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H. Nagano · S. Kawasaki · Y. Kishima · Y. Sano

Structural differences in the vicinity of the *waxy* locus among the *Oryza* species with the AA-genome: identification of variable regions

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Abstract We constructed a fine physical map for a 260-kb rice BAC contig surrounding the *waxy* locus. In order to identify variable regions within this 260-kb as to the restriction fragments length polymorphisms and copy numbers, sixty overlapping fragments derived from the 260-kb contig were used as probes to compare their corresponding structures among the *Oryza* species with AA-genome. According to the hybridization patterns, each fragment was classified into four types; true single copy (class 1), single copy with a smear background (class 2), multiple copy without a smear background (class 3), and only a smear background (class 4). Out of 16 single copy (class 1 and class 2) regions obtained in this map, the one site corresponding to *wx* gave rise to remarkable polymorphisms among AA-genome species in *Oryza*. In most of the fragments observed as repetitive segments (class 4), we could not find obvious differences in the hybridization pattern. However, interestingly, one site sorted into class-3 showed copy numbers varying among the lines. The lines belonging to *O. sativa O. rufipogon*, *O. meridionalis*, and *O. longistaminata* possessed high-copy numbers of this fragment, whereas only a few bands were detected in the lines from *O. glaberrima*, *O. barthii*, and *O. glumaepatula*. The two variable regions found within the AA-genome species represented genomic dynamisms.

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H. Nagano · Y. Sano (✉)
Laboratory of Plant Breeding, Faculty of Agriculture,
Hokkaido University, 060-8589 Sapporo, Japan
e-mail: rysano@abs.agr.hokudai.ac.jp
Fax: +81-11-706-2439

S. Kawasaki
National Institute of Agricultural Resources,
Kan'non-dai 2-1-2, Ibaraki 305, Japan

Y. Kishima
Laboratory of Genetic Engineering, Faculty of Agriculture,
Hokkaido University, Sapporo 060-8589, Japan

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Introduction

In grasses, genome studies have been rapidly developed in a few years. The comparative studies at the map and megabase level showed that the genome constituents such as gene content and orders are unexpectedly conserved among the grass genomes (Gale and Devos 1998). On the other hand, the intergenic region between the *sh1* and *a1* genes are remarkablely different among maize, sorghum and rice (Chen and Bennetzen 1996; Chen et al. 1998). The physical distance between the two genes were reported to be about 140 kb, 20 kb, and 20 kb in maize, sorghum and rice, respectively (Chen et al. 1997). These studies suggested that upon investigating process for the speciation between near-related species, intergenic region is more suitable than genic region. The intergenic region is comprised by sequences with different origins, and these different sequences adjoin each other (Barakat et al. 1997; Bennetzen 1997; McCouch 1998). A complex of genomic sequences derived from various sources have not been comprehensively compared among closely related species.

The *waxy* (*wx*) locus in rice has been genetically well characterized, since its gene expression affects seed quality, especially amylose content (Sano 1984; Hirano and Sano 1991). A number of the rice varieties carrying low amylose content have been isolated, and some are actually being utilized for rice production in several countries (Grist 1986). These low amylose lines occurred at the *wx* locus or the other loci controlling the *wx* gene expression (Okuno et al. 1983, Yano et al. 1985; Sano 1984; Cai et al. 1998; Hirano et al. 1998; Isshiki et al. 1998). Here we payed attention to the *wx* locus and its proximal region that might be differentiated in the course of speciation in the *Oryza* species and subsequent domestication of rice varieties. We constructed an elaborate physical map covering a 260 kb

around the *wx* locus using BAC clones from the Japonica rice (Nagano et al. 1997; Nagano et al. unpublish data). Sixty-four *Hin*dIII sites were mapped within this 260-kb region, and these fragments were divided into 60 fragments ranging from 1.4 kb to 10 kb, with an average size of 5.7 kb. Such fine fragmentations of this region made possible to compare their corresponding genomic structures among the various *Oryza* species. This study was conducted in order to identify variable regions in the *wx* vicinity in the AA-genomes of 14 lines from 7 *Oryza* species. Southern hybridizations were undertaken using the 60 fragments as probes. We found two sites to be polymorphic within the 14 lines; one from single copy region and other from repeated sequences. These regions represent a dynamism of the genome sequence in *Oryza* species.

