	AB374037		700	*Eco*RV	C	
Os02g03890	151a-10242		AU245678	440	*Hae*III	A	7
Os02g13840	151a-14521			989	*Afa*I	C	6
Os02g17870		AB374017		337	*Taq*I	B	6
Os02g29530	151a-19310			1,134	*Dde*I	A	2
Os02g30800	151a-7022783	AB374041		1,116	*Afa*I	B	6
Os02g36740		AB373997		420	*Afa*I	A	6
Os02g43350		AB373999		226	*Dde*I	A	6
Os02g47310	151a-3922780		AU250356	672	*Afa*I	B	
Os02g57160		AB374013		317	*Dde*I	B	6
Os02g58650	151a-2841		AU248431	488	*Eco*RI	B	2
Os03g07300	151a-16122776		AU248992	749	*Afa*I	A	4
Os03g29950	151a-11031			404	*Afa*I	B	4
Os03g36750	151a-21234		AU247613	886	*Afa*I	A	7
Os03g43760	151a-5521			500	*Sau*3AI	A	4
Os03g56300	151a-4849		AU246242	714	*Dde*I	A	4
Os03g60090		AB374008		232	*Afa*I	A	4
Os04g27860	151a-11843			343	*Dde*I	A	2
Os04g32950		AB374016		176	*Alu*I	A	6
Os04g37619	151a-21261		AU249112	557	*Afa*I	B	2
Os04g43220		AB374011		220	*Afa*I	A	2
Os04g48230	151a-15235			597	*Eco*RI	A	2
Os04g58350	151a-22419			490	*Mse*I	C	
Os05g04190	151a-10266			791	*Afa*I	B	
Os05g06330	151a-19926	AB374038		573	*Taq*I	B	
Os05g13780	151a-19822779			658	*Afa*I	B	4
Os05g14170	151a-22783	AB374030		787	*Mse*I	B	
Os05g28280	151a-20982	AB374039		739	*Hae*III	A	1
Os05g38330		AB374032		410	*Dde*I	B	
Os05g43360	151a-8417			591	*Afa*I	B	1
Os06g04280	151a-10522781			1,217	*Dde*I	A	2
Os06g15420	151a-14422775	AB374009		1,810	*Afa*I	A	5
Os06g21560		AB374015		587	*Alu*I	A	7
Os07g01480	151a-21485		AU246805	230	*Dde*I	B	
Os07g01930	151a-22185			363	*Ssp*I	A	7
Os07g19030	151a-21936			361	*Mse*I	A	
Os07g30774	151a-3739	AB374018		424	*Mse*I	A	2

Table 3 continued

Marker name/corresponding rice locus number in TIGR pseudomolecule 4	Original EST clones derived from			1st hit score of Blastn vs. rice gene	Restriction enzymes	Species-specificity class[a]	Locating chromosome
	F. arundinacea	*L. perenne*	*L. multiflorum*				
Os07g30840	151a-6713		AU246553	511	*Taq*I	A	2
Os07g38620		AB374007		315	*Afa*I	A	2
Os07g39630	151a-14846			938	*Afa*I	A	2
Os07g48920	151a-14380			698	*Mse*I	A	2
Os08g01350	151a-7598			424	*Dde*I	A	
Os08g03390	151a-6922778			761	*Afa*I	B	
Os08g09940	151a-1826			1,088	*Taq*I	A	7
Os08g15080	151a-15741			613	*Dde*I	B	
Os08g23320	151a-17976			363	*Dde*I	A	3
Os08g27010	151a-4075			609	*Taq*I	B	
Os08g31810	151a-140	AB374033		353	*Taq*I	A	7
Os08g33630	151a-19216		AU251138	585	*Taq*I	A	7
Os08g41830	151a-6777		AU249538	694	*Taq*I	A	7
Os08g44930		AB374029		333	*Alu*I	A	7
Os09g15820	151a-5753		AU247715	936	*Ssp*I	B	5
Os09g20640	151a-8322775	AB374042		872	*Afa*I	B	5
Os09g20880	151a-6386			575	*Afa*I	A	5
Os10g02980	151a-1926			595	*Dde*I	A	
Os10g10244	151a-3074			783	*Dde*I	A	1
Os10g11140	151a-689			567	*Taq*I	B	1
Os10g17280	151a-14292		AU247907	801	*Sau*3AI	A	
Os10g39930	151a-12822782			712	*Taq*I	B	
Os11g29380	151a-8765	AB374026		831	*Afa*I	A	4
Os11g34130		AB374028		424	*Taq*I	A	4
Os11g38020	151a-17528			726	*Taq*I	B	4
Os11g43900		AB374024		208	*Afa*I	A	4
Os11g47710	151a-11294		AU247573	545	*Afa*I	A	4
Os11g48040	151a-3012		AU246430	345	*Taq*I	B	4
Os12g02390	151a-5855			668	*Mse*I	B	
Os12g08810	151a-22213		AU248740	1,080	*Dde*I	B	5
Os12g13320	151a-3188			369	*Afa*I	A	
Os12g23180	151a-5701		AU246589	993	*Dde*I	A	4
Os12g27830	151a-15369			383	*Dde*I	A	4
Os12g40550	151a-1022779			862	*Afa*I	B	5
Os12g42980		AB374019		220	*Dde*I	B	

