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Genome Mapping and Molecular Breeding in *Lolium* and *Festuca*

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ABSTRACT

Of the 8 species in the genus *Lolium*, Italian ryegrass (*Lolium multiflorum* Lam.), is one of the most important cool-season forage grasses and is the most widely cultivated annual forage grass in Japan. Another important *Lolium* species is perennial ryegrass (*L. perenne* L.); it is cultivated mainly for forage and turf in the British Isles, Europe, USA, Australia, and New Zealand. Both Italian ryegrass and perennial ryegrass are outcrossing species, and each has a relatively large genome (1 C ≈ 2000 Mb). In recent years, many molecular markers, including amplified fragment length polymorphism (AFLP) markers, restriction fragment length polymorphism (RFLP) markers, simple sequence repeat (SSR) markers, and expressed sequence tag (EST) markers, have been developed for Italian ryegrass and perennial ryegrass, and several types of molecular linkage map have been constructed. In addition, markers closely linked to genes for resistance to diseases such as crown rust, gray leaf spot, and bacterial wilt have been identified. Quantitative trait loci (QTLs) for flowering time, winter hardiness, forage quality, and other important traits have also been analyzed. In this paper, we review recent progress in genome mapping and QTL analysis in *Lolium* spp. We also include studies on tall fescue (*Festuca arundinacea* Schreb.) and meadow fescue (*F. pratensis* Huds.), two species closely related to *Lolium*.

Keywords: fescue, linkage map, molecular marker, QTL, resistance gene, ryegrass

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INTRODUCTION

Italian ryegrass (*Lolium multiflorum* Lam.), an important grass species belong to genus *Lolium*, *Poaceae* family, also called annual ryegrass, is an upright annual species that behaves like a biennial or even a short-lived perennial depending on environmental conditions. Italian ryegrass is widely used as animal fodder, i.e. hay, pasturage or silage and turf grasses worldwide. It is also used for quick cover in erosion control plantings. Another important species in *Lolium*, perennial ryegrass (*L. perenne* L.), which is also used mainly as forage and turf, is cultivated mainly in the British Isles, Europe, USA, Australia, and New Zealand. Both Italian ryegrass and perennial ryegrass are naturally diploid, outcrossing species, with 2n=14, having a moderate genome size (1 C ≈2000 Mb), but many tetraploid commercial cultivars were produced. A genus closely related to *Lolium* is *Festuca*, which includes two important forage grasses: tall fescue (*F. arundinacea* Schreb.) and meadow fescue (*F. pratensis* Huds.). Tall fescue is cultivated throughout temperate regions for use as hay, pasturage, and silage, and in erosion control. Tall fescue originated in Europe and has a large genome of 5929 Mb. Meadow fescue is a diploid ($2n = 2x = 14$), obligate outbreeding forage grass

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species. It is cultivated throughout temperate regions worldwide. The DNA content is about 1800 Mb per haploid genome (Seal 1983) – close to the genome size of Italian ryegrass and perennial ryegrass.

MARKER DEVELOPMENT

The genomic study of most forage crops has lagged behind that of other major crops such as rice and maize in terms of genomic sequencing, compilation of expressed sequence tags (ESTs), and development of markers, including restriction-fragment-length polymorphism (RFLP) markers, simple sequence repeat (SSR) markers, In/Del markers, and single nucleotide polymorphism (SNP) markers. The main reasons for this are the outcrossing nature of forage species, the relatively large genome, and the relatively low economic value of forage crops compared with those of other crops.

However, many molecular markers, including RFLP markers, SSR markers, and EST markers, have recently been developed for *Lolium* and *Festuca*, especially for perennial ryegrass.

RFLP markers

The advantages of RFLP markers over other types, such as random amplified polymorphic DNA (RAPD) and amplified-fragment-length polymorphism (AFLP) markers, include their co-dominant nature and the ease with which map information can be transferred to a different mapping population. Although RFLP analysis requires a large amount of genomic DNA and is time-consuming and costly, an informative RFLP linkage map is useful for analyzing the structural organization of genomes (Berhan *et al.* 1993) and for generating physical maps of specific chromosomes through *in situ* hybridization using DNA markers (Werner *et al.* 1992; Wanous and Gustafson 1995). In addition, comparative RFLP mapping of related species has the potential to provide important insights into the evolution of plant genomes (Ahn and Tanksley 1993; Huang and Kochert 1994). The RFLP marker set for the Poaceae family in particular (mainly rice, oats, and barley) has been a very useful tool for comparative genome mapping (van Deynze *et al.* 1998).

Inoue *et al.* (2004a) developed 239 RFLP probes from a *Pst-I* genomic library of Italian ryegrass; 74% (239 of 396) of these showed single- or low-copy hybridization patterns. In perennial ryegrass, Faville *et al.* (2004) selected 157 cDNAs assigned to 8 different functional categories associated with agronomically important biological processes to detect polymorphic EST-RFLP loci in an F_1 ($NA_6 \times AU_6$) population.

RFLP markers were also developed from a tall fescue *Pst-I* genomic DNA library (Xu *et al.* 1991). The genomic clones were evaluated using 9 genotypes from 3 species of *Festuca*; 174 probes gave readable results, of which 79% were single-copy. The single-copy probes revealed good polymorphism in tall fescue, but approximately 21% of the probes did not cross-hybridize to any of the diploids or tetraploids, and these represented genome-specific clones.

