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ORIGINAL PAPER

Development of simple sequence repeat (SSR) markers and construction of an SSR-based linkage map in Italian ryegrass (*Lolium multiflorum* Lam.)

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Abstract In order to develop simple sequence repeat (SSR) markers in Italian ryegrass, we constructed a genomic library enriched for (CA)n-containing SSR repeats. A total of 1,544 clones were sequenced, of which 1,044 (67.6%) contained SSR motifs, and 395 unique clones were chosen for primer design. Three hundred and fifty-seven of these clones amplified products of the expected size in both parents of a two-way pseudo-testcross F_1 mapping population, and 260 primer pairs detected genetic polymorphism in the F_1 population. Genetic loci detected by a total of 218 primer pairs were assigned to locations on seven linkage groups, representing the seven chromosomes of the haploid Italian ryegrass karyotype. The SSR markers covered 887.8 cM of the female map and 795.8 cM of the male map. The average distance between two flanking SSR markers was 3.2 cM. The SSR markers developed in this study will be useful in cultivar discrimination, linkage analysis, and marker-assisted selection of Italian ryegrass and closely related species.

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Introduction

Italian ryegrass (IRG, *Lolium multiflorum* Lam.) is one of the most important forage grasses and is widely cultivated in Asia, North and South America, Europe, and New Zealand. It is the most widely cultivated annual forage grass in Japan. Genetically, IRG is an outcrossing species and is generally observed as a diploid taxon with the chromosome constitution $2n = 2x = 14$.

Molecular markers such as restriction fragment length polymorphism (RFLP), randomly amplified polymorphic DNA (RAPD) (Hayward et al. 1994, 1998), amplified fragment length polymorphism (AFLP) (Bert et al. 1999), and simple sequence repeat (SSR) markers (Jones et al. 2001, 2002a, b) have been used for establishing genetic maps in perennial ryegrass (PRG, *L. perenne* L.). However, only RFLP and AFLP markers have been used in linkage map construction in IRG (Inoue et al. 2004). SSRs, or microsatellites, are tandemly repeated units of 2–6 nucleotides (for example (CA)n). They are widely distributed in eukaryotic genomes, highly abundant and highly polymorphic. Variation in the repeat number of SSR units

can be detected in PCR product lengths. Although the development of SSR markers is time-consuming and costly, they have advantages over RFLP, RAPD, and AFLP markers, such as being PCR-based, multi-allelic, highly reproducible, and co-dominant. Due to the complicated nature of detecting techniques of RFLP and AFLP, the development of SSR markers in IRG and assignment of these markers to an IRG linkage map is required.

There are several methods of developing SSR markers. The classic method is screening of genomic libraries using SSR motifs as probes, but this method usually has very low efficiency for generation of SSR markers. The use of SSR-enriched libraries can give a very high rate of SSRs containing clones, but also gives a very high rate of redundancy. Computational approaches that search for SSR-containing sequences in public databases are also used (Wang et al. 1994; Cardle et al. 2000). As a result, SSR markers have been developed in many plant species, including rice (Wu and Tanksley 1993), maize (Taramino and Tingey 1996), wheat (Röder et al. 1995), barley (Ramsay et al. 2000), sorghum (Peng et al. 1999), PRG (Jones et al. 2001), tall fescue (Saha et al. 2005), and timothy (Cai et al. 2003). SSRs have been used in many studies such as linkage map construction (Röder et al. 1998), gene tagging (Fahima et al. 1998), map-based cloning (Liu et al. 2002), genetic diversity (Chen et al. 2002) and evolutionary studies (Buchanan et al. 1994).

In this paper, we describe the development of SSR markers using a (CA)n-enriched SSR library and construction of an SSR-based linkage map using a two-way pseudo-testcross (Grattapaglia and Sederoff 1994) F_1 population in IRG.

Materials and methods

Plant materials

For linkage analysis of SSR markers, a two-way pseudo-testcross F_1 population consisting of 60 individuals derived from a single cross between two IRG individuals, 11S2 (a male-sterile plant carrying the CMS cytoplasm, as female parent) and 11F3 (a male-fertile plant carrying the CMS cytoplasm, as male parent) was used. Genomic DNA of all plants was extracted from young leaves by using the CTAB method (Murray and Thompson 1980). To test the cross-species amplification of IRG SSRs in related species, the DNA from one random selected individual of commercial variety Kiyosato (PRG), Nannryou (tall fescue, TF), and Tomosakae (meadow fescue, MF) were used.