Materials and methods

Plant materials and DNA isolation for genomic hybridization

Fourteen lines from seven rice species (Table 1) were used for genomic hybridization experiments. Total genomic DNAs were isolated as described by Hirano et al. (1989). Each DNA digested with *Hin*dIII was run in 0.8% agarose gel electrophoresis. The resultant DNA was transferred to nylon membranes (Pall: Biodyne B), and was hybridized using the ECL gene detection system (Amersham) to the probes prepared from all the subcloned fragments. The membranes were washed twice in the standard ECL condition with 0.5 *M* NaCl at 42°C for 20 min.

Screening for clones with the *wx* locus from the BAC library

The BAC library comprising 21,504 clones that was constructed from the Japonica rice protoplasts of cv 'Shimokita' as mentioned by Nakamura et al. (1997) was supplied for the screening. Seven high-density matrix replicas arraying the BAC clones were hybridized by a DNA probe containing the *wx* genomic sequence (Hirano and Sano 1991). Southern hybridization was carried out using the ECL gene detection system (Amersham).

Subcloning of the BAC clones

We attempted to obtain overlapping subclones with lambda DASH vector (Stratagene). The DNAs from the two representative BAC clones (125-G1 and 120-H10) were partially digested with *Sau*3AI and 10- to 23-kb fractions were purified from agarose gel (Fig. 1). The fractionated DNA was ligated with *Bam*HI digests of the phage vector, lambda DASH. Packaging was performed according to the manufacturer's protocol (Amersham). Three hundreds of the phages were picked up from total resultants. These phage clones were employed for the mapping because of continuously overlapping. Purification of the phage clones were carried out as the methods described by Sambrook et al. (1989). The probes from 60 overlapping fragments, which included the entire 260 kb region were prepared from *Hin*dIII and/or *Eco*RI digests of the lambda phage subclones.

Table 1 The samples of *Oryza* species with the AA genome used in the present study

Sample no	Species[a]	Cultivar or accession	Remarks
1	*O. sativa*	Shimokita	Japonica type from Japan
2	*O. sativa*	T65 *wx*	Near-isogenic line of Taichung 65 with *wx* from Kinoshitamochi (BC_{12})
3	*O. sativa*	221	Javanica type from Indonesia
4	*O. sativa*	PTB10	Indica type from India
5	*O. rufipogon*	W107	Annual type from India
6	*O. rufipogon*	W120	Perennial type from India
7	*O. rufipogon*	W1717	Perennial type from China (through IRRI)
8	*O. rufipogon*	W1718	Perennial type from China (through IRRI)
9	*O. glaberrima*	W025	From Guinea
10	*O. barthii*	W1592	From Cameroun
11	*O. glumaepatula*	W1185	From Surinam
12	*O. meridionalis*	W1625	From Australia
13	*O. longistaminata*	W1034	From Nigeria
14	*O. longistaminata*	W1572	From Nigeria

[a] *Note:* The nomenclature for identifying *Oryza* species was made according to Sano and Sano (1990) and Morishima et al. (1992), although their nomenclature is confusing in the literature. Based on their sexual affinities, *O. glumaepatula* and *O. meridionalis* are regarded as distinct species and *O. nivara* as an annual type of *O. rufipogon.* All the materials are preserved at the National Institut of Genetics, Mishima, and Plant Breeding Laboratory, Hokkaido University, Sapporo

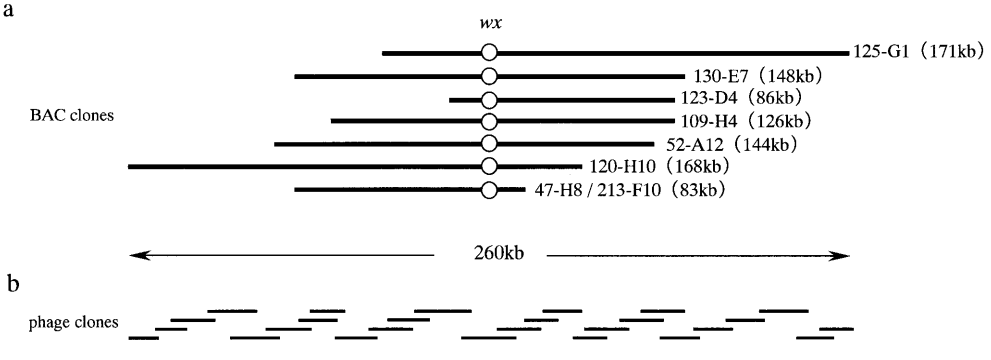

Fig. 1a, b Relative locations of the BAC clones and their overlapping lambda subclones which were isolated by probing the *wx* gene (Hirano and Sano 1991). **a** Of the 8 clones obtained, 47-H8 and 213-F10 were identical. *Numbers* in *parentheses* indicates size of each clone. **b** Twenty-six overlapping lambda phage subclones constituting 260 kb in the BAC contig were mapped below