SSR markers

SSRs or microsatellites are tandem repeat units of 2 to 6 nucleotides. They are highly abundant and polymorphic and are widely distributed in the genomes of eukaryotes. Although the development of SSR markers takes time and money, SSR markers have a number of advantages (such as being PCR-based, multi-allelic, highly reproducible, and co-dominant) over other markers, including RFLP, RAPD, and AFLP markers. Therefore, SSRs have been widely used in constructing linkage maps (Röder *et al.* 1998) and in gene tagging (Fahima *et al.* 1998), map-based cloning (Liu *et al.* 2002), studies of genetic diversity (Chen *et al.* 2002), and evolutionary studies (Buchanan *et al.* 1994).

Hirata *et al.* (2006) constructed a genomic library enriched for (CA)*n*-containing SSR repeats to develop SSR primers for Italian ryegrass. After the sequencing of a total of 1544 clones, 1044 (67.6%) were found to contain SSR motifs. Of 395 primers designed from unique clones, 357 primer pairs could amplify products of the expected size in both parents of a 2-way pseudo-testcross F_1 mapping population, and genetic loci detected by a total of 218 primer pairs were assigned to locations on 7 linkage groups. To increase the available number of SSR markers in Italian ryegrass, Inoue *et al.* (2005) developed 851 new polymorphic primers from 4 Italian ryegrass SSR-enriched genomic libraries, following evaluation across a screening panel consisting of 5 individual specimens of Italian ryegrass, 1 of perennial ryegrass, 1 meadow fescue, and 1 tall fescue. Following re-screening of the SSR markers from Hirata *et al.* (2006), some tall fescue EST-SSR primers (Saha *et al.* 2004), and other published perennial ryegrass SSR markers (Jones *et al.* 2001), a total of 1172 working primers, including the aforementioned newly developed SSR markers, were found to be useful for Italian ryegrass. Of these, 679, 581, and 682 primers successfully amplified SSR sites in perennial ryegrass, meadow fescue, and tall fescue, respectively (Hongwei Cai, Nana Yuyama, and Maiko Inoue, unpublished data).

Jones *et al.* (2001) developed 366 SSR primers from 2 SSR-enriched libraries from perennial ryegrass. After sequencing 1853 clones, they identified 859 SSR-containing clones, of which 718 were unique. From the 366 clones that allowed the design of the SSR primers, 100 selected SSR primer pairs were evaluated for amplification and genetic polymorphism across a panel of diverse genotypes. The results showed a high efficiency of amplification (81%) and a relatively high level of polymorphism (67%), with a range of 2 to 7 alleles per locus. In addition, cross-species amplification was detected in a number of related pasture and turfgrass species, with high levels of transfer to other *Lolium* species and members of the related genus *Festuca*. The identities of putative SSR ortholoci in these related species were confirmed by DNA sequence analysis. A perennial ryegrass EST collection has also been used for the development of EST-SSR primer pairs: Faville *et al.* (2004) analyzed 14 767 unigenes and developed 310 EST-SSR primer pairs showing efficient amplification and detecting 113 polymorphic loci.

Jensen *et al.* (2005b) have reported on the characterization and mapping of 76 SSR markers for perennial ryegrass. These markers were publicly available or were obtained either from genomic libraries enriched for SSR motifs or from perennial ryegrass expressed sequence tag (EST) clones. Of the reference SSR markers, 65 were mapped to 4 perennial ryegrass mapping populations. Jensen *et al.* (2007) have also tested the amplification of 105 perennial ryegrass SSR markers in 23 grass species representing 7 tribes from 3 subfamilies of *Poaceae*. Between 2% and 96% of the SSR markers could be amplified within a given species. A subset of 8 SSR markers was evaluated for polymorphism across 9 of the 23 grass species: 4 to 7 of the markers were polymorphic within each species, with an average detection rate of 2.4 alleles per species. Gill *et al.* (2006) has designed 563 and 931 SSR primers from GeneThresher and SSR-enriched library, respectively. And they detected 258 and 355 polymorphic primer pairs from the two libraries in their screening panel consisting of eight *L. perenne* cultivars.

More recently, Asp *et al.* (2007) mined perennial ryegrass SSRs from 25 744 ESTs, representing 8.53 Mb of nucleotide information from 3 genotypes of perennial ryegrass. Their results showed that 1458 ESTs (5.7%) contained 1 or more SSRs. Of these SSRs, 955 (3.7%) were non-redundant. Tri-nucleotide repeats were the most abundant type of repeat, followed by di- and tetra-nucleotide repeats. Most of the SSR motifs in the 3 genotypes of perennial ryegrass showed no polymorphisms (97.7%), whereas only 22 EST-SSRs showed allelic or genotypic polymorphisms, or both. Comparative analysis of the peren-

nial ryegrass EST-SSRs with sequences of *F. arundinacea*, *Brachypodium distachyon*, and *Oryza sativa* identified 19 clusters of sequences that were orthologous between these 4 species. Analysis of the clusters showed that the SSR motifs are generally conserved in the closely related species *F. arundinacea*, but the lengths of the SSR motifs often differ. In contrast, SSR motifs are often lost in the more distantly related species *B. distachyon* and *O. sativa*. Studer et al. (2008) used these 955 SSR containing ESTs to designed 744 primers. Primer amplification was tested in 8 genotypes of *L. perenne* and *L. multiflorum* representing (grand-) parents of 4 mapping populations and resulted in 464 successfully amplified EST SSRs. Three hundred and six primer pairs successfully amplified products in the mapping population VrnA derived from 2 of the 8 genotypes included in the original screening and revealed SSR polymorphisms for 143 ESTs.

