Theor Appl Genet (2000) 100:447–453

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Genetic mapping of hypervariable minisatellite sequences in rice (*Oryza sativa* L.)

Received: 1 March 1999 / Accepted: 22 June 1999

Abstract Minisatellites, or DNA fingerprinting sequences, have been utilized in animal linkage studies for several years but have not been used as markers for plant genome mapping. In animal genome mapping they have resulted in limited success because they are evenly dispersed in some species but are often clustered near telomeric regions, as observed on human chromosomes. The purpose of the present study was to generate DNA fingerprints utilizing several rice-derived minisatellites containing different core sequences and numbers of repeat units, followed by assessing their potential for use as genetic markers when mapped to a rice recombinant inbred line (RIL) population. Sites of segregating minisatellite loci were mapped onto 11 of the 12 rice RIL linkage maps. The implications for the use of rice minisatellite core sequences as genetic markers on linkage maps in rice are discussed.

Key words Linkage maps · Minisatellites · DNA fingerprinting · Rice

Communicated by G. Wenzel

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Introduction

The world-wide use of restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPD), simple sequence repeats (SSRs or microsatellites), amplified fragment length polymorphisms (AFLPs), and various other molecular systems has made possible the tremendous advancements in the production of high-density linkage maps and in the power of utilizing linkage studies for localizing genes in plants and animals. Although the density of mapped loci in plants has dramatically increased in the past few years, the markers commonly used to create these maps have mainly been dimorphic, and not highly polymorphic in all populations, thus causing some of the analyzed dimorphic markers to be noninformative. For example, in hexaploid wheat (*Triticum aestivum* L. em Thell.) and in rice (*Oryza sativa* L.) only about 10% of all markers tested have been shown to be polymorphic. In addition, studies are time-consuming and require the use of many different markers and restriction enzymes.

Minisatellites in humans (Jeffreys et al. 1985) and in rice (Winberg et al. 1993; Zhou and Gustafson 1995; Zhou et al. 1997) are comprised of arrays of short, tandemly repeated sequences, subject to germline recombination, which are throught to occur through unequal crossing over (Jeffreys et al. 1988) and replication slippage of exchanges between sister chromatids (Jeffreys et al. 1990). Any such changes will potentially result in large differences in the repeat unit number present at any particular locus and, therefore, large differences in length polymorphism. These differences between minisatellite loci and the resulting high degree of heterozygosity offer the potential for making minisatellites excellent tools for use as multilocus markers.

Minisatellites have been utilized in animals linkage studies for several years (Jeffreys et al. 1987; Georges et al. 1991; Wells et al. 1989; Julier et al. 1990; Blanchetot and Gooding 1994). Jeffreys et al. (1987) examined the segregation of minisatellite bands in several different inbred mouse strains and deteced at least 13 loci, of which

8 could be assigned to positions on chromosomes by linkage. Using 11 minisatellite probes Julier et al. (1990) established that mouse chromosome assigments could be made for 181 (52%) of the 346 polymorphic minisatellite bands studied. Blanchetot and Gooding (1994) detected an average of 11.2 bands per fly (*Glossina morsitans morsitans*), inherited in a Mendelian pattern, with an average of 9 loci of which 40% were polymorphic in each line. All authors to date have emphasized that human minisatellite probes are capable of providing large numbers of mappable polymorphisms. One major stated advantage of utilizing minisatellite markers is that large sections of a genome can be mapped with a relatively small number of markers.

Minisatellites, or DNA fingerprinting sequences, have not generally been used as markers for genome mapping. In animal genome mapping, they have resulted in some success because they are evenly dispersed in some species such as mice, *Bovidae* and in plants such as *Arabidopsis* (Georges et al. 1990 and 1991; Julier et al. 1990; Tourmente et al. 1998), whereas in humans they are often clustered near telomeric regions of the chromosomes (Royle et al. 1987; Wells et al. 1989). Wells et al. (1989) found that 18 of the 31 mapped bands clustered near telomeres. If present in other species, this clustering observed in humans would severely limit the usefuness of minisatellites as genetic markers. In tomato (*Lycopersicon esculentum* L.), no clustering of minisatellite loci was observed using the human 33.6 and 33.15 minisatellites (Broun and Tanksley 1993).

