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Microsatellite DNA markers for rice chromosomes

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Abstract We found 369 complete microsatellites, of which $(CGG/GCC)_n$ was the most frequent, in 11 798 rice sequences in the database. Of these microsatellites, 35 out of 45 could be successfully converted into microsatellite DNA markers using sequence information in their flanking regions. Thus, the time and labor used to develop new microsatellite DNA markers could be saved by using these published sequences. Twenty eight polymorphic markers between Asominori (japonica) and IR24 (indica) have been correctly mapped on the rice genome and microsatellites appear to be randomly distributed in the rice chromosomes. Integration of these markers with the published microsatellite DNA markers showed that about 35% of the rice chromosomes were covered by the 56 microsatellite DNA markers. These microsatellites were hypervariable and were easily to assay by PCR; they were distributed to all chromosomes and therefore, one can easily select plants carrying desired chromosome regions using these microsatellite DNA markers. Thus, microsatellite maps should aid the development of new breeds of rice saving time, labor, and money.

Key words Microsatellite (simple sequence repeats) · Polymorphisms · Rice · Genetic linkage maps · PCR

Introduction

Genetic linkage maps will contribute greatly to practical breeding programs and genetic research. Recently, in addition to classical genetic maps based on phenotypic and isozyme markers, molecular maps based on RFLP markers have been developed for several crops. For rice, the RFLP map was initially constructed by McCouch et al. (1988). More recently, several RFLP linkage maps, including high-density maps, have been developed (Saito et al. 1991; Causse et al. 1994; Kurata et al. 1994).

These RFLP linkage maps have a number of advantages over classical genetic maps for genetic research and breeding. However, the RFLP technique, based on Southern blotting, is not very convenient in practical breeding programs because it requires large amounts of purified DNAs. Since the number of alleles per locus for RFLPs is limited, it is often difficult to find polymorphisms within a subspecies, and genotypes for breeding materials which can be applied to RFLP are limited (Wu and Tanksley 1993; Rongwen et al. 1995). Therefore, highly polymorphic and easily assayable DNA markers are needed to aid the development of new breeds.

Microsatellites consist of tandemly arrayed di-, tri-, and tetra-nucleotide repeats, which are hypervariable and ubiquitously distributed throughout eukaryotic genomes. Microsatellite DNA markers, which can be directly amplified by PCR, have been developed using the unique sequences that flank microsatellites (Litt and Luty 1989; Tautz 1989; Weber and May 1989). In several crop plants, including soybean (Akkaya et al. 1992), rice (Wu and Tanksley 1993), maize (Senior and Heum 1993), barley (Becker and Heun 1995), wheat (Röder et al. 1995) and brassica (Lagercrantz et al. 1993), specific amplification of microsatellite loci indicated that microsatellite DNA markers are more variable than RFLPs. Thus, microsatellite DNA markers, which are highly polymorphic and easily assayed by PCR with small samples of genomic DNA, should be of value for breeding programs. More recently, in both the human and mouse genome, extremely high-density molecular maps based on microsatellite DNA markers have been constructed (Dib et al. 1996; Dietrich et al. 1996). In addition to the above special feature of microsatellite DNA markers, information about these loci could be quite valuable, because they can be used as co-dominant landmarks of chromosomes.

In general, microsatellites have been isolated from DNA libraries and published sequence-data. In the mouse microsatellite map, 225 of the microsatellites were developed

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from published sequence data (Dietrich et al. 1996). In rice, a few microsatellites have been found in the published sequences (Wu and Tanksley 1993; Inoue et al. 1994; Wang et al. 1994). However, numerous sequence data including the EST (*E*xpressed *S*equence *T*agged) sites of rice are registered in the databank. There may be microsatellites in these sequences which will enable researchers to easily develop new microsatellite DNA markers.

We have been developing several DNA markers via PCR to aid the rice breeding program (Akagi et al. 1995). In the present study, we have found many microsatellites within published sequences, and converted some of them into microsatellite DNA markers. Mapping these markers with the published ones indicated that about 35% of the rice chromosomes were covered with these microsatellite DNA markers.