Construction of SSR-enriched libraries and sequencing of SSR-enriched clones

An SSR genomic library enriched for CA repeats was produced by Genetic Identification Services (GIS, Chatsworth, CA, USA). The library was constructed from a single individual randomly selected from progeny of IRG breeding line CL1. The enriched DNA fragments were cloned into the *Hin*dIII cut site of the pUC19 plasmid. The recombinant plasmids were transformed into Competent High JM109 *Escherichia coli* cells (Toyobo, Tokyo, Japan), which were then plated onto LB agar plates containing 50 μg/ml ampicillin, 100 μg/ml X-galactosidase, and 0.5 mM isopropyl βD-thiogalactopyranoside. The plates were incubated overnight at 37°C. Only white colonies were used for further analysis. The inserts were amplified by colony PCR. PCR was carried out in 10 μl reaction mix containing 1.0 μl 10 × ExTaq buffer (20 mM Mg^{2+}), 0.8 μl 2.5 mM each dNTP, 0.16 μl 100 μM p7 primer (5′-CGCCAGGGTTTTCCCAGTCACGAC-3′), 0.16 μl 100 μM p8 primer (5′-AGCGGATAACAATTTCACACAGGAAAC-3′), and 0.025 μl 5 U/μl Takara Ex Taq (Takara Bio Inc., Tokyo, Japan). The PCR conditions were as follows: 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 60 s; 72°C for 5 min; and holding at 4°C.

The colony PCR products were purified on a MultiScreen-FB plate (Millipore, Billerica, MA, USA), and then directly sequenced using the SQ1 primer (5′-ACGACGGCCAGTGAATTCGA-3′) or the SQ3 primer (5′-AGGAAACAGCTATGACCATGATTAC-3′) with a BigDye Terminator Cycle Sequencing Kit 2.0 (PE Applied Biosystems, Foster City, CA, USA). The PCR conditions for sequencing were as follows: 96°C for 1 min; 35 cycles of 96°C for 10 s and 60°C for 4 min, and holding at 4°C. After purification of the products with a MultiScreen-HV plate, the sequence was carried out on an ABI377 sequencer according to the protocol of PE Applied Biosystems.

Primer design and SSR analysis

Before the design of amplification primers, the primary sequences were processed as follows: (1) Selected sequences including an SSR region (≥ 8 repeats). (2) Deleted vector sequences. (3) Excluded clones that are unsuitable for primer design: either the 5′ or the 3′ sequence flanking the SSR motif was too short (< 20 bp); either the 5′ or the 3′ sequence flanking the SSR motif could not be sequenced; the GC contents of the primer design regions were too low (< 30%); or the SSR motifs were scattered. (4) Connected forward and

reverse sequences with AutoAssembler software (PE Applied Biosystems). (5) Eliminated redundant sequences (≥ 95% similarity) with DNASIS v3.5 software (Hitachi Software Engineering Co., Ltd., Tokyo, Japan). For the clones suitable for primer design, two forward and two reverse primers were designed with PRIMER 0.5 software (Lander, Cambridge, MA, USA). The parameters used in the primer design were as follows: (1) primer length between 18 and 30 bp; (2) T_m values of the primers between 60 and 70°C (optimal T_m is 65°C); (3) percentage of GC between 40% and 60% and (4) predicted PCR products from 100 to 450 bp.