However, all agree that DNA fingerprinting minisatellite sequences are rather complex because of the frequent occurrence of problems associated with distinguishing between alleles and that the use of multilocus probes have some limitations in genetic mapping. In addition, it is well-known that a high degree of somatic mutations can and do occur at hypervariable loci in both animals and plants, which could lead to problems in linkage analysis depending on the observed mutation frequency for that particular locus (Armour et al. 1989; Jeffreys et al. 1988; Rogstad 1994). Due to the occurrence of new mutations, new bands not present in either parent have been found in some mapping populations (Julier et al. 1990).

When dealing with minisatellite sequences or any SS-type marker, the possibility that differences in heterogeneity between loci does exist, which can translate into differences between groups of loci as well as between individual loci within a single group. There is a tendency to place SSRs or minisatellites together, and indicate that they have the same mutation rate as other regions of the genome, even though it is becoming clear that some SSR or minisatellite loci mutate at vastly different rates than others (Mahtani and Willard 1998; Rogstad 1994; Talbot et al. 1995). There is no doubt but what heterogeneity between minisatellite loci exists. If we add allelic heterogeneity in mutation rate, as shown at some SSR and minisatellite loci, it is clear that mutation rate no longer appears as an invariant ”physical constant” but can vary depending on the region of the genome where one is working. At best, the mutation rate should be considered as an average of many values, each being the result of the DNA region in which the mutation takes place.

Also, the occurrence of undetected new mutations could result in causing segregation distortion to occur in one region of the genome and not another or could cause the data to appear to contain errors, all of which can have an impact on the interpretation and appearance of a map. Except in the cases of some cancers, there is no evidence of somatic stability (Thein et al. 1987; Dejong et al. 1988). Frequently, SSR or minisatellite loci contain tandem repetitive sequences and have several different alleles, thus resulting in high levels of heterozygosity in animals and plants. An additional problem associated with these polymorphisms might result from the presence of co-migating bands from different loci, which show up as heterogeneity in linkage studies. However, in the animals studies to date, none of the above-mentioned problems has resulted in any serious difficulties.

Julier et al. (1990) used 11 minisatellite sequences and were able to show linkage and a wide distribution of chromosome assignments for 181 (52%) of the polymorphic bands in their study of the mouse genome. Their conclusions were that a large segment of the mouse genome could be studied utilizing only a small number of minisatellite sequences (6 probes covered 80% of the linkage groups and combinations of two enzymes provided polymorphic markers for 90% of the linkage groups).

The purpose of the study described here was to generate DNA fingerprints, utilizing several rice (*Oryza sativa* L.)-derived minisatellites (Winberg et al. 1993; Zhou and Gustafson 1995; Zhou et al. 1997) containing different core sequences and numbers of repeat units and to subsequently assess their potential for use as genetic markers when mapped to the Japanese Rice Genome Project (RGP) rice recombinant inbred line (RIL) population. Sites of segregating minisatellite loci were mapped onto 11 of the 12 rice RIL linkages maps. The implications for the use of rice minisatellite core sequences as polymerase chain reaction (PCR)-based genetic markers on linkage maps are discussed.

Materials and methods

Population and map

The Japanese Rice Genome Project recombinant inbred line (RGP-RIL) mapping population was developed by selfing the progeny of an F_2 population from a Japonica-Indica cross involving ‘Asominori’/‘IR24’ utilizing the single-seed-decent technique until the F_7 generation (Tsunematsu et al. 1996). Among 165 F_6 lines obtained from 227 F_2 plants, 71 random lines were identified to make up the mapping population. The RFLP markers used to create the rice map were those previously mapped by Saito et al. (1991) and Kurata et al. (1994). In addition, newly created rice genomic clones (*Ky*) from a rice cultivar ‘IR24’/*Pst*I library were also mapped.