Materials and methods

Search of the rice sequences in the DDBJ database for microsatellites

A total of 11 798 rice nucleotide sequences were obtained from the DDBJ database (rel. 24). The sequences containing microsatellites were screened for all possible types of di-, tri-, and four types of tetra-nucleotide repeats, as shown in Table 1. The homology searches of the rice squences for these repeats were performed using the FASTA computer program.

Plant materials and isolation of DNA

Seventy one of the recombinant inbred lines, together with the parental lines Asominori and IR24, were used for mapping polymorphisms of microsatellites (Tsunematsu et al. 1993; Tsunematsu et al. 1996). The seeds of these lines were kindly provided by Dr. Atsushi Yoshimura (Kyusyu University). Crude DNA was extracted from the leaf tissue of parental and recombinant inbred lines according to the method described by Edwards et al. (1991).

Amplification of microsatellites and detection of their polymorphisms

The nucleotide sequences of the primer pairs used for each microsatellite are shown in Table 2. For amplification of published microsatellites, primers were synthesized according to the data in the original reports (Wu and Tanksley 1993; McCouch et al. 1995).

PCR amplification was performed in 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.5 or 1 unit of TAKARA *Taq* (TAKARA), 4 nmole of dNTP, 10 pmole of primer, with 10 ng of genomic DNA per 20 μl using a Thermal Cycler 9600 (Perkin-Elmer). Thirty five PCR cycles were performed, with 30 s of denaturation at 94°C, 30 s of annealing at 55°C, and 1 min of polymerization at 72°C. Polymorphisms in the PCR products were detected by ethidium bromide staining after electrophoresis on 3% MetaPhor agarose gels (FMC).

Construction of a linkage map

Polymorphisms in each of the microsatellites were typed according to the recombinant inbred population which consisted of 71 plants. This population was used to construct an RFLP/RAPD linkage map (Tsunematsu et al. 1993; Tsunematsu et al. 1996). Linkage analysis was performed with the RFLP mapping data using Mapmaker version 3.0 (Lander et al. 1987). Mapping-data for the RFLPs of this population were supplied by the Rice Genome Research Program of Japan.

Results and discussion

Abundance of microsatellites in rice nucleotide sequences

New microsatellites were screened from published rice sequences in the DDBJ databank, because numerous sequence data, including that of EST sites, is available in this database. Homology searches were performed with possible di-, tri-, and with four types of tetra-nucleotide repeats. Sequences which included complete nucleotide repeats over 20 bp were selected. A number of them are shown in Table 1. Out of 11 798 sequences, 369 contained complete repeats.

Most types of repeat sequence, except $(GC/CG)_n$, $(AGT/TCA)_n$ and $(GACC/CTGG)_n$, were found. The sequence containing $(CGG/GCC)_n$ was the most frequently present in the published rice sequences, followed by $(GAG/CTC)_n$ (Table 1). Poly (CGG) was abundant and found throughout the rice genome (Zhao and Kochert 1992). The results observed here might represent this feature of the rice genome. Most $(CGG/GCC)_n$ repeats were found in the EST sites, though plaque hybridization with oligonucleotides revealed no poly(CGG) in the cDNAs (Panaud et al. 1995). As discussed by Panaud et al. (1995), this difference may be brought about by the washing stringency used for a very high GC% probe.

Only a limited number of the published rice sequences contained the $(AT/TA)_n$ sequence, although it is generally abundant in plant genomes (Lagercrantz et al. 1993; Wang et al. 1994). In the rice sequences, $(AT/TA)_n$ was found in intron or 5′ non-coding regions. Since most of the sequences analyzed here were EST sites, which have a high GC%, few sequences containing repeats consisting of A or T were found.

Sequence information on both regions flanking the microsatellites is required to convert microsatellites into DNA markers. Many sequences containing microsatellites and their flanking sequences were available. Therefore,

Table 1 Frequencies of microsatellites in the rice sequences registered in the database

Repeat unit	Number of sequences	Repeat unit	Number of sequence
CGG/GCC	137	GA/CT	55
GAG/CTC	67	AT/TA	5
CTT/GAA	26	GT/CA	2
CTG/GAC	25	GC/CG	0
ACG/TGC	20		
TGG/ACC	18	GATA/CTAT	2
ATC/TAG	3	GTAG/CATC	2
CAG/GTC	2	CTTT/GAAA	2
TTG/AAC	2	GACC/CTGG	0
ATT/TAA	1		
AGT/TCA	0		