Four combinations between the above two forward and two reverse primers were tested in the two parents (11S2 and 11F3) for mapping. PCR for SSR amplification was carried out in 10 µl reaction mix containing 20 ng genomic DNA, 1.0 µl GeneAmp 10 × PCR Gold Buffer, 1.0 µl 25 mM $MgCl_2$, 0.8 µl GeneAmp dNTP Mix (2 mM each dNTP), 3.2 µl 5 µM each forward and reverse primer, and 0.05 µl 5 U/µl AmpliTaq Gold (PE Applied Biosystems). The PCR conditions were as follows: 95°C for 10 min; 35 cycles of 94°C for 30 s, 60 or 55°C for 30 s; 72°C for 7 min, and holding at 4°C. Electrophoresis was carried out on a GenePhor Electrophoresis Unit (Amersham Biosciences, Freiburg, Germany) with a GeneGel Clean 15/24 Kit (Amersham Biosciences) according to the user's manual. The electrophoresis conditions were 25°C, 200 V, 12 mA, 3 W for 10 min, then 500 V, 15 mA, 8 W for 80 min. After staining with Vistra Green, PCR products were detected with a Molecular Imager FX (Bio-Rad, Hercules, CA, USA). Primer combinations that gave a good signal in the parents were selected and used for the analysis of the F_1 mapping population. The primers selected for mapping were fluorescence-labeled with FAM or HEX. The PCR conditions using fluorescence-labeled primers were as follows: 95°C for 10 min; 35 cycles of 94°C for 30 s, 60 or 55°C for 30 s, and 72°C for 60 s; 72°C for 7 min, and holding at 4°C. Electrophoresis was carried out on an ABI377 sequencer (PE Applied Biosystems), and the data were collected and scored using GeneScan and Genotyper software (PE Applied Biosystems).

Genetic mapping of SSR markers

A high-density genetic linkage map based on AFLPs and RFLPs using 124 individuals from the 11F2 × 11S3 population had already constructed and the AFLP linkage map consists of seven linkage groups (LG), corresponding to the seven cytological chromosomes (Fujimori et al. 2000). In this study, the first 60 individuals of above AFLP mapping population were used for mapping SSR markers. To integrate SSR markers into this linkage map, we selected 129 AFLP markers (including terminal markers on all LG) spaced about 10 cM apart and all RFLP markers from the two parental linkage maps to construct a framework genetic map.

The conformance of SSR marker segregation in the F_1 population to Mendelian inheritance was analyzed using chi-square statistics. Segregating markers were classified into three segregation types: (1) band present in female parent (11S2) and absent in male parent (11F3) and segregating 1:1 (ab × aa); (2) band absent in female parent (11S2) and present in male parent (11F3) and segregating 1:1 (aa × ab) and (3) band present in both parents and segregating 3:1 (ab × ab; the genotypes of bb and b- could not be distinguished.). The linkage maps were constructed from type 1 and 3 SSR markers, selected AFLP markers and RFLP markers for the female (11S2) map, and from type 2 and 3 SSR markers, selected AFLP markers and RFLP markers for the male (11F3) map. Multiple bands derived from the same primer pair and deduced to be obtained from the same genetic locus on the basis of exclusive segregation and map location were considered to be co-dominant markers (ab × ab; the genotypes of bb and b- could be distinguished, ab × ac, ab × cd). The linkage map was constructed with JoinMap software (Stam 1993) at LOD = 4.0, and map distances were calculated with the Kosambi mapping function (Kosambi 1944). The linkage map was drawn using MapChart 2.1 (Voorrips 2002).

Results

Characterization of the SSR-enriched libraries and clones sequenced

Of 1,544 clones sequenced in total, 1,044 (67.6%) contained SSR motifs (Table 1). Among them, 202 clones were unsuitable for primer design for the following reasons: in 114 clones, either the 5′ or the 3′ sequence flanking the SSR motif was too short (< 20 bp); in 79 clones, either the 5′ or the 3′ sequence flanking the SSR motif could not be sequenced; in other 9 clones, the GC contents of the primer design regions were too low (< 30%). The remaining 842 clones were suitable for design of primer, and of this set, 447 clones showing ≥ 95% similarity to other clones were not used. In total, 395 unique clones (25.6%) were used to design primers.

The 395 unique SSR clones were classified into four categories following Jones et al. (2001)—perfect, imperfect, interrupted, and compound (CA and other

Table 1 Clones sequenced and SSR markers developed in Italian ryegrass

	Number	Percentage[a]
Clones sequenced	1,544	100.0
Clones containing SSR motif	1,044	67.6
Clones suitable for primer design	842	54.5 (80.7)
Unique SSR colnes	395	25.6 (46.9)
Working primers	357	23.1 (90.4)
Polymorphic primers in the F_1 population	260	16.8 (72.8)

[a] Percentage of clones sequenced. Values in parentheses are the percentages of the above item

motif) repeat by SSR motif types. The frequency and repeat number of each motif are shown in Table 2. The library consisted of 91.3% CA-repeat-containing clones in all four categories (30.9, 13.4, 31.6, and 15.4%). In addition two types of trinucleotide repeat, CAA and CAT (7.4%), were found in the library (2.5, 0.3, 4.3, and 0.3% in four categories, respectively). The repeat number of trinucleotides (27.8) was higher than that of dinucleotides (20.7), unlike that reported in PRG (17.0 vs. 19.0, Jones et al. 2002a). The proportion of clones containing perfect repeats (33.3%) was lower than that reported in PRG (54%, Jones et al. 2002a) and in timothy (78.1%, Cai et al. 2003). The average number of CA repeat units (21.4) was slightly larger than that in PRG (19.0, Jones et al. 2002a).