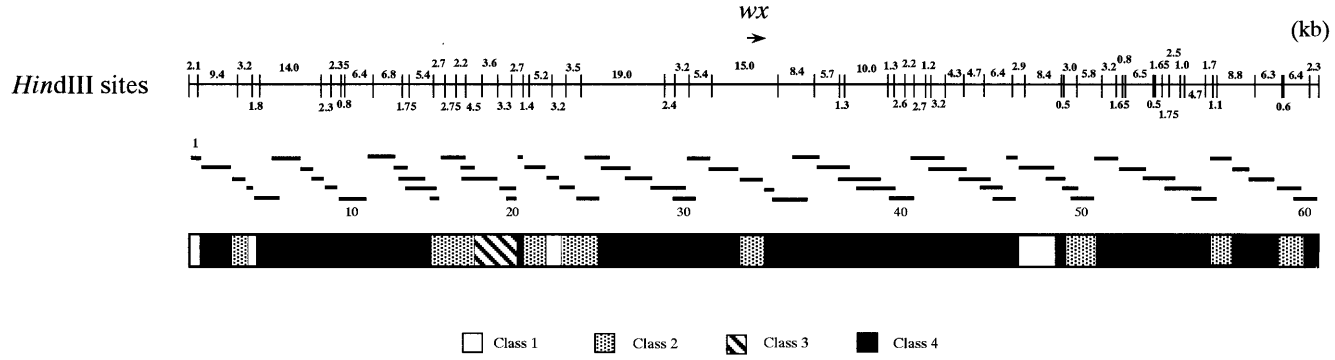

Fig. 2 Physical map constructed based on *Hin*dIII restriction sites, 60 overlapping fragments covering the 260-kb contig, and classification of their DNA fragments. The *wx* locus is present in the center of the map, and the arrow shows an orientation of the 5' to the 3' of the *wx* gene. *Numbers above* the map indicate sizes of the *Hin*dIII fragments which are demarcated by *vertical lines*. Sixty overlapping segments (*black lines* with *numbers*) covering the 260 kb contig were used for the probes. The numbers of these segments indicate an order from the *left side* of the contig. The resultant hybridization patterns were classified as Class 1–4 displayed in the wide box with the four patterns *below* the map

Results

Selection of the waxy clones from the BAC library and construction of the restriction map

The *wx* probe was employed to screen the rice BAC library containing 21,504 clones (Nakamura et al. 1997). Eight clones were selected and were found to originate from the vicinity of *wx* which is a single locus in rice chromosome (Fig. 1). Genetic analysis using a F_2 population derived from crossing Japonica and Indica rices showed that some single-copy segments [restriction fragment length polymorphism (RFLP) markers] obtained from these clones were tightly linked with the *wx* locus, indicating that these clones originated from the vicinity of *wx* (data not shown). Two out of the eight clones remained fragments which do not correspond to those of rest of the clones. Therefore, the two BAC clones, 120-H10 and 125-G1, were localized in either side of the contiguous clones (contig). One hundred of the phage subclones were obtained from each of the two representative BAC clones (Nagano et al. unpublished data). Firstly, the subclones containing the *wx* sequence were selected, and distal *Hin*dIII fragments among these clones were employed as the next probes to look for neighboring clones. This procedure was reiterated to connect the subclones and to determine their positions in the contig. At the same time, we located *Hin*dIII sites on the phage clones. Since the subclones were distributed evenly within the contig, new *Hin*dIII sites were mapped every few clones by the Southern blots (Fig.2). In some *Hin*dIII fragments with more than 10 kb in size, *Eco*RI sites were determined by double digestions of the clones (data not shown).

Classification of the segments in the 260-kb contig

We divided the 260 kb contig into overlapping 60 fragments to analyze genome organization (Table 2). The fragments were prepared from *Hin*dIII and/or *Eco*RI digests of the lambda phage subclones. These 60 overlapping fragments ranging from 1.4 kb to 10 kb were used as probe for Southern blottings (Table 2 and Fig. 2). The obtained hybridization patterns were able to be classified into four types; the patterns with discrete single copy band(s) (class 1), discrete single copy band(s) with a smeared background (class 2), multiple discrete bands without a smeared background (class 3), and patterns consisting solely of smeared bands (class 4) (Fig. 2 and Fig. 3). These classifications in class 1 to 4 were interpreted as single-copy sequences, single-copy sequences with short repetitive sequences, multiple copy sequences and repetitive sequences in the rice genome, respectively.