Table 2 The primer sequences used to amplify microsatellite markers in rice sequences in the databank

Marker	Forward primer	Reverse primer	Repeat sequence	Chromosomal position	Genes	Accession
OSR1	accatggatggtaccaactc	ttcgcttgcatctactatgc	(at)(ct)	11	ω-3 fatty acid desaturase	D78506
OSR2	agcatgcattgcaagctagcg	gatctgctgatcagttacacg	(at)	1	Homeotic gene (MADS3)	L37528
OSR3	agctaaggtctgggagaaacc	aagtaggatggggcacaagctc	(at)	1	Oryza cysteine	M29259
OSR4	gagtgcagttggcatcaaag	taccaccaatgaatgccggag	(at)		β-amylase	L10346
OSR5	cctatcccattagccaaacattgc	gatttacctcgacgccaacctg	(at)	3	Phytochrome 18	X14172
OSR6	ccaagggaaagatgcgacaa	gtggacgctttatatatggg	(at)	11	RFLP marker	D25465
OSR7	aactcatttgtcacacgcaca	aagcctttcctcgtaacacg	(at)	8	Heat-shock protein 82	Z11920
OSR8	tcttctttctctactccctg	tgcttcctgccttcctcca	(ga)	2	Intrinsic protein	D17443
OSR9A	ccaacatctccgtcagaatc	acaaagtcaacgtcggcgtc	(ga)	2	RFLP marker	D16340
OSR9B	gtcagaatcaagcgagatcg	gtcgtacgtctctctctc	(ga)	2	RFLP marker	D16340
OSR11	ccccttcctctcgcgtcgtac	ccgtcctcctcctcctgcga	(ga)	2	EST	D39059
OSR12	cgtcttccccaacgctaaaa	cgccgggcttcgcttgtc	(ga)	9	EST	D25487
OSR13	catttgtgcgtcacggagta	agccacagcgcccatctctc	(ga)		EST	D46417
OSR14	aaatccacgcacactttgcg	aggtaaacgagcttgaggtg	(ga)		EST	D39674
OSR15	caaccaacgccaaaagctac	gtggtactcgccctgcatga	(ga)	4	EST	D49014
OSR16	aaaactagcttgcaaagggga	tgccggctgatcttgttctc	(ga)		Homeotic gene (MADS1)	L34271
OSR17	gctggttgattcagctagtc	gcctcgttgtcgttccacac	(aatt)		Catalase A	D29966
OSR18	ccccaaggataatatcaggag	tgcgtatgtttgttcccaag	(ac)	6	EST	D48213
OSR19	ctctctcaccattccttcag	gatctgaataagaggggaaac	(ct)	6	Waxy	X65183
OSR20	tggtcaagtgacttaggtgg	agagctccaactctttacaag	(ct)	12	Serine carboxypeptidase I	D17586
OSR21	atttctttggccacaggcga	cccagattcggaacaagaagaac	(ct)	6	Starch branching enzyme	D10838
OSR22	ctgagtctcctgtcctcatc	cttgaatctctgcactgcac	(ct)	7	EST	D48899
OSR23	tgatacgtggtacgtgacgc	taatcgcttccctacccctg	(ct)	1	Sucrose phosphate synthase	D45890
OSR24	tccggctccctctcccccc	acctcgttgtccttaacctc	(ct)		EST	D24943
OSR25	ccaacagaaaccacacaccacc	aagcctccgctccgttcttttc	(gc)	6	Waxy promoter	[a]
OSR26	gacagagctttggatgtctg	aaagaagctgcctttgacgc	(tg)	2	EST	D48106
OSR27	gggtgatttcttggaagcga	tctcggagagcttctccatc	(aga)	1	EST	D40151
OSR28	agcagctatagcttagctgg	actgcacatgagcagagaca	(aga)	9	EST	D40471
OSR29	gctagcagctatagcttagc	agactgcctgtgagatcaca	(gaa)	9	EST	D40093
OSR30	tcaccgtcgaatcgaatcca	agtcgaggaaggagaagttc	(ggc)	8	EST	D15442
OSR31	tcgcttcgcttctactggag	ttctccagcttgtcgacgtc	(cgg)	3	EST	D41754
OSR32	ctccagcttcggcaacgtca	cttcttgatgccctcaatcgt	(ttc)	12	Rubisco small subunit	X07515
OSR33	aacgcgaggacacgtacttac	acgagatacgtacgcctttg	(gatg)	10	OSRRf	D84275
OSR34	gaaaccaccacacctcaccg	ccgtagaccttcttgaagtag	(ggc)	5	EST	D22858
OSR35	gcctcgagcatcatcatcag	atcaacctgcacttgcctgg	(gaa)	8	EST	D48916