Primer design, polymorphism survey and cross-species amplification of IRG SSRs to other related species

Two forward and two reverse primers were designed for each unique clone, and the best primer combinations that could amplify products in expected size in the parents were chosen. Of the 395 unique clones, sequences from 357 could be amplified as SSR products in the parents by using at least one pair of primers. In addition, using these 357 SSR primers, we found that about 67% could be amplified in PRG, 50% in MF, and 71% in TF (see Electronic Supplementary Material).

These 357 primer pairs were used to screen polymorphisms between the two parents of the F_1 mapping population, and 260 primer pairs detected polymorphism. Finally, the loci amplified by 218 primer pairs were mapped on the seven IRG LG (see details in next section), in these 218 primer pairs, 137 pairs amplified less than three bands in the parents of the F_1 mapping population and they amplified a single locus in the mapping population. In contrast, the other 81 primer pairs amplified more than two bands in the parents, of these, 20 pairs were mapped as single locus and other 61 pairs were mapped as multiple loci on different LG.

The information for the 218 primer pairs mapped on the seven IRG LG is shown in the Electronic Supplementary Material.

Genetic mapping of SSR markers

Twenty-eight out of 260 primer pairs were not used for linkage analysis because of amplification of indistinct bands. The other 232 primer pairs generated 429 polymorphic markers in the F_1 population. Of these 429 polymorphic markers, 134, 140, and 155 markers were classified into the segregation types 1, 2, and 3 described as in the Materials and methods section, respectively (Table 3). Linkage analysis was performed using 289 SSR markers (188 primer combinations) consisting of types 1 and 3 for the 11S2 map based on 79

Table 2 Frequency and repeat numbers of SSR motifs in the unique clones

SSR category	SSR motifs	Number (%[a]) of primer pairs screened	Repeat number average (range)	Number (%[b]) of primer pairs showing successful amplification	Number (%[c]) of polymorphic primer pairs
Perfect SSRs	CA	122 (30.9)	15.8 (8–41)	112 (91.8)	88 (78.6)
	CAA	10 (2.5)	28.3 (17–44)	10 (100.0)	7 (70.0)
Imperfect SSRs	CA	53 (13.4)	15.7 (8–43)	48 (90.6)	35 (72.9)
	CAA	1 (0.3)	17.0 (17)	1 (100.0)	0 (0.0)
Interrupted SSRs	CA	125 (31.6)	23.9 (11–77)	110 (88.0)	80 (72.7)
	CAA	17 (4.3)	27.8 (10–71)	17 (100.0)	10 (58.8)
	CAT	1 (0.3)	37.0 (37)	1 (100.0)	0 (0.0)
	Other	3 (0.8)	35.0 (9–50)	1 (33.3)	1 (100.0)
Compound SSRs	CA + other	61 (15.4)	29.0 (8–70)	55 (90.2)	37 (67.3)
	Other	2 (0.5)	20.0 (15–25)	2 (100.0)	2 (100.0)
Total		395	21.4 (8–77)	357 (90.4)	260 (72.8)

[a] Percentage of unique clones

[b] Percentage of primer pairs screened

[c] Percentage of successful amplification

selected AFLP markers and 56 RFLP markers; and 295 SSR markers (194 primer combinations) consisting of types 2 and 3 for the 11F3 map based on 79 selected AFLP markers and 53 RFLP markers. Markers in the parental maps were distributed across seven LG, reNSpresenting the seven haploid chromosomes of IRG (Fig. 1). The numbering of LG follows that of PRG (Jones et al. 2002a, b), because synteny between IRG and PRG was confirmed by using Poaceae anchor probes (Van Deynze et al. 1998) and IRG genomic probes (Inoue et al. 2004) in our mapping population (Table 4).