In tall fescue, Saha *et al.* (2004) designed 157 EST-SSR primer pairs from tall fescue ESTs and tested them on 11 genotypes representing 7 grass species. Nearly 92% of the primer pairs produced characteristic SSR bands in at least 1 species. A large proportion of the primer pairs produced clear reproducible bands in other grass species, with most success in the close taxonomic relatives of tall fescue. A high level of marker polymorphism was observed in tall fescue and ryegrass (66%). Furthermore, Saha *et al.* (2006) developed 511 SSR primers pairs from a tall fescue genomic library enriched in (GA/CT)*n* repeats. The parents and a subset of a tall fescue mapping population were used to select primer pairs for mapping in tall fescue. Their survey results revealed that 48% (in rice) to 66% (in tall fescue) of the primer pairs produced clear SSR-type amplification products in divergent grass species. Polymorphism rates were higher in tall fescue (68%) than in other species (46% in ryegrass, 39% in wheat, and 34% in rice). A set of 194 SSR loci (38%) was identified; these loci amplified across all 6 species.

Other markers: STS, CAPS, RGA, and SNP

Inoue and Cai (2004) converted RFLP markers of Italian ryegrass to sequenced tag site (STS) markers. They end-sequenced 93 previously mapped single- or low-copy RFLP probes; of these, 87 clones gave acceptable results from both forward and reverse directions, and 71 contigs were detected. Other clones could not be sequenced completely because their fragments were too long. STS primers were designed for each of the 93 clones, and 66 primers amplified a single band of the expected size. Of the 67 STS primer pairs, 57 (85%) could amplify products in perennial ryegrass, 47 (70%) in meadow fescue, and 55 (82%) in tall fescue; 40% of the STS primers detected the within-cultivar length or the presence/absence of polymorphisms.

Ikeda *et al.* (2004) generated about 6000 ESTs from 7 Italian ryegrass cDNA libraries. From those cDNA clones, Miura *et al.* (2007) converted EST markers into cleaved amplified polymorphic sequence (CAPS) markers. Of 260 EST primer pairs that amplified a single band, 74 generated loci that showed clear polymorphisms among individuals of an F1 mapping population, and 69 were mapped onto a previously constructed Italian ryegrass linkage map (Hirata *et al.* 2006). In perennial ryegrass, Sawbridge *et al.* (2003) reported the generation and analysis of an EST and cDNA microarray resource for perennial ryegrass. From 29 cDNA libraries of perennial ryegrass representing a range of plant organs and developmental stages, over 44,000 ESTs were produced, analyzed by BLAST searches, categorized functionally, and subjected to cluster analysis, leading to the identification of a unigene set corresponding to 14,767 genes. This unigene set, representing approximately 1/3 to 1/2 of all expressed sequences in ryegrass, was compared with the *Arabidopsis* and rice proteomes and used to develop a unigene cDNA microarray for genome-wide gene expression analysis in grasses.

The majority of plant disease resistance genes encode a predicted nucleotide-binding site (NBS) and leucine-rich repeat (LRR) (Takken and Joosten 2000). The conserved motifs are widely used for isolating resistance gene analogues (RGA). Large-scale sequencing of disease RGA has been carried out in Italian ryegrass (Ikeda 2005). A total of 24,000 PCR clones amplified on the basis of NBS-LRR degenerate primers and a nested PCR strategy were subjected to single-pass sequencing. Of these clones, 9344 (41.2%) showed marked levels of identity to either known NBS-LRR resistance genes or RGA derived from other plant species. These clones were grouped into 115 clusters, and of 115 representative clones, 62 had a continuous open reading frame and were defined as potentially functional RGA from Italian ryegrass. Specific STS primer sets were designed for these 62 clones, and 50 RGA sequences were subsequently amplified from genomic DNA of Italian ryegrass in single-step PCR. A subset of these STS primers was also able to amplify PCR products of the expected size from genomic DNA of tall fescue and meadow fescue.

Cogan *et al.* (2006) surveyed SNP variation across 100 candidate genes by means of amplicon cloning and sequencing in perennial ryegrass. The putative SNPs were identified within and between the parents of the F_1 ($NA_6 \times AU_6$) genetic mapping family and were validated among individual progeny. The results revealed a high incidence of SNP (approx. 1 per 54 bp), with similar proportions in exons and introns. About 50% of the validated genic SNPs were assigned to the F_1 genetic map, showing high levels of coincidence with previously mapped RFLP loci. Ponting *et al.* (2007) identified SNPs from full-length genes corresponding to well-defined biochemical functions such as lignin biosynthesis and oligosaccharide metabolism.

LINKAGE MAP CONSTRUCTION

Almost all forage crops are outcrossing species with a self-incompatibility nature that usually prevents the development of inbred lines. So, unlike in most inbred species such as wheat, rice, and other major crops, the most popular types of segregating population, including F_2, back-cross (BC_1), double-haploid (DH), and recombinant inbred (RI) lines, are difficult to produce in the case of most forage crops. Therefore, several unique population types are usually used in constructing linkage maps of forage crops. The most commonly used population type is a pseudo-testcross F_1 population. In this method, F_1 progeny generated from the cross between unrelated multiple heterozygous individuals can be used (1-way pseudo-testcross by Ritter *et al.* 1990; 2-way pseudo-testcross by Grattapaglia and Sederoff 1994). Working with the F_1 generation of a cross between 2 heterozygous individuals has allowed the direct analysis of segregation and the construction of genetic maps (Viruel *et al.* 1995; Maliepaard *et al.* 1998). But with this method, segregation tests and calculation of the recombination values may be complex and difficult, because different types of marker such as BC_1 and F_2 markers are mixed. Furthermore, a locus may have multiple alleles that segregate to 3 or more in an individual. Therefore, construction of a linkage map usually requires special software such as JoinMap (Stam 1993). Other types of mapping population such as full-sib family and half-sib family can also be used in linkage map construction for outcrossing forage crops.