[a] This sequence data was obtained from the parent database, which was coded in the same gene as OSR19

these published sequences should help in the development of new microsatellite DNA markers.

Development of microsatellite DNA markers

In order to develop new microsatellite DNA markers based on the published rice sequences, specific primer pairs for 45 sequences containing microsatellites were synthesized. Half of these sequences were selected from EST sites, and the other half from genomic sequences. Thirty five of these primer pairs gave specifically amplified microsatellites, while 11 failed to do so. Amplification failure might be due to the use of unsuitable primer pairs, because another pair of microsatellite primers used for EST sites (D48213) resulted in amplification, whereas microsatellites in EST sites (D22450 and D47071) were not observed with other primer pairs. The sequences of primer pairs which successfully amplified rice microsatellite markers are shown in Table 2.

Polymorphisms of the 35 microsatellite DNA markers were examined in the Asominori (japonica) and IR24 (indica) lines. Seven out of thirty five markers were identical between these lines (Fig. 1). The microsatellite DNA marker OSR6 (*O*ryza *S*imple sequence *R*epeat 6) was only amplified in the japonica cultivar Asominori. However, OSR6 was highly polymorphic within the species of japonica cultivar (Akagi et al., unpublished data). The microsatellite in OSR5 has already been investigated by Wu and Tanksley (1993) and failed to amplify. This may be due to the fact that *O. sativa* without cv. 36 and some *O. rufipogon* was contained in an insertion within the microsatellite (Akagi et al., unpublished data). Polymorphisms, which were located at both ends of the insertion (Fig. 2), were detected after digestion with *Sma*I or *Nco*I because the size of the amplicon was too large to detect differences in the length of the microsatellite. Microsatellites on both sides of the insertion were polymorphic (Fig. 2, arrow).

Mapping of microsatellites on chromosomes

Polymorphic microsatellite DNA markers were mapped by using a population of 71 inbred recombinant lines (Fig. 3).

Microsatellite DNA markers were mapped on all of the chromosomes.

OSR9A and OSR9B contain the same microsatellite, *Xnbp357*, found in the RFLP probe which was developed by Saito et al. (1991). These 2 microsatellite DNA markers mapped to exactly the same locus of chromosome 2 where the RFLP probe had also been mapped. Polymorphisms of this microsatellite have been mapped to the same locus with different primer pairs (Inoue et al. 1994). Similarly, the loci of OSR21 and OSR23 were consistent with the loci of the original sequences – the starch branching enzyme-I gene and the sucrose phosphate-synthase gene, respectively – which had been mapped by RFLP (Nakamura et al. 1994; Sakamoto et al. 1995). This indicates that polymorphisms in microsatellites can be used to confirm map position. In the present study, the locus of several genes was determined by polymorphisms in the microsatellites within these genes.