Of the markers derived from the female parent 11S2, 263 SSR markers (126 markers segregating 1:1, 137 3:1), 53 RFLP and 79 AFLP markers were distributed into the seven groups. Of these, 236 SSR markers (116 1:1, 120 3:1), 34 RFLP and 69 AFLP markers were mapped on the 11S2 map, which covers a genome size of 887.8 cM, with an average map distance of 2.6 cM between markers. Of the markers derived from the male parent 11F3, 273 SSR markers (137 1:1, 136 3:1), 51 RFLP markers and 80 AFLP markers were distributed into the seven groups. Of these, 251 SSR markers (134 1:1, 117 3:1), 32 RFLP markers and 78 AFLP markers were mapped on the 11F3 map, which covers a genome size of 795.8 cM, with an average map distance of 2.2 cM between markers. Finally, 378 SSR markers (212 primer combinations), 48 RFLP markers and 121 AFLP markers were mapped on the two parental linkage maps, which cover a genome size of 1,683.6 cM, with an average map distance of 3.1 cM. Large gaps (21.6 and 21.5 cM) appeared on LG 1 of 11S2 and LG 6 of 11S2, respectively. As shown in Table 5, LG 1 had the fewest SSR markers (29), and LG 4 had the most (75); the average number of SSRs mapped per LG was 54.

Genome coverage of SSR markers and comparison of SSR and AFLP based maps

Compared with the high-density AFLP linkage map constructed from the same F_1 population (Fujimori et al. 2000), the maps constructed using the SSR, RFLP and selected AFLP markers in this study cover most regions of the AFLP maps. If we use only the SSR markers to construct linkage maps, they would cover 91.5% (812.1 out of 887.8 cM) of the female linkage map and 91.9% (731.0 out of 795.8 cM) of the male linkage map. However, except in LG 6 of 11F3, the lengths of the linkage maps were extended by the SSR markers at one extreme of each LG at least. And the total length of SSR-based map was 887.8 and 795.8 cM for male and female maps, respectively, larger than the AFLP-based maps (769.9 and 735.8 cM for male and female maps, respectively). In addition, the number of mapped co-dominant (or ab × ab type) bridge SSR markers, 121 (10–29 for each LG, showed in Fig. 1), is about two times of the number of bridge AFLP markers (ab × ab type) in the original AFLP map (64 in total, 4–16 for each LG), the mapping information of these markers will be more useful than AFLP markers in the construction of new maps.

SSR clusters on the linkage maps

Several SSR clusters were found on LG 2, LG 5, LG 6 and LG 7 for both parental maps (Fig. 1). Five SSR markers were mapped to a narrow region of 53.8–54.2 cM on LG 2 of the female map, and ten were mapped to 46.8–53.3 cM on LG 2 of the male map. Six SSR markers were mapped to 67.6–72.3 cM on LG 5 of female map, and seven were mapped to a narrow region of 56.7–58.9 cM on LG 5 of the male map. Nine SSR markers were mapped to 60.8–66.5 cM on LG 6 of female map, and eight were mapped to 59.5–68.6 cM on LG 6 of the male map. Twelve SSR markers were mapped to 49.4–56.8 cM on LG 7 of the female map, and 10 were mapped to 46.9–54.0 cM on LG 7 of the male map.

Discussion

Usefulness of CA repeat-contained SSR markers

The dinucleotide CA is the most commonly observed SSR array in mammalian genomes (Hamada and Kakunaga 1982; Stallings et al. 1991). However, the most common dinucleotide repeats in plants are AT, GA, and then CA (Cardle et al. 2000). CA-enriched SSR libraries were used to develop SSR markers in maize (Sharopova et al. 2002), rice (Wu and Tanksley 1993; Panaud et al. 1995), wheat (Röder et al. 1995), barley (Ramsay et al. 2000), white clover (Kolliker et al. 2001), sunflower (Tang et al. 2002), and timothy (Cai et al. 2003). Tang et al. (2002) have developed 381 SSR markers containing CA repeats in sunflower, and Cai et al. (2003) have developed 276 SSRs containing CA repeats in timothy. Jones et al. (2001) developed about 400 SSRs, mostly derived from CA-containing clones, of which 93 were assigned to genetic map locations. In IRG, we developed 357 SSRs from a CA-enriched SSR library, and mapped 218 on all seven LG of IRG. The results suggest that it is possible to construct a linkage map covering most of the genome by using SSR markers developed from a library enriched for only one SSR motif.