Table 1 Characteristics and polymorphism differences between the rice-derived minisatellite sequences analyzed

Sequence	Size (kb)	Number of bands	Number of segregating bands	Number of segregating bands mapped	Restriction enzyme
pOs 6.1S	0.38	0	0	0	*Hin*dIII
pOs 6.2H	0.69	50	28	22	*Hin*dIII
pOs 6.8H	0.60	2	2	2	*Dra*I
pOs 6.5A	0.70	8	0	0	*Hin*dIII
pOs 6.6	0.60	3	1	0	*Hin*dIII
pOs 6.10H	0.75	26	4	2	*Dra*I
pOm 6.1	0.35	14	7	7	*Hin*dIII
Total		103	42 (41%)	33 (79%)	

DNA extraction

The DNA extraction was done in Japan by taking fresh leaves from single plants of each of the 71 RILs and grinding them in liquid nitrogen or dry-ice. DNA was extracted from the tissue using the CTAB (cetyltrimethyl ammonium bromide) technique of Murray and Thompson (1980).

Minisatellite sequences

The minisatellite sequences were previously isolated and characterized at the University of Missouri (Winberg et al. 1993; Zhou and Gustafson 1995; Zhou et al. 1997) (Table 1). Minisatellite clones pOs 6.1S, pOs 6.2H, pOs 6.5A, pOs 6.6H, pOs 6.8H, and pOs 6.10H were isolated from *Oryza sativa*, while pOm 6.1 was isolated from *O. meridionalis.*

Southern hybridization

The DNA was sent to Missouri where it was incorporated into mapping blots. Standard Southern hybridizations were done in Missouri. The restriction enzyme showing the most polymorphism was utilized for each sequence (Table 1).

Linkage analysis.

All segregating minisatellite bands present in the DNA fingerprint were scored either as "A" for homozygous for 'Asominori', "B" for homozygous for 'IR24', and missing data were scored as "–". All autorad readings were done in Missouri, and all linkage analyses were performed in Missouri and Japan. A two-point linkage analysis was performed, and pairs of loci were considered linked only if the LOD score exceeded 3.0, depending on the minisatellite band being analyzed. A multi-point analysis was performed in order to determine the marker order within each linkage group. All map distances and recombinant frequencies were estimated using *JoinMap* (Stam 1993; Stam and Van Ooijen 1995).

Results and discussion

Because rice DNA fingerprints obtained by using minisatellite sequences were very complex, and some allele distribution tended to be distorted toward short sequences resulting in a smear of bands among the low-molecular-weight level, it was impossible to score bands as being allelic. Therefore, we analyzed all bands as either present or absent. This implies that a band of a specific length is a single allele at a single locus and not two co-migrating alleles from separate loci. This is impossible to verify, but likely to have only a minor effect, because the frequency of shared bands between unrelated individuals has been shown to be low, especially among the higher molecular-weight bands (Jeffreys et al. 1985).

The DNA fingerprints were scored where each segregating band within a fingerprint was scored as a presence/absence (+/–) or dominant marker. It was impossible to assess any segregating band for the presence of codominance. The accuracy of scoring of the DNA fingerprints can be subject to the vagaries of many factors, including electrophoretic band shifts and gel unevenness, all of which were minimized by use of careful laboratory techniques and molecular markers on every gel. Below approximately 3 kb in band size, as has been noted previously, it was no longer possible to score individual bands because they appeared somewhat diffuse and blended together. Also, any shift in band intensity could occasionally be a problem, and this problem would be intensified if the band in question was composed of different alleles from several loci or of loci of the same length but having different internal structures. Occasionally, bands could be detected that came from neither parent, which obviously would be the result of a mutation at that particular locus, thus creating a new band. The occurrence of new bands will vary from locus to locus and plant to plant because, as observed in the past, different mutation rates can occur for different loci (Rogstad 1996).

Five of the seven (71%) rice-derived minisatellites evaluated exhibited segregating banding patterns when hybridized to the rice RIL mapping population. Three of the seven probes (pOs 6.6H, pOs 6.5A, and pOs 6.8H) exhibited a limited banding pattern, while three probes (pOs 6.2H, pOs 6.10H, and pOm 6.1) exhibited widely diverse multiple banding patterns. Only pOs 6.1S showed a smear with no discernible banding pattern. Probe pOs 6.5A showed a banding pattern, however, none of the bands segregated in this population.