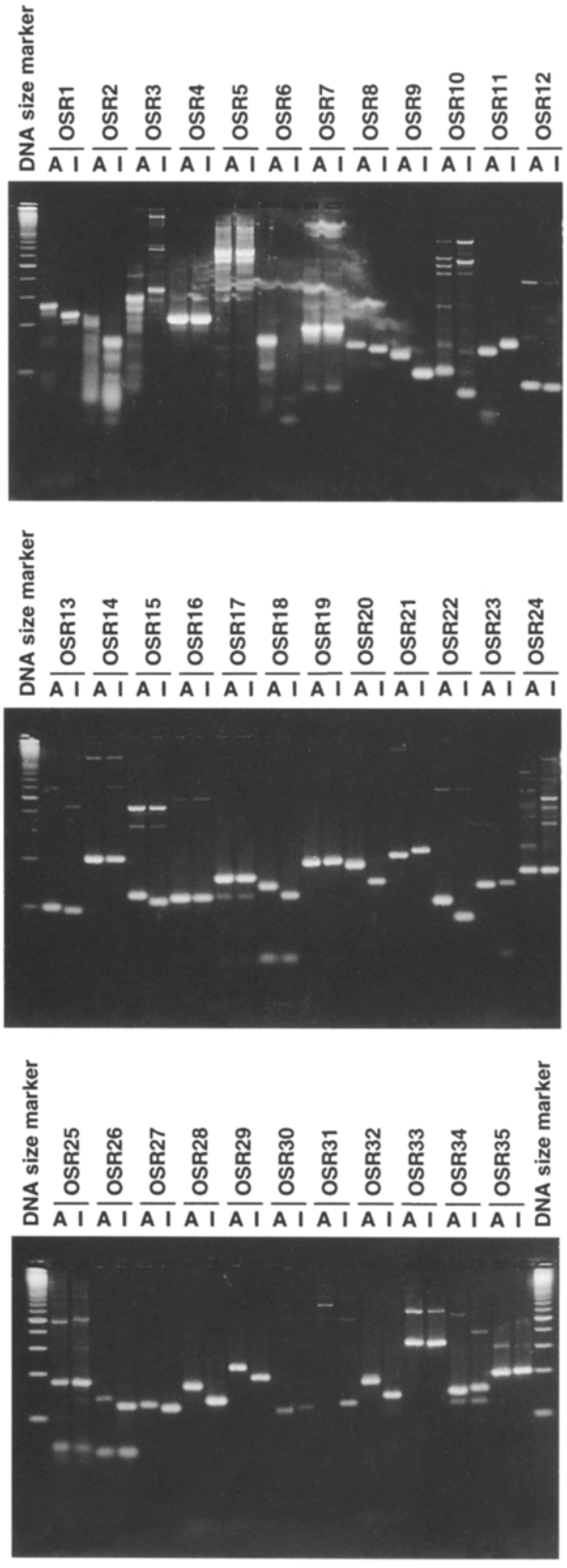


Fig. 1 Polymorphisms of PCR-amplified microsatellite DNA markers (*OSR*) between Asominori (japonica) and IR24 (indica). Amplicons were detected by ethidium bromide staining after electrophoresis on 3% MetaPhor agarose gels (FMC). *Lanes A and I* contain PCR product amplified from the DNA of Asominori and IR24, respectively. DNA size-marker lanes contain a 100-bp/500-bp DNA ladder