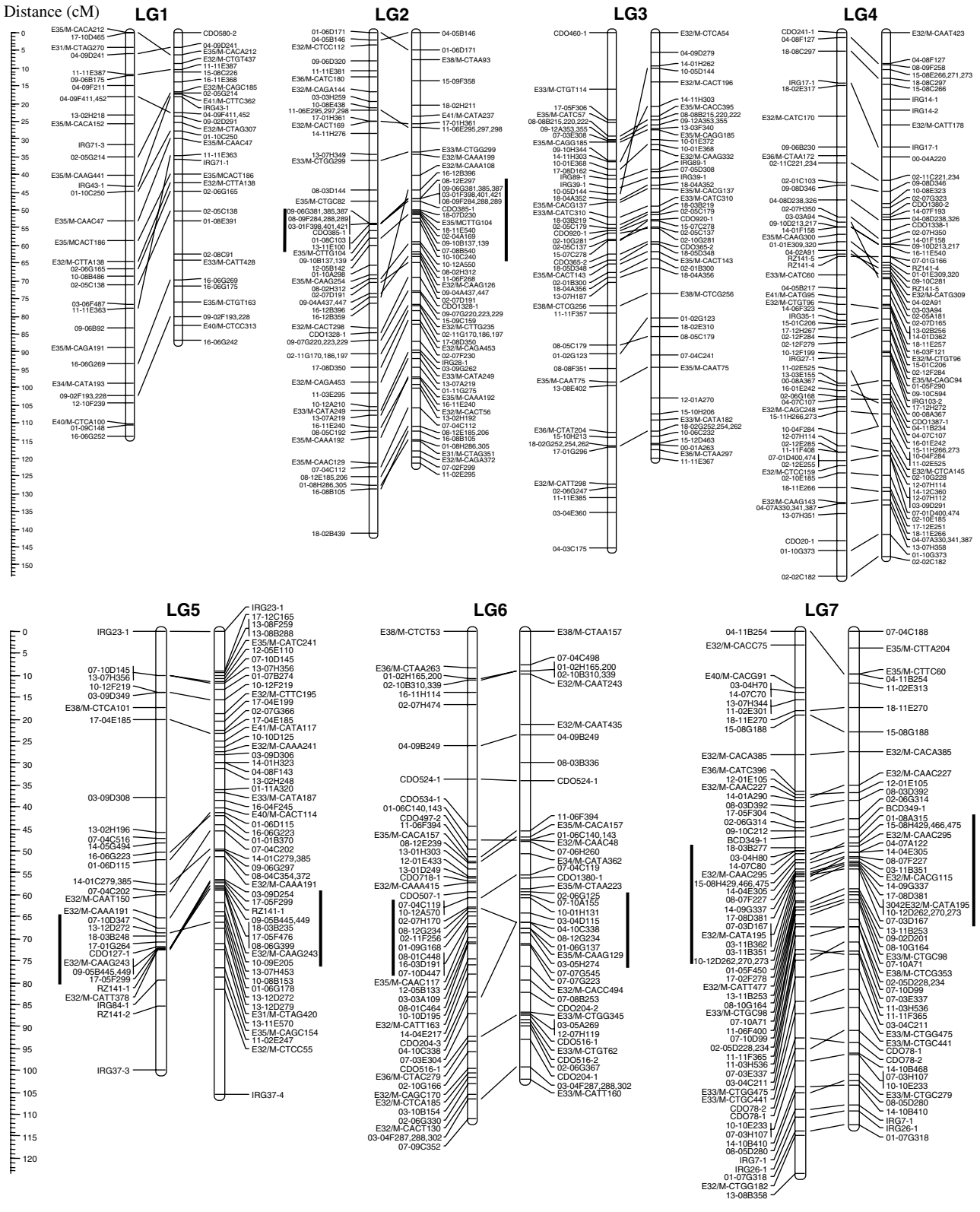

Fig. 1 Linkage map of Italian ryegrass constructed based on the segregation data of SSR, RFLP and selected AFLP markers in a two-way pseudo-testcross F_1 population 11S2 × 11F3. The linkage groups are numbered at the *top*. The female parent map (11S2) is shown on the *left*, and the male parent map (11F3) is shown on the *right* in each pair. IRG genomic RFLP, barley, oat, and rice anchor markers are indicated as IRG*x*, BCD*x*, CDO*x*, and RZ*x*, respectively. AFLP markers are indicated by a combination of *Eco*RI/*Mse*I primer pair and *amplified product size*. SSR markers are indicated by a combination of *clone name* and *amplified product size* (last three digits show size in bp). *Bars* show SSR clusters