The probes were hybridized to nylon membranes containing the parents and the 71 members of the rice RIL mapping population. The total number of bands present was counted as were the number of segregating bands (Table 1). The total number of 42 polymorphic bands scored ranged from 28 using pOs 6.2H to 1 using pOs 6.6H. Nine of the segregating bands were not mapable, but the remaining 33 (79%) segregating bands were mapped to linkage groups in the RIL population.

Table 2 Number of rice minisatellite bands mapped per rice chromosome

Rice chromosome	Number of minisatellite bands mapped	Segregation distortion[a]	LOD value
1	3	–	2.0
2	4	–	1.5
3	4	IR24	2.5
4	4	IR24	2.5
5	3	IR24	2.0
6	0	IR24	2.5
7	1	–	3.0
8	4	–	3.0
9	2	–	3.0
10	3	–	1.5
11	1	IR24	1.5
12	5	IR24	3.0

[a] Significant segregation distortion was observed in the parent named

Segregation patterns of the six rice minisatellite probes did not reveal any new mutations, resulting in bands being observed in the progeny that were not present in the parents. However, because of the presence of considerable smearing of bands at the lower molecular levels, it was not possible to distinguish bands and, therefore, any new low-molcular-weight bands resulting from a mutation would go undetected.

The data were examined for the presence of any segregation distortion due to the additional presence of co-migrating bands and/or the influence of one parent over the other. Segregation distortion as examined using JOINMAP indicated the presence of segregation distortion on 6 of the chromosomes (chromosomes 3, 4, 5, 6, 11, and 12). This distortion was noted in both Japanese cDNA probes and some of the rice-derived minisatellite bands. In all of the cases observed, the distortion was skewed toward the 'IR24' parent. The skewedness was scattered over the chromosomes and did not appear to be located near either the telomeres or the centromeres. The inheritance of the rice minisatellite bands appeared to be similar in nature to that for all other loci examined, and no distinct differences could be observed between cDNA and minisatellite clones.

As stated in the Introduction, major differences in heterogeneity between loci can exist, which also translates into differences between groups of loci as well as between individual loci within a single group. From existing literature, it is known that some minisatellites, as well as SSRs, are capable of mutating at significantly different rates than others, and this factor could be the cause of some of the observed variation. If it were possible to take into account this observed heterogeneity in mutation rate, is becomes quite clear that mutation rate is not a constant but is variable, depending on the region of the chromosome being studied.

Also, the occurrence of any undetected new mutations could influence the presence of segregation distortion occurring in one region of the genome and not another, which could have an impact on the interpretation and appearance of a linkage map. Therefore, when placing minisatellite or SSR loci onto a RFLP map one should take these factors into consideration. An additional problem could result from the presence of co-migrating bands from different loci, which show up as heterogeneity in linkage studies.

It was clear from the beginning that because of the above factors, not all minisatellite bands were mappable at the same LOD score. The LOD scores, which allowed us to map minisatellite loci, ranged from a low of 1.5 to 3.0 (Table 2). In all cases, the minisatellite bands fit onto the existing rice RIL linkage maps without either increasing the centiMorgan distance of the map (in many cases the map was reduced in centiMorgan distance) or changing any of the pre-existing cDNA clone map order.