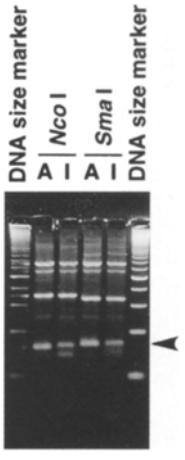
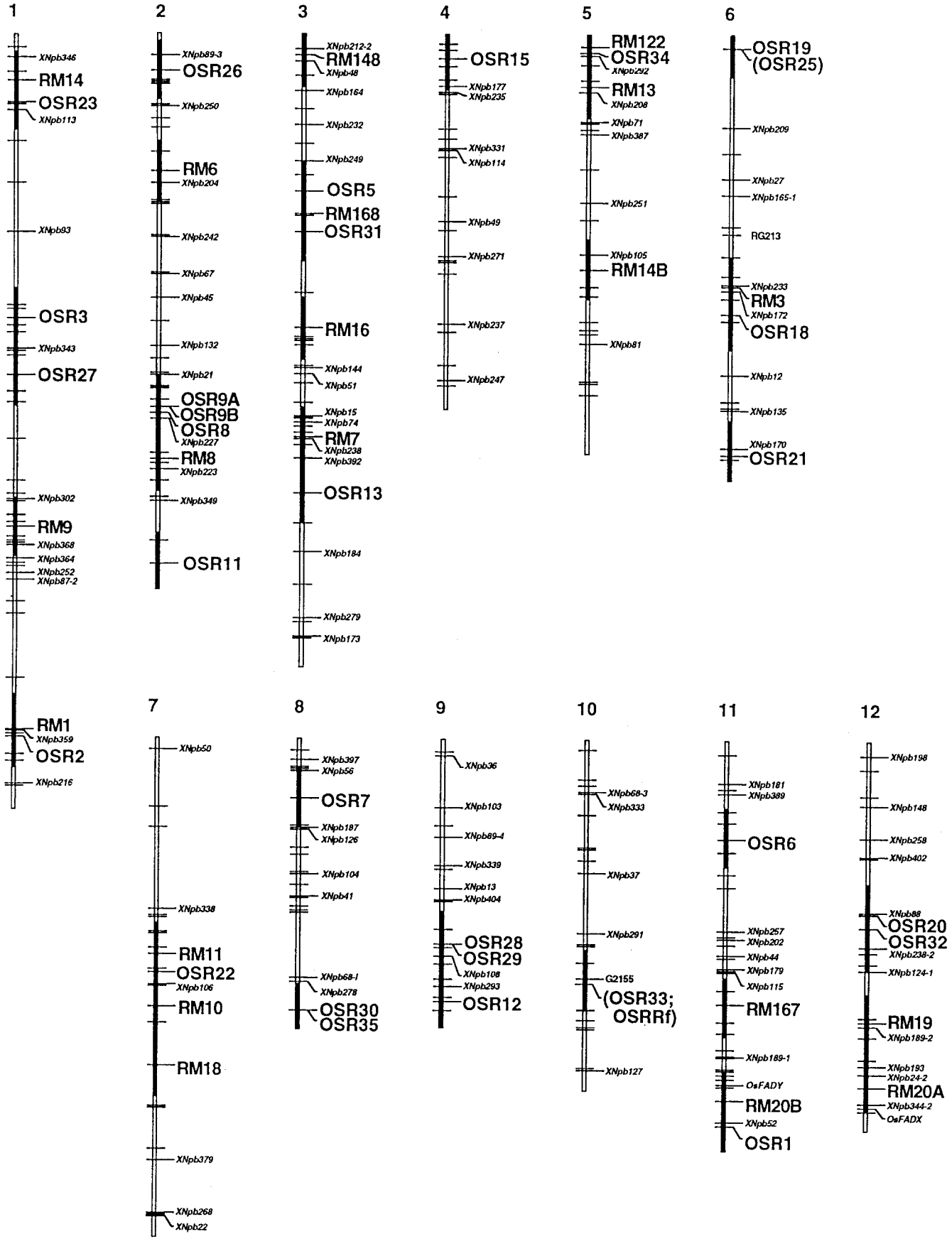


Fig. 2 Polymorphisms of cleavage down from amplified fragments in OSR5. PCR product of OSR5 were electrophoresed on 3% MetaPhor agarose gels (FMC) followed by digestion with *Nco*I or *Sma*I. *Lanes A and I* contain PCR product amplified from DNA of Asominori and IR24, respectively. DNA size-marker lanes contain a 100-bp/500-bp DNA ladder. The bands containing microsatellites are indicated by an *arrow*

Fig. 3 The loci of 56 microsatellite DNA markers in the rice genome. *OSR* indicates the microsatellite markers developed in this study, while *RM* indicates those that were developed by other laboratories (Wu and Tanksley 1993; McCouch et al. 1995). Locations of RFLP markers which were mapped by the Rice Genome Research Program of Japan are indicated by *short vertical bars*. Only the names of the RFLP markers developed by Saito et al. (1991) are shown on the right hand side of the map in *small italic letters*. Loci of non-polymorphic microsatellite DNA markers, OSR25 and OSR33, are also indicated in the map. OSR25 was mapped to the same locus as OSR19, since these two markers existed in the same gene. The OSR33 locus was evaluated using data from another population (Akagi et al. unpublished data). The *black region* of the chromosomes represents a 10-cM region adjacent to each microsatellite DNA marker