[a] Species specificity was evaluated by genotyping four bulks of genomic DNA of *L. perenne* and *F. pratensis* respectively, the bulks being composed of 8 individuals of the same variety or accession. Class A, banding pattern with specific fragments from both species; class B, with specific fragments from only one species; class C, with no specific fragment

using the *Lolium/Festuca* monosomic substitution line, 23 of the 24 indel-type markers with a S_{Fp} value of 1.0 were successfully mapped. In contrast, of the 13 markers with a S_{Fp} value of less than 0.80, only three markers were mapped (Table 2). In the case of the CAPS markers, 43 out of 49 (87.8%) with specificity class A were successfully allocated to individual chromosomes. However, the success rate of locating markers with specificity classes B and C was only 54.5% (Table 3). Although accurate evaluation of marker species specificity might require the genotyping of populations with a wider genetic divergence within *Lolium* and *Festuca*, the results do indicate that the markers estimated to have a high species-specificity in this study have the potential to identify the genomes of *Lolium* and *Festuca*. The results also suggest that the species specificity herein estimated could be of some value for general

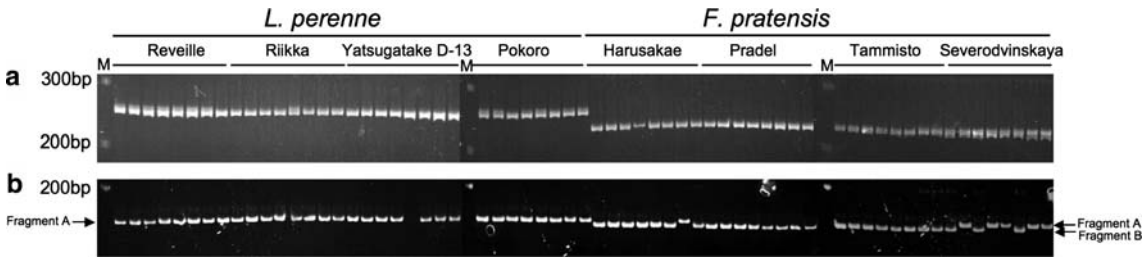

Fig. 2 Electrophoretograms of PCR fragments of indel-type markers amplified from *L. perenne* and *F. pratensis* on 6% acrylamide gels. *M* indicates 100 bp molecular ladder. Values of specificity to *L. perenne* (S_{Lp}) and *F. pratensis* (S_{Fp}) respectively were scored as 1.00 on Os01g43070 marker (**a**). On Os06g06090 marker, S_{Lp} was computed as the difference of the frequency of the fragment A in *L. prenne* and *F. pratensis* population, i.e. $S_{Lp} = 31/32 - 6/32 = 0.78$. On the other hand, S_{Fp} was calculated as the difference of the frequency of the fragment B: 26/32 – 0/32 = 0.81 (**b**)