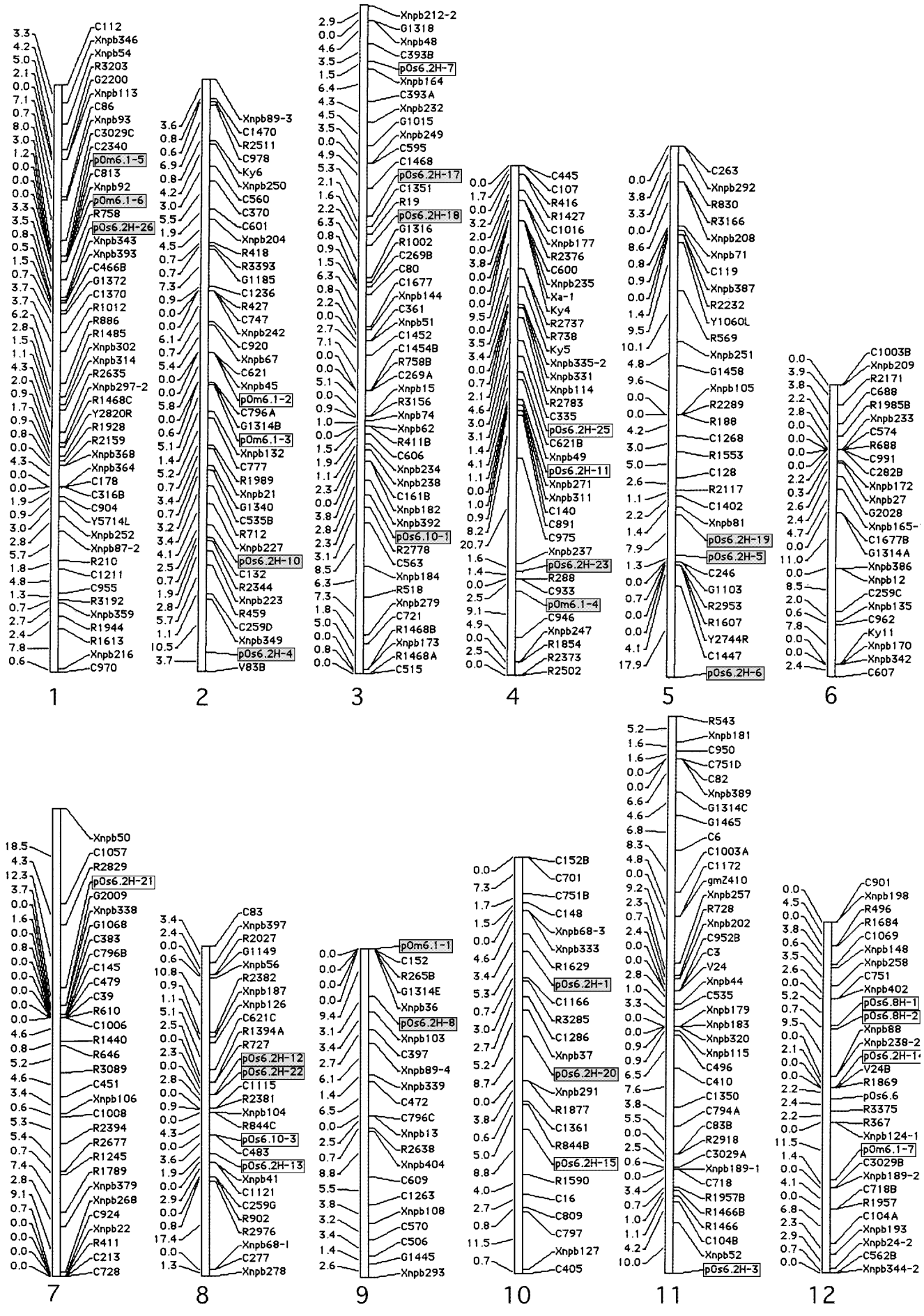
Assigning rice minisatellite polymorphisms to various locations on the rice linkage map clearly confirmed the scattered distribution of these rice minisatellites throughout the genome. Only on chromosome 8 did any of the minisatellite bands show linkage to the same site. Bands pOs 6.2H-12 and pOs 6.2H-22 from the same minisatellite mapped to the same locus, which could mean that they are allelic.

The rice-derived minisatellite bands did not show the same degree of telomeric clustering as noted in humans and other animals; the general indication was that they were scattered throughout the rice genome (Fig. 1). This is in agreement with the use of pOs 6.2H to create DNA fingerprints of a series of Japanese rice YAC clones covering all 12 rice chromosomes from one end of the other (Kurata et al. 1997). However, three of the rice minisatellite bands were located at or near the telomeric regions of rice chromosomes 5, 9, and 11. This is considerably less clustering at the telomeres as compared to that noted in human studies.

Rice chromosome 6 appeared to be different from all others in that it contained no mappable minisatellite bands in contrast to the other 11 rice chromosomes. This was very surprising in view of the fact that pOs 6.2H was used to create DNA fingerprints of rice YAG clones from one end of chromosome 6 to the other (Fig. 2; N. Kurata, unpublished data). There is no clear reason for this variation. However, one possibility could be that the minisatellite loci located on chromosome 6 are low in molecular weight and that when viewed in terms of the entire genome, they are only present on the autorad as smears at the lower end of the molecular-weight scale.