Table 3 Number of SSR markers used for the linkage analysis

Categories	Parental genotypes[a] (11S2 × 11F3)	Expected ratios[b] and plant genotypes in progeny	Number of markers	Linked markers		Mapped markers	
				11S map	11F map	11S map	11F map
1	ab × aa	1:1 (ab: aa)	134	126	–	116	–
2	aa × ab	1:1 (ab: aa)	140	–	137	–	134
3	ab × ab	3:1 (bb, b-: aa)	155	137	136	120	117
Total			429	263	273	236	251
				401		378	

[a] b, The presence of distinct marker alleles; a, the absence of marker alleles

[b] Presence versus absence of the marker

Table 4 Comparison of the RFLP's map location in IRG, PRG, and hexaploid wheat

Marker name	Linkage group[a]	IRG[b]	PRG[c]	Hexaploid wheat arms[d]
CDO580	LG 1	LG 1	LG 1	1S
CDO385	LG 2	–	LG 2,7	2S,5L
CDO1328	LG 2	–	LG 2	2L
IRG28	LG 2	LG 2	–	–
CDO365	LG 3	–	LG 2	6S
CDO460	LG 3	LG 3	LG 3	3S
CDO920	LG 3	LG 3	LG 3	2L,3L,7L
IRG39	LG 3	LG 3,4	–	–
CDO20	LG 4	LG 4	LG 4	4L
CDO241	LG 4	LG 5	LG 4	6L
CDO1338	LG 4	–	–	4S,5S
CDO1387	LG 4	LG 4	LG 4	4L
IRG14	LG 4	LG 4	–	–
CDO127	LG 5	–	–	1S,4L,5L
IRG23	LG 5	LG 5	–	–
IRG37	LG 5	LG 5	–	–
IRG84	LG 5	LG 5	–	–
CDO204	LG 6	LG 6	LG 6	–
CDO497	LG 6	LG 6	LG 6	6L
CDO507	LG 6	–	–	6L
CDO516	LG 6	LG 6	LG 6	6
CDO524	LG 6	LG 6	–	6S
CDO534	LG 6	LG 6	–	1S,3LS,6S
CDO718	LG 6	–	–	3L
CDO1380	LG 6,4	LG 6	LG 6	6L
CDO78	LG 7	LG 1	–	7L
BCD349	LG 7	–	LG 7	7S
IRG7	LG 7	LG 7	–	–
IRG26	LG 7	LG 7	–	–

[a] Linkage group of Italian ryegrass in this study

[b] Linkage group of Italian ryegrass in Inoue et al. (2004)

[c] Linkage group of perennial ryegrass in Jones et al. (2002a)

[d] Linkage group of wheat in Van Deynze et al. (1998)

Redundancy of library

Our level of redundancy was 42.8% (447/1,044), almost the same as that detected in the hexaploid timothy (Cai et al. 2003). However, it was higher than that in PRG (16.4%, Jones et al. 2001). Our high level of redundancy could be due to two reasons. (1) The PRG SSR-enriched clone collection contained sequences from both (CA)n-enriched and multiplex repeat enriched libraries, but our IRG SSR library used only CA repeats for enrichment. (2) We sequenced more clones than in PRG. In fact, when only CA-containing clones were counted, the redundancy in PRG was 24.4% (48/197). The redundancy of our library was 22.2% (55/248) when we sequenced the first 248 clones. These two rates are almost identical.