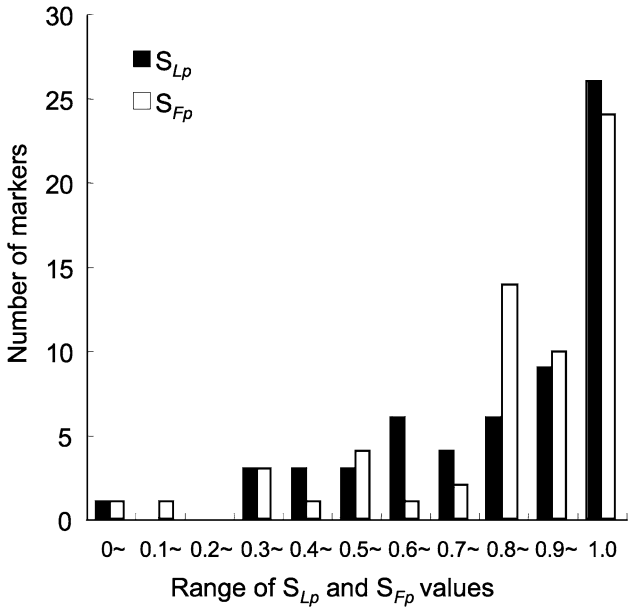

Fig. 3 Distribution of species-specificity *S* values of 61 indel-type markers in the combination of *L. perenne* (S_{Lp}) and *F. pratensis* (S_{Fp}), see *S* values at each marker/species in Table 2

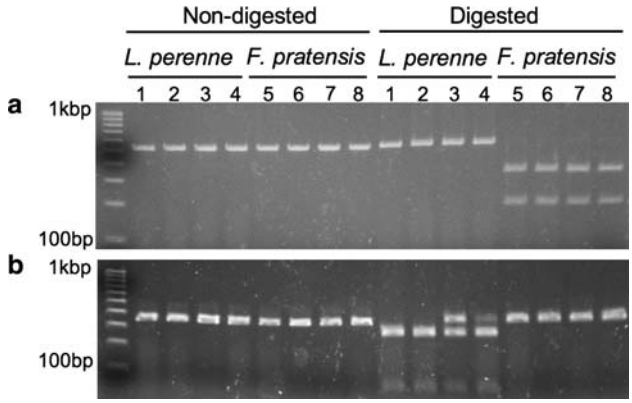

Fig. 4 Electrophoretogram patterns of CAPS markers using bulked genome DNA of *L. perenne* and *F. pratensis* on 4% agarose gels. *1* Reveille, *2* Riikka, *3* Yatsugatake D-13, *4* Pokoro, *5* Harusakae, *6* Pradel, *7* Tammisto, *8* Severodvinskaya, *M* 100 bp ladder. Following species specificity of banding pattern in CAPS as defined in "Materials and methods," Os04g48230 marker digested with *Eco*RI is of class A marker (**a**), while Os05g38330 marker digested with *Dde*I is a class B marker (**b**)

Table 4 Mean specificity values of 61 indel-type markers in the all-possible combinations of four *Lolium/Festuca* species

Contrastive species	Mean species-specificity values			
	S_{Lp}^{a}	S_{Lm}	S_{Fp}	S_{Fa}
L. perenne	–	0.37	0.84	0.68
L. multiflorum	0.38	–	0.72	0.59
F. pratensis	0.83	0.68	–	0.59
F. arundinacea	0.56	0.46	0.45	–

[a] S_{Lp}, S_{Lm}, S_{Fp} and S_{Fa} mean the specificity values to *L. perenne*, *L. multiflorum, F. pratensis* and *F. arundinacea*, respectively

utilization in *Lolium/Festuca* genetics. For the species-specificity estimation, the indel-type markers were tested on individuals while the CAPS markers were tested on bulks of DNA. It is possible that the bulk assay could lead to an over-estimation of the species specificity compared to the individual assay, e.g. un-amplified genotypes included in bulks due to polymorphisms with the primer site could result in mis-scoring.

Species specificities of the markers within combinations of other *Lolium/Festuca* species including *L. multiflorum* and *F. arundinacea* were lower than those in the combination between *L. perenne* and *F. pratensis* (Table 4). Hence, some indel-type markers do not show specificity between genera but do show polymorphisms within the same genus. Although these differences of species specificity among the species should be validated by further investigation from wider sampling, we suppose that higher species specificity in the combination between *L. perenne* and *F. pratensis* might be attributable to the markers being specifically developed to show polymorphisms between *L. perenne* and *F. pratensis*. The diploid genome (PP) of *F. pratensis* has been proposed as one of the progenitors of the hexaploid genome ($PPG_1G_1G_2G_2$) of *F. arundinacea*