Italian ryegrass linkage maps

A first-generation map of *Lolium* was constructed using a segregating population derived from an F_1 hybrid of perennial ryegrass × Italian ryegrass, crossed with a doubled haploid perennial ryegrass (Hayward *et al.* 1998). A total of 106 markers (17 isozyme, 48 RAPD, and 41 RFLP markers) are mapped to 7 linkage groups. This map has a total length of 692 cM, with lengths of linkage groups ranging from 67 to 155 cM.

Recently, many molecular markers, including AFLP, SSR, and SNP markers, have been developed in *Lolium* and

Festuca, and high-density, high-resolution comparative linkage maps have been constructed.

Inoue *et al.* (2004a) constructed a high-density linkage map for Italian ryegrass using RFLP, AFLP, and telomeric-repeat–associated sequence markers. A 2-way pseudo-testcross F_1 population consisting of 82 individuals was used to analyze the above 3 types of marker. The final map includes 385 markers, which are separated into 7 major linkage groups. The total map length is 1244.4 cM, and the average interval between markers is 3.7 cM. In this map, the linkage groups LG1, LG2, LG3, LG4, and LG6 are comparable with those on the perennial ryegrass map reported by Jones *et al.* (2002b). Furthermore, the locations of some of the anchor probes on this map were shown to be common to those in the homologous groups in wheat. Hirata *et al.* (2006) mapped 218 SSR markers to 7 linkage groups, representing the 7 chromosomes of the haploid Italian ryegrass karyotype. The SSR markers cover 887.8 cM of the female map and 795.8 cM of the male map. The average distance between 2 flanking SSR markers is 3.2 cM.

Studer *et al.* (2006) constructed a linkage map of Italian ryegrass using AFLP and SSR markers for mapping QTLs for bacterial wilt resistance. Seventeen AFLP primer combinations and 233 SSR primer pairs were screened using a 2-way pseudo-testcross population consisting of 297 individuals. A total of 368 loci were finally used to construct a genetic map with a total length of 804 cM and an average distance between loci of 1.9 cM. The resulting 7 linkage groups contain 38 to 68 markers, with at least 2 biparental SSR markers present on each linkage group. The length of linkage groups ranged from 87 to 154 cM, with an average of 115 cM.

Perennial ryegrass linkage maps

Bert *et al.* (1999) constructed a high-density AFLP linkage map of perennial ryegrass using a progeny set of 95 plants from a testcross involving a doubled-haploid tester. This genetic map covered 930 cM in 7 linkage groups and was based on 463 AFLP markers using 17 primer pairs, 3 isozymes, and 5 EST markers. The average density of markers is approximately 1 per 2.0 cM. Strong clustering of AFLP markers is observed at putative centromeric regions. Overall, 272 markers cover about 137 cM, whereas the remaining 199 markers cover approximately 793 cM.

A molecular-marker linkage map has also been constructed for perennial ryegrass by using a 1-way pseudo-testcross population based on the mating of a multiple heterozygous individual with a doubled-haploid genotype (Jones *et al.* 2002a). RFLP, AFLP, isozyme, and EST data were combined to produce an integrated genetic map containing 240 loci covering 811 cM on 7 linkage groups. The map contains 124 co-dominant markers, of which 109 are heterozygous anchor-RFLP probes from wheat, barley, oats, and rice, allowing inference of comparative relationships between perennial ryegrass and other Poaceae species. The genetic maps of perennial ryegrass and the *Triticeae* are highly conserved in terms of synteny and colinearity. In addition, Jones *et al.* (2002b) also constructed an SSR-based linkage map using the same 1-way pseudo-testcross reference population. Ninety-three loci were assigned to positions on 7 linkage groups. The SSR locus data were integrated with selected data for RFLP, AFLP, and other loci mapped in the same population to produce a composite map containing 258 loci.

Armstead *et al.* (2002) constructed 2 linkage maps of perennial ryegrass from F_2 and BC_1-type populations using, predominantly, RFLP data based on heterologous probes used in mapping other grass species. The maps identified 7 linkage groups, which covered a total of 515 cM (F_2) and 565 cM (BC_1). The 38 markers common to both populations were mapped within a distance of 446 cM in the F_2 population and 327 cM in the BC_1 population, reflecting a higher recombination frequency in the former, although the difference was not evenly spread over the 7 linkage groups.

Faville *et al.* (2004) constructed a functionally associated molecular genetic marker map of perennial ryegrass. In this map, 2 parental genetic maps were produced: the NA_6 genetic map contains 88 EST-RFLP and 71 EST-SSR loci with a total map length of 963 cM, whereas the AU_6 genetic map contains 67 EST-RFLP and 58 EST-SSR loci with a total map length of 757 cM. Bridging loci permitted the alignment of homologous chromosomes between the parental maps, and a subset of genomic DNA-derived SSRs was used to relate linkage groups to the perennial ryegrass reference map.

Jensen *et al.* (2005b) reported the mapping of a reference SSR marker set in perennial ryegrass. Four perennial ryegrass mapping populations were used to map the SSR markers. A consensus linkage map of the 4 mapping populations contains 65 of the SSR markers, and the SSR markers identify all 7 perennial ryegrass linkage groups. Gill *et al*. (2006) has constructed a moderate-density framework linkage map for *Lolium perenne* based on 376 SSR markers. The map consists of 81 SSR markers spread over 7 linkage groups, and most of the remaining 295 SSR markers have been placed into their most likely interval on the framework map.

Male and female molecular-marker linkage maps of an interspecific annual × perennial ryegrass mapping population were developed to determine the map location of the seedling root florescence (SRF) character and to identify additional genomic regions useful for species separation (Warnke *et al.* 2004). 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 these linkage maps. The lengths of the male and female maps differed: the male map was 537 cM and the female map was 712 cM.