Comparison of segments in the 260-kb among Oryza species with the AA-genome

We investigated whether the constituents in this region vary in different species with the AA genome. Hybridization experiments should be better solution to address this issue. We undertook hybridization experiments using DNAs from the 14 lines of seven *Oryza* species against the 60 probes (Fig. 3). Most of the hybridization patterns in each line followed a classification obtained from 'Shimokita' line. However, detailed comparisons of these patterns RFLP and differences in copy numbers.

We examined segments sorted into class 1 and 2 which showed a few discrete fragments in the hybridization patterns. The RFLP found in the discrete bands observed among the 14 lines were summarized in Table 3. Although the generation of RFLP tends toward increasing between the distantly related species, the degree of the polymorphisms varies among the fragments belonging to class 1 and 2. Less variations among the 14 lines were observed in the nos. 1, 3, 4, 22, 23, and 24 fragments, while the nos. 16, 17, 25, 33, 50, and 59 fragments gave rise to highly different hybridization patterns within the 14 lines. Among the 6 fragments having diverged RFLP patterns, it was noteworthy that no. 33

Table 2 Characteristics of the 60 fragments used as probes for the genomic hybridization analysis

Fragment no.[a]	Size (kb)	Corresponding *Hin*dIII fragments[b]						Class[c]
1	2.1	2.1						1
2	9.4	9.4						4
3	3.3	3.3						2
4	1.8	1.8						1
5	6.0	14.0						4
6	6.9	14.0						4
7	3.2	14.0						4
8	3.0	14.0	2.3					4
9	3.0	2.3	2.35	0.8				4
10	6.4	6.4						4
11	6.8	6.8						4
12	2.9	6.8	1.75	5.4				4
13	6.4	1.75	5.4					4
14	7.5	5.4	2.7					4
15	2.5	2.7						2
16	6.0	2.75	2.2	4.5				2
17	4.5	4.5						2
18	9.0	4.5	3.6	3.3				3
19	4.2	3.3	2.7					3
20	2.7	2.7						3
21	1.4	1.4						4
22	5.2	5.2						2
23	3.2	3.2						1
24	3.5	3.5						2
25	5.5	19.0						2
26	6.0	19.0						4
27	7.1	19.0						4
28	6.6	19.0						4
29	8.4	19.0	2.4	3.2				4
30	5.6	3.2	5.4					4
31	5.6	5.4	15.0					4
32	7.0	15.0						4
33	5.8	15.0						2
34	2.6	15.0	8.4					4
35	8.4	8.4						4
36	6.5	8.4	5.7					4
37	7.9	5.7	1.3	10.0				4
38	10.0	10.0						4
39	9.2	10.0	1.3	2.6				4
40	6.0	2.6	2.2	2.7				4
41	8.0	2.7	1.2	3.2	4.3			4
42	9.0	3.2	4.3	4.7				4
43	7.7	4.7	6.4					4
44	5.7	4.7	6.4					4
45	5.7	6.4	2.9					4
46	2.9	2.9						4
47	8.4	8.4						1
48	4.8	8.4	0.5	3.0				4
49	4.1	3.0	5.8					2
50	5.8	5.8						2
51	5.8	3.2	1.65	0.8				4
52	6.4	6.4						4
53	7.9	6.5	0.5	165	1.75	2.5	1.0	4
54	8.7	2.5	1.0	4.7	1.7			4
55	6.1	4.7	1.7	1.1	8.8			4
56	5.2	1.1	8.8					2
57	4.0	8.8	6.3					4
58	6.3	6.3						4
59	6.4	6.4						2
60	5.8	6.4	2.3					4

[a] All the fragments were mapped in Fig. 2
[b] The list indicates *Hin*dIII fragment(s) that were overlapped completely or partially with each of the 60 fragments
[c] Classification was based on a comparison with the pattern of 'Shimokita' genomic DNA as represented in Figs. 2 and 3

fragment corresponding to the *wx* locus was only a probe exhibited polymorphisms within the 4 *O. sativa* lines (Fig. 4). A 6-kb *Eco*RI fragment is present within the *wx* coding region of a Japonica cultivar, 'Shimokita', and