The data clearly indicate the rice-derived minisatellites are capable of providing an additional source of markers for linkage studies in rice. They also indicated that a significant proportion of the observed polymorphisms were mappable. The results suggest that one of the main advantages of the minisatellite polymorphism observed is that most of the rice genome can be analyzed with only a small number of probe/restriction en-

Fig. 1 The Japanese Rice Genome Project RIL linkage map containing rice minisatellite loci (in *grey*) ▶


1
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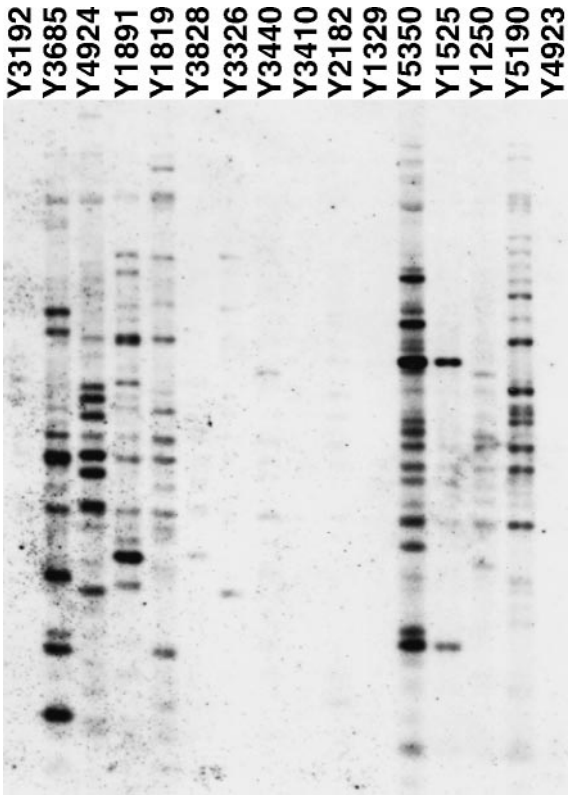

Fig. 2 DNA fingerprint patterns of rice YACs from rice chromosome 6 when hybridized to pOs 6.2H (with permission from N. Kurata)

zyme combinations. Six probes and two restriction enzymes provided 33 polymorphic markers that were present on 11 of the 12 linkage groups. In fact, rice minisatellite probe pOs 6.2H and one restriction enzyme alone provided polymorphisms on 11 of the 12 linkage groups.

At present, we can think of only two potential problems associated with the utilization of minisatellite polymorphisms in rice linkage studies, and these problems could also be problems in any linkage study involving the use of minisatellite or microsatellite markers in either animals or plants. First, occasionally a hypervariable locus can be unstable, and variations in mutation rates have been observed (Jeffreys and Morton 1987; Jeffreys et al. 1988, 1990; Rogstad 1996). Second, and the one more likely to occur, would be the presence of co-migrating bands from different loci. These co-migrating bands could affect the results in any mapping population, whether plant or animals, by introducing false heterogeneity in any linkage study where they occurred. However, if one allows for the introduction of the possibility of lowering the LOD scores in some specific instances, then the above problems can be minimized. If the locus fits into a linkage group without changing the existing map order or increasing the centiMorgan distance, then the locus probably belongs in that linkage group. If one rechecks the autorads and eliminates any errors in reading, then the reading score is correct, and any vagaries are probably due to one of the above two problems. However, we do not believe that either problem will create any serious difficulties. Segregation distortion was observed to be present in both the minisatellite and the cDNA clones of the rice RIL mapping population. There did not appear to be any more segregation distortion in the minisatellite bands than what occurred in the cDNA bands. Most of the segregation distortion occurred for rice chromosomes 1, 3, 4, 5, 6, 11, and 12. Only rice chromosome 2 showed absolutely no segregation distortion coming from either set of markers.

The application of minisatellite markers as dominant loci should be useful in extending linkage maps for a much wider coverage of the genome in either plants or animals.

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