Meadow fescue and tall fescue linkage maps

Xu *et al.* (1995) were the first to construct a RFLP linkage map of hexaploid tall fescue. This map was generated from an F_2 population of HD28-56 × Kentucky-31 and contains 108 RFLP markers. The map covers 1274 cM over 19 linkage groups, with an average of 5 loci per linkage group (LG) and 17.9 cM between loci. Mapping of the homoeologous loci detected by the same probe allowed them to identify 5 homoeologous groups, within which the gene orders were found to be generally conserved among homoeologous chromosomes. Using 12 genome-specific probes, they assigned several of the 19 linkage groups to 1 of the 3 tall fescue genomes (PG_1G_2). Comparative genome mapping with maize probes indicated that homoeologous group 2 and 3 in tall fescue corresponded to maize chromosome 1.

Saha *et al.* (2005) reported a genetic map of tall fescue constructed with PCR-based markers (AFLP and EST-SSRs). Two parental maps were initially constructed by using a 2-way pseudo-testcross mapping strategy. The female map included 558 loci located in 22 linkage groups and covered 2013 cM of the genome. In the male map, 579 loci were grouped in 22 linkage groups with a total map length of 1722 cM. The distances between markers in the 2 maps ranged from 3.61 cM (female parent) to 2.97 cM (male parent). Markers that revealed polymorphism within both parents and showed a 3:1 segregation ratio were used as bridging loci to integrate the 2 parental maps into a biparental consensus map. The integrated map covers 1841 cM over 17 linkage groups, with an average of 54 loci per linkage group, and has an average of 1 marker every 2 cM. Homoeologous relationships among linkage groups were identified in 6 of the 7 predicted homoeologous groups.

Chen *et al.* (1998) constructed the first genetic linkage map of meadow fescue and compared its genomic relationship with tall fescue. Heterologous RFLP markers originally isolated from a tall fescue *Pst-I* genomic DNA library (Xu *et al.* 1991, 1995) were used in the linkage map construction; 66 markers were mapped on 7 linkage groups with a

total length of 280.1 cM. Of those, 33 were mapped in both species, and 70% were located in corresponding linkage groups in meadow fescue and tall fescue. Alm *et al.* (2003) constructed a high-density genetic linkage map of meadow fescue by using a full-sib family of a cross between a genotype from a Norwegian population (HF2) and a genotype from a Yugoslavian cultivar (B14). A total of 550 loci have been mapped by using homologous and heterologous RFLPs, AFLPs, isozymes, and SSRs. The map consists of 466 markers and has a total length of 658.8 cM, with an average marker density of 1 marker every 1.4 cM.

Comparative genome mapping using anchor probe sets for the *Poaceae* family

Anchor probes that can hybridize between various species have become efficient, powerful tools for comparative genome mapping or the study of synteny. van Deynze *et al.* (1998) screened an RFLP marker set for the *Poaceae* family; it was selected from cDNA libraries developed from rice, oats, and barley. A total of 1800 probes were screened on garden blots containing the DNA of rice, maize, sorghum, sugarcane, wheat, barley, and oats, and 152 of them were selected as "anchors" because (1) they hybridized to the majority of target grass species in Southern blot analysis, (2) they appeared to be low- or single-copy sequences in rice, and (3) they helped provide reasonably good genome coverage of all species. The 152 probes were then screened for polymorphism in mapping parents, and polymorphic markers were mapped in rice, oats, maize, and wheat. The use of anchor probes for comparative mapping is an efficient way of establishing genetic relationships for comparisons among all the species and genera being studied (Ahn and Tanksley 1993; Ahn *et al.* 1993; van Deynze *et al.* 1995a, 1995b, 1995c).

Jones *et al.* (2001) conducted the first comparative genome analysis in perennial ryegrass using 109 heterologous anchor RFLP probes from wheat, barley, oats, and rice. The genetic maps of perennial ryegrass and the *Triticeae* cereals are highly conserved in terms of synteny and colinearity. This is supported by the general agreement of the syntenic relationships between perennial ryegrass, oats, and rice and the syntenic relationships between the *Triticeae* and these species. A lower level of synteny and colinearity was observed between perennial ryegrass and oats than in the *Triticeae*, despite the closer taxonomic affinity between these two species. Jones *et al*. (2001) proposed that the linkage groups of perennial ryegrass be numbered in accordance with these syntenic relationships, to correspond to the homoeologous groups of the *Triticeae* cereals.

Sim *et al.* (2005b) constructed an RFLP-based genetic map of ryegrass based on an interspecific population that was derived by crossing perennial ryegrass and Italian ryegrass, for comparative mapping with other *Poaceae* species using heterologous anchor probes. First, a genetic map containing 235 AFLP, 81 RAPD, 160 SSR, 2 isozyme, 16 RFLP, and 2 morphological markers was constructed (Warnke *et al.* 2004). Next, they reconstructed a second linkage map using only RFLP markers for comparative mapping. This map covers a total map distance of 573 cM with a total of 123 loci, including 16 loci previously reported in Warnke *et al.* (2004). Of the 123 loci, 112 were common to the Triticeae consensus linkage map, 82 to the oat linkage map, and 108 to the rice linkage map (Sim *et al.* 2005b). The 7 ryegrass linkage groups were represented by 10 syntenic segments on *Triticeae* chromosomes, 12 syntenic segments on oat chromosomes, and 16 syntenic segments on rice chromosomes, suggesting that the ryegrass genome has a high degree of genome conservation in relation to the *Triticeae*, oats, and rice.