(Humphreys et al. 1995). One possible reason why the S_{Fa} against *Lolium* (0.68 and 0.59 against *L. perenne* and *L. multiflorum*, respectively) is less than the S_{Fp} with regard to *Lolium* (0.84 and 0.72) is the possible divergence of the P genome during evolution in the hexaploid from its original diploid progenitor (Chen et al. 1998). Therefore, to develop markers for the genetic analysis of *Lolium*/*Festuca* hybrids containing *L. multiflorum* or *F. arundinacea*, screening genome DNA of *L. multiflorum* or *F. arundinacea* would be more efficient. However, we were able to confirm that some of the markers in this study showed a high species specificity in *Lolium*/*Festuca* combinations including *L. multiflorum* and *F. arundinacea* (data not shown).

A comparison at the sequence level between *F. pratensis* and *L. perenne* genes revealed that 27.8% of the indels consisted of SSRs, but these were all composed of less than 4 bp (Fig. 1). This therefore implies that many of the indel-type markers developed in this study contained non-SSR indels. Generally, SSR sites are highly polymorphic, even within a species, and are often used for genotyping at the individual level (Morgante and Olivieri 1993), although several SSR markers showing species specificity have been reported (Momotaz et al. 2004). *Festulolium* cultivars are often bred as polyploids (Yamada et al. 2005). Hence, when discrimination of same-species alleles in polyploid *Festulolium* is required, highly polymorphic markers such as some SSR markers would be more useful than species-specific markers. However, highly species-specific markers, e.g. some indel-type markers developed in this study, seem to be more reliable for identifying a particular species in a mixed population or in interspecific or intergeneric hybrids with unknown parents. Many of the CAPS markers also showed a high species specificity and were able to identify the *F. pratensis* allele in the substitution genotypes (Table 3). Some of the CAPS markers seemed to be based on single nucleotide polymorphisms (SNPs). This indicates the possibility of the development of species-specific SNP markers.

Estimating the position of marker loci within a genome is an important facet of marker design. Markers maybe required around particular loci such as quantitative trait loci (QTL) but in order to map introgressed segments they would be required to be distributed throughout the whole genome. Chromosome mapping using the monosomic substitution genotypes indicates that the comparative relationship between *Lolium*/*Festuca* and rice is largely consistent with previous report (Jones et al. 2002; Alm et al. 2003; Sim et al. 2005). For example, with a few exceptions, rice chromosome 1 corresponds to *Lolium*/*Festuca* chromosome 3 (King et al. 2007), and rice chromosome 3 corresponds to *Lolium*/*Festuca* chromosome 4 (Tables 1, 2). This result suggests that this marker system could be used to develop markers covering the whole *Lolium*/*Festuca* genome by designing primer sets corresponding to rice unique genes distributed throughout the whole rice genome. In this study on the monosomic substitution plants, chromosomes 1, 5 and 6 were somewhat under-represented. However, designing more markers homologous to unique rice genes locating the corresponding chromosomes should ensure a more even marker distribution. Although the locus order of markers on each chromosome has not been determined in this study, the linear order (colinearity) of genetic markers and genes has previously been shown to be very well conserved between different grass genomes (Feuillet and Keller 2002). Recently, King et al. (2007) reported that the linear order of BAC clones on *Lolium*/*Festuca* chromosome 3 and rice chromosome 1 was virtually the same with only two minor disruptions. This suggests the possibility of targeting markers to a particular locus in *Lolium*/*Festuca* using the syntenic relationship with rice.

In this study, we confirmed the advantages of the intron-flanking EST marker system developed using rice genomic information to the *Lolium*/*Festuca* complex. (1) high frequency of polymorphism in intron regions; (2) high specificity of marker polymorphisms to distinguish between *Lolium* and *Festuca*; and (3) The ability to derive marker loci from the rice genome applicable to the *Lolium*/*Festuca* genomes. Armstead et al. (2006, 2007) identified the staygreen mutation in *Lolium*/*Festuca* using genomic-loci and the annotation information of rice. Functionally annotated information of genes, which is being rapidly accumulated in species such as rice and other major monocot crop studies, can be advantageously used in the development of markers linked to biochemically and physiologically related phenotypic traits in key crops such as grass where to date less genomic research has been undertaken. By adding functionally annotated information, the intron-flanking EST marker system could contribute further to the development of useful markers for the introgression mapping of the *Lolium*/*Festuca* complex.

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