1
XNpb346
RM14
OSR23
XNpb113
XNpb93
OSR3
XNpb343
OSR27
XNpb302
RM9
XNpb368
XNpb364
XNpb252
XNpb87-2
RM1
XNpb359
OSR2
XNpb216
2
XNpb89-3
OSR26
XNpb250
RM6
XNpb204
XNpb242
XNpb67
XNpb45
XNpb132
XNpb21
OSR9A
OSR9B
OSR8
XNpb227
RM8
XNpb223
XNpb349
OSR11
3
XNpb212-2
RM148
XNpb48
XNpb164
XNpb232
XNpb249
OSR5
RM168
OSR31
RM16
XNpb144
XNpb51
XNpb15
XNpb74
RM7
XNpb238
XNpb392
OSR13
XNpb184
XNpb279
XNpb173
4
OSR15
XNpb177
XNpb235
XNpb331
XNpb114
XNpb49
XNpb271
XNpb237
XNpb247
5
RM122
OSR34
XNpb292
RM13
XNpb208
XNpb71
XNpb387
XNpb251
XNpb105
RM14B
XNpb81
6
OSR19
(OSR25)
XNpb209
XNpb27
XNpb165-1
RG213
XNpb233
RM3
XNpb172
OSR18
XNpb12
XNpb135
XNpb170
OSR21
7
XNpb50
XNpb338
RM11
OSR22
XNpb106
RM10
RM18
XNpb379
XNpb268
XNpb22
8
XNpb397
XNpb56
OSR7
XNpb187
XNpb126
XNpb104
XNpb41
XNpb68-1
XNpb278
OSR30
OSR35
9
XNpb36
XNpb103
XNpb89-4
XNpb339
XNpb13
XNpb404
OSR28
OSR29
XNpb108
XNpb293
OSR12
10
XNpb68-3
XNpb333
XNpb37
XNpb291
G2155
(OSR33;
OSRRf)
XNpb127
11
XNpb181
XNpb389
OSR6
XNpb257
XNpb202
XNpb44
XNpb179
XNpb115
RM167
XNpb189-1
OsFADY
RM20B
XNpb52
OSR1
12
XNpb198
XNpb148
XNpb258
XNpb402
XNpb88
OSR20
OSR32
XNpb238-2
XNpb124-1
RM19
XNpb189-2
XNpb193
XNpb24-2
RM20A
XNpb344-2
OsFADX

OSR6, which was found in the *Not*I-linking clone L612, was mapped on to a different chromosome by RFLP. L612 had been mapped on chromosome 1 with RFLP, while OSR6 mapped to chromosome 11 (Kurata et al. 1994). In the detailed map developed by the Rice Genome Research Program of Japan, many minor RFLP bands were also mapped. There may be a region homologous to the L612 clone, which contains OSR6, on chromosome 11.

Construction of a microsatellite map

To construct a molecular map based on microsatellites, we also mapped the published microsatellite DNA markers (Wu and Tanksley 1993; McCouch et al. 1995) using the same recombinant inbred population. A total of 56 microsatellite DNA markers (35 newly developed, and 23 already published) were mapped and these markers were distributed on all of the chromosomes (Fig. 3).

We think that microsatellite DNA markers every 20 cM are necessary to aid practical breeding, because the frequency of double crossing-over between two markers is less than 1%. The total length of all the rice chromosomes was estimated to be about 1800 cM; therefore, at least 90 microsatellite DNA markers should be developed for the construction of a map. Black regions on the chromosomes represent 10-cM regions adjacent to each microsatellite DNA marker (Fig. 3). About 35% of all chromosomes was covered by 56 microsatellite DNA markers. This suggested that about another 130 markers will be needed for the construction of a microsatellite map.

In general, new microsatellites have been isolated either from DNA libraries or else from sequences in databanks. In the present study, we found many sequences containing microsatellites and we were able to develope new microsatellite DNA markers using these sequences. This method could save time and labor when producing such DNA markers, although there were still only a limited number of sequences containing microsatellites. Microsatellite DNA markers developed in other laboratories should be used in the construction of a molecular map, because newly developed microsatellite DNA markers can be easily integrated into the existing data.

Microsatellite DNA markers were more polymorphic than RFLPs. In rice, microsatellite DNA markers showed polymorphism both between species and within subspecies (Wu and Tanksley 1993). We found that some of the microsatellites developed here can be used to distinguish between the highly related japonica cultivars (Akagi et al., unpublished data). Since polymorphisms of microsatellites can be detected by PCR, a great number of plants can be analyzed at the seedling stage. This will enable researchers to easily select plants carrying desired chromosome regions using microsatellite DNA markers. Therefore, a molecular map based on microsatellites should promote the practical breeding of rice by saving time and money.

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