Comparative genome analysis in meadow fescue was conducted by Alm *et al.* (2003) using homologous and heterologous RFLPs. Their results showed conserved syntenic relationships between the meadow fescue linkage groups and the maps of *Lolium* ssp., the *Triticeae*, oats, rice, maize, and sorghum. For *Lolium* ssp., 46 loci on the meadow fescue map have equivalent map locations in perennial ryegrass, representing 62% genome coverage. The meadow fescue maps contain 117 loci with known map locations in the *Triticeae*. Of the 72% of the meadow fescue genome covered by *Triticeae* markers, 94% were orthologous, and a high degree of orthology was observed. The relationship between fescue and oats was inferred from the known relationships between the *Triticeae* and oats (van Deynze *et al.* 1995c). The 48 loci with map locations in oats represented genome coverage of only 48%, of which 90% was orthologous between oats and meadow fescue.

MAPPING IMPORTANT GENES (CHARACTERS) AND QTLs

Recently, some major genes including disease-resistance genes and self-incompatibility genes have been mapped and the markers closely linked to those genes identified. In addition, agriculturally important characters have also been analyzed by QTL analysis.

Mapping of disease-resistance genes and other major genes

Crown rust

Crown rust, which is caused by *Puccinia coronata* f. sp. *lolii*, infests a broad range of hosts, including many genera of forage grasses such as *Lolium*, *Festuca*, *Agropyron*, *Agrostis*, *Paspalum*, *Phleum*, *Poa*, and *Puccinellia* (Smiley *et al.* 1992).

A major resistance gene for crown rust in Italian ryegrass has been demonstrated at the molecular level (Fujimori *et al.* 2003). A resistance-gene locus designated *Pc1* was detected from Yamaiku 130, a breeding line highly resistant to crown rust; 3 AFLP markers were found tightly linked to *Pc1* within a map distance of 0.9 cM, another 3 were found on the opposite side within a distance of 1.8 cM, and AFLP marker ATC-CATG153 co-segregated with *Pc1*. Hirata *et al.* (2003) identified another major resistance gene designated *Pc2* in the cultivar Harukaze. Linkage analysis with Italian ryegrass SSR markers was used to assign these resistance genes into a reference map of Italian ryegrass, and the mapping data suggested that *Pc1* and *Pc2* are located on LG4 and LG6, respectively (Hirata *et al.* 2003). Studer *et al.* (2007) analyzed the QTLs of crown rust resistance in Italian ryegrass. Disease scores obtained through leaf segment test evaluations from glasshouse plants were found to be correlated highly with scores from a multisite field assessment, thus confirming the suitability of this method for crown rust investigations. Two QTLs were consistently detected on LG1 and LG2. Equally in perennial ryegrass, major QTLs mapped to genomic regions known to control crown rust resistance, particularly on LG1 and LG2 (Roderick *et al.* 2000; Thorogood *et al.* 2001; Dumsday *et al.* 2003; Forster *et al.* 2004; Muylle *et al.* 2005). The SSR marker LPSSRH03F03 (Jones *et al.* 2002b), which is closely linked to crown rust resistance in perennial ryegrass (Dumsday *et al.* 2003), was mapped in the Italian ryegrass population at position 68 cM on LG2, clearly separate from the major QTL located at one end of this linkage group.

Dumsday *et al.* (2003) demonstrated a major-effect locus in perennial ryegrass by using bulked segregate analysis and QTL analysis: a resistance gene locus, *LpPc1*, conferring a major effect, was located on LG2. Comparative genetic analysis revealed a conserved syntenic relationship between LG2 of perennial ryegrass and linkage group B of *Avena*, which is the location of a cluster of genes for resistance to crown rust (Yu and Wise, 2000). Roderick *et al.* (2002) also identified some QTLs for crown-rust resistance by using an F_2 perennial ryegrass mapping family and 4 single-pustule isolates. By using crown-rust isolates 1 and 2, two QTLs with moderate effect were identified on LG5 and 2 QTLs with minor effect were identified on LG2 and LG3.

By using isolates 3 and 4, they detected 2 QTLs on LG5 and 2 QTLs on LG6 that had moderate effect. At least 9 additional putative QTLs exhibiting minor effects were identified by using these isolates. Muylle *et al.* (2005a, 2005b) detected 4 QTLs for crown-rust resistance that explained 45% of the variations; these 4 loci were located on 2 different linkage groups. The locus with the strongest effect explained 30% of the phenotypic variation. Sim *et al.* (2005a) evaluated the phenotypic segregation of crown-rust resistance at 2 geographically and environmentally different locations. Two common QTLs were detected on LG2 and LG3, and 2 location-specific QTLs were detected on LG6 and LG7.

Blast or gray leaf spot resistance

Blast, also called gray leaf spot, is a serious fungal disease recently reported on ryegrass. It is caused by *Pyricularia* sp., which also causes rice blast and many other grass diseases. Miura *et al.* (2005) screened EST-derived CAPS and AFLP markers linked to a gene for resistance to ryegrass blast in Italian ryegrass. They analyzed the segregation of resistance in an F_1 population derived from a cross between a resistant and a susceptible cultivar; the results suggested that resistance is controlled by a single dominant gene (*LmPil*) located on LG5 of Italian ryegrass. Of the 30 EST-CAPS markers screened, 1 marker, p56, flanking the *LmPil* locus was identified. The restriction pattern of p56 amplification showed a unique fragment corresponding to the resistance allele at the *LmPil* locus.