Cross-species amplification of IRG SSRs to other related species

IRG belongs to the *Lolium–Festuca* complex. This complex includes four closely related forage grasses: IRG, PRG, TF, and MF. Most studies suggest that effective cross-amplification is limited to members of the same genus or related genera in both SSR and STS markers (Lagercrantz et al. 1993; Talbert et al. 1994, 1996; Erpelding et al. 1996; White and Powell 1997; Whitton et al. 1997; Peakall et al. 1998; Devey et al. 1999; Echt et al. 1999; Mano et al. 1999). However, in the *Lolium–Festuca* complex, the rate of cross-amplification seems to be high (Jones et al. 2001; Lem and Lallemand 2003; Inoue and Cai 2004; Saha et al. 2004). Jones et al. (2001) demonstrated that about 71% of PRG SSR markers could be transferred to IRG when they tested 100 primer pairs. Inoue and Cai (2004) reported that about 85% of IRG STS markers could be transferred to PRG, 70% to MF, and 82% to TF. Using the SSR primers developed in this study, we found that about 67% could be amplified in PRG, 50% in MF, and 71% in TF (see Electronic Supplementary Material). These results suggest that markers developed from one member of the *Lolium–Festuca* complex can be extensively used in other members of this complex.

Multiple loci amplified by same primer pair

In most diploid species, one SSR primer usually amplifies only one locus. For example, in PRG, the closely related species to IRG, Jones et al. (2002a) reported

Table 5 SSR markers mapped on each linkage group of the F_1 mapping population

Linkage group	Mapped SSR markers			Map length (cM)		SSR marker interval (cM)
	11S2	11F3	Total	11S2	11F3	
LG 1	19	17	29	113.8	86.9	5.4
LG 2	43	44	70	141.2	121.6	3.0
LG 3	32	31	48	145.3	120.0	3.7
LG 4	43	56	75	153.2	147.7	2.9
LG 5	20	41	50	99.8	105.3	2.2
LG 6	33	25	52	111.1	101.9	3.3
LG 7	46	37	54	123.4	112.4	2.8
Total	236	251	378	887.8	795.8	
Average	34	36	54	126.8	113.7	3.2

that in their mapping population, of the 89 primer pairs, 83 produced single-locus amplification patterns, while six (7%) detected more than one locus, with five detecting duplicate loci and one detecting a triplicate locus. In rice, Temnykh et al. (2000) also reported that six (3%) multiple loci in 188 mapped SSR markers. In this study, of 218 SSR primer pairs showing polymorphism between the parents of the mapping population, 81 primer pairs amplified more than two bands in the parents of which 20 pairs were mapped as single locus. Therefore, 61 (28.0%) pairs were mapped as multiple loci on different LG. This rate is very high when compared with that of in PRG. However, most the 61 primer pairs amplifying multiple loci were amplified products with a different size to that expected. The presence of multiple loci amplified by single primer pair may indicate that the partial homologous LG in Italian ryegrass genome. However, using Italian ryegrass genomic RFLP markers, Inoue and Cai (2004) have reported 8.4% (8/95) of RFLP markers were mapped as multiple loci; this rate was very close to the rate of multiple SSR loci reported by Jones et al. (2002a) in PRG. As the amplification of SSR products was affected by many factors such as primer designed, PCR condition, and Taq polymerase used, the primers amplifying multiple loci in this report may need to be redesigned to reduce the number of amplified loci, and the presence of homologous LG in IRG will be need to study.

SSR clusters on the LG

SSR clustering or non-random physical distribution is usually found in linkage maps of plant species (Schmidt and Heslop-Harrison 1996; Ramsay et al. 1999; Elsik and Williams 2001; Tang et al. 2002). Ramsay et al. (2000) reported some SSR clusters in centromeric regions of barley. Our results showed that there are SSR clusters on LG 2, LG 5, LG 6 and LG 7. However, the presence of SSR clusters in the centromeric regions of LG needs to be confirmed.

The population size usually affects marker distance, marker orders and marker clusters in linkage map. For mapping of large numbers of markers, especially RFLP markers, because complicated nature of detecting, sometimes small population size was used, for example, Davis et al. (1999) used 54 individuals of an F_2 population to mapped 1736 RFLP markers. In our study, only 60 individuals of the F_1 AFLP mapping population were used for mapping of the SSR markers; however, the map order of selected AFLP markers detected using 60 individuals and using all of 124 individuals were same (our unpublished data), and this may suggest that SSR marker orders in this study can be reliable. But the SSR marker distance may be changed when using more large mapping population and the SSR clusters still need to confirm by using large mapping population.

In conclusion, we developed 357 SSR markers from a CA-enriched SSR library and mapped 260 SSR loci on the seven LG in IRG with an average distance of 3.2 cM. These SSR markers will be useful in cultivar discrimination, linkage analysis, and marker-assisted selection in IRG and related species such as PRG.

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