Curley *et al.* (2005) used an Italian × perennial ryegrass population to analyze the QTLs for gray leaf spot resistance. This mapping population consisted of 156 progenies derived from the cross between 2 heterozygous ryegrass clones, MFA and MFB (Warnke *et al.* 2004). The inoculation test was performed in a greenhouse using 2 strains: one was isolated from ryegrass, and the other was rice-infecting. A total of 3 QTLs that most strongly affected gray leaf spot resistance were detected: the single QTL detected by using the ryegrass isolate was located on LG3 of the MFB parent, explaining between 20 and 37% of the phenotypic variance; the 2 QTLs detected by using the rice-infecting isolate were located on LG2 of the MFA parent and LG4 of the MFB parent, each explaining about 10% of the phenotypic variance. The QTLs on LG3 and LG4 appear syntenic to blast resistance loci in rice. For the QTL on LG3 of the MFB parent, there are several syntenous blast resistance genes and QTLs in rice. Major blast resistance genes *Pi-t* and *Pi-24(t)* (Sallaud *et al.* 2003), *Pi-sh*, and *Pi-27(t)* (Zhu *et al.* 2004) have been mapped to rice chromosome 1, which is syntenic with perennial ryegrass LG3 (Jones *et al.* 2002a; Sim *et al.* 2005a).

Other resistance genes and traits

Bacterial wilt caused by *Xanthomonas translucens* pv. *graminis* (*Xtg*) is a major disease of ryegrasses and fescues. Studer *et al.* (2006) detected a major QTL of wilt resistance by using a 2-way pseudo-testcross population consisting of 306 F_1 individuals. This population was derived from a reciprocal cross between 2 highly heterozygous Italian ryegrass genotypes with contrasting levels of resistance to bacterial wilt. Highly correlated data between trial locations demonstrated the suitability of glasshouse screens for phenotypic selection. A high-density genetic linkage map including 368 markers (AFLP and SSR) was constructed. QTL analysis of both glasshouse and field resistance data was performed and a single major QTL and a minor QTL were observed on LG4 and LG5, respectively.

In perennial ryegrass, the 2 self-incompatibility loci map to LG1 and LG2, respectively, in accordance with the *Triticeae* consensus map. The *S* and *Z* loci of perennial ryegrass show conserved synteny with the equivalent loci in rye. On the other hand, when self-compatibility (SC) was investigated in an F_2 family, distorted segregation ratios of markers on LG5 were found, indicating the possible presence of a gametophytic *SC* locus. Interval linkage analysis of pollen compatibility after selfing confirmed that this distortion was due to a locus (*T*) analogous to the *S5* locus of rye (Thorogood *et al.* 2002, 2004).

Seedling root florescence (SRF) is an important indicator for distinguishing between perennial ryegrass and Italian ryegrass; it was mapped to LG1 by using an annual × perennial ryegrass (MFA × MFB) pseudo-F_2 population (Warnke *et al.* 2004).

QTL analyses

Recently, many quantitative traits, including yield, plant height, heading date, disease resistance, and stress tolerance, have been studied in several forage species. The results of QTL analyses provide basic information for quantitative gene targeting, cloning, and gene isolation, and they also provide markers for marker-assisted selection of important agronomic characters.

Inoue *et al.* (2004b) reported on the chromosomal positions and the contribution of putative QTLs affecting lodging resistance and related traits in Italian ryegrass. Traits included 7 quantitative characters – heading date, plant height, culm weight, culm diameter, culm strength, tiller number, and culm pushing resistance. They evaluated lodging scores in the field in a 2-way pseudo-testcross F_1 population. Their results revealed 17 QTLs on 6 of the 7 linkage groups for all traits except culm weight. This was accomplished by simple interval mapping using a cross-pollination algorithm. Thirty-three independent QTLs were also detected by composite interval mapping from both male and female parental linkage maps. In addition, up to 18 QTLs for lodging scores evaluated at 9 different times were detected on all linkage groups.

Xiong *et al.* (2006) conducted a QTL analysis of fiber components and crude protein using an annual × perennial ryegrass interspecific hybrid population. Fiber components (neutral detergent fiber [NDF] and acid detergent fiber [ADF]), acid detergent lignin (ADL), and crude protein (CP) were included in the study. Fiber components were all correlated positively with each other and were negatively correlated with CP. A total of 63 QTLs were detected from both the female and male maps for the 4 traits measured over 3 harvests. Coincident QTLs were detected on linkage groups LG2, LG6, and LG7 for NDF; on LG1, LG2, and LG7 for ADF; on LG6 and LG7 for ADL; and on LG2 for CP. Coincident QTLs were also detected on LG2, LG6, and LG7 for NDF and ADF, providing evidence of the genetic basis of the observed high level of phenotypic correlation. The QTLs on LG2, LG6, and possibly LG7 for the fiber components were co-located on the same linkage group as several lignin biosynthetic genes from perennial ryegrass.

Humphreys *et al.* (2003) reported QTLs in perennial ryegrass for forage quality, including water-soluble carbohydrate content (WSC), neutral detergent fiber (NDF), plant size, leaf extension rate, and regrowth rate. QTLs were found on all 7 linkage groups for morphological and growth traits, and explained between 23% and 40% of the phenotypic variation in the traits. QTLs that explained around 20% to 25% of trait variation for nutritive quality traits (total WSC, crude protein, and NDF) were found on 4 linkage groups. QTLs for individual WSC components were found on 6 linkage groups. The QTL for total WSC usually coincided with the QTL for high-molecular-weight fructan. The QTL for NDF coincided with the QTL for WSC on LG1 and LG2.

Armstead *et al.* (2004) identified a genomic region with a major effect on heading date in perennial ryegrass and identified the orthologous region in the fully sequenced rice genome. By comparing the relative positions of RFLP probes CDO545, RZ144, R2869, and C764 in perennial ryegrass and rice, they found that this region of perennial ryegrass LG7 showed synteny with rice chromosome 6, which covers the region of the rice genome containing the

Hd3 locus (Yamamoto *et al.* 1998) or the *Hd3a* and *b* loci (Monna *et al.* 2002). QTL analysis revealed the genotype–trait association between C764 on LG7 of perennial ryegrass and days-to-heading. This major QTL represented up to 70% of the variance of heading date in perennial ryegrass.

Cogan *et al.* (2005) reported QTLs for 5 herbage quality traits, including crude protein content, estimated *in vivo* dry-matter digestibility, NDF content, estimated metabolizable energy, and water-soluble carbohydrate content in perennial ryegrass. They measured these through near-infrared reflectance spectroscopy analysis in the p150/112 reference genetic mapping population (183 individuals, Jones *et al*. 2002a). The samples were prepared for herbage quality analyses from individual plants at 6 different times or locations. A total of 42 QTLs from 6 different sampling experiments varying by developmental stage (anthesis or vegetative growth), location, or year were detected, and some coincident QTLs were detected on LG3, LG5, and LG7.

Jensen *et al.* (2005a) mapped the QTLs for the vernalization response in perennial ryegrass. A total of 5 QTLs for the vernalization response, measured as days-to-heading, were identified and mapped to LG2, LG4, LG6, and LG7. These QTLs each explained 5.4% to 28.0% of the total phenotypic variation, and the overall contribution of these 5 QTLs was 80% of the total phenotypic variation.

Humphreys *et al.* (2004) mapped the gene for drought-resistance transferred from *Festuca* into *Lolium*. Following the initial hybridization of a synthetic autotetraploid of Italian ryegrass ($2n = 4x = 28$) with *F. glaucescens*, the F_1 hybrid was backcrossed twice onto diploid Italian ryegrass ($2n = 2x = 14$) to produce a diploid *Lolium* genotype with a single *F. glaucescens* introgression located distally on the nucleolar-organizer region arm of chromosome 3.

Moore *et al.* (2005) transferred a mutant "stay-green" gene conferring a disrupted leaf-senescence phenotype from an *F. pratensis* chromosome segment into Italian ryegrass. The genetic location within the introgressed *F. pratensis* segment of the senescence gene was mapped by using 29 AFLPs generated by 22 selected primer combinations. The final genetic distance of the *F. pratensis* chromosome segment between the terminal *F. pratensis*-derived AFLP markers was estimated to be 19.8 cM, and the stay-green gene (*sid*) mutation was located at 9.8 cM. The closest flanking markers to *sid* were at 0.6 cM and 1.3 cM.

CONCLUSIONS AND PERSPECTIVES

As mentioned above, several species in *Lolium* and *Festuca* are widely used as animal fodder (hay, pasturage, silage), and for turf and erosion control. The genomic study of these forage crops has lagged behind that of other major crops because of their out-crossing nature, relatively large genomes, and relatively low economic value compared with other crops. However, many countries have undertaken studies of the breeding and mapping of forage crops using molecular markers in the last decade, and of forage crops studied, perennial ryegrass, Italian ryegrass, tall fescue, and meadow fescue have received special attention as well as other important legumes including alfalfa, *Medicago truncatula*, and clover. Researchers have reported many achievements in this field: the development of several kinds of molecular marker; construction of high-density molecular maps (both genetic and physical); generation of a large number of ESTs; and QTL analysis of important agricultural traits.

Until now, no any genes have been isolated through map-based cloning in *Lolium* and *Festuca*; however, the basic tools for map-based cloning in ryegrass are complete. BAC libraries have been constructed for both Italian ryegrass and perennial ryegrass (Fujimori *et al.* 2004; Farrar *et al*. 2007). Many RFLP and SSR markers are mapped on the seven linkage groups (Jones *et al*. 2001; Inoue *et al*. 2004a; Hirata *et al*. 2006), and more than 50 000 ESTs have been developed by various institutes (Sawbridge *et al*. 2003; Ikeda *et al*. 2004). Theses information and resources allow us to isolate important genes such as disease resistant genes in *Lolium* and *Festuca* in the future. And for isolate genes in *Lolium* and *Festuca*, more efficient, larger number of molecular markers covers all genome regions will be needed to develop, for example, SNP marker.

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JAPANESE ABSTRACT

Lolium 属の 8 草種の中でイタリアンライグラス(*Lolium multiflorum* Lam.)は最も重要な寒地型牧草の一つであり、日本で最も広く栽培されている一年生牧草である。*Lolium* 属の中でもう一つ重要な草種であるペレニアルライグラス(*L. perenne* L.)は主にイギリス、ヨーロッパ大陸、アメリカ、オーストラリアおよびニュージーランドで牧草や芝草として栽培されている。イタリアンライグラスとペレニアルライグラス両草種ともに他殖性で、比較的大きいゲノムサイズ(1 C ≈ 2000 Mb)を持っている。最近では数種類の分子マーカー、例えば、増幅断片長多型（AFLP）、制限酵素断片長多型（RFLP）、単純配列反復（SSR）および EST（expressed sequence tag）マーカーが両草種において開発され、それらを用いた連鎖地図も作製されている。加えて、冠さび病、いもち病および青枯病（bacterial wilt）等病害抵抗性遺伝子に緊密に連鎖する分子マーカーも検出されている。さらに、開花期、越冬性、飼料品質およびその他の重要形質の量的形質遺伝子座（QTL）解析も行われている。ここでは *Lolium* 属におけるゲノムマッピングおよび QTL 解析の最近の成果についてまとめる。また、*Lolium* 属に近縁の二草種、トールフェスク（*Festuca arundinacea* Schreb.）およびメドウフェスク（*F. pratensis* Huds.）についてのゲノム解析の成果も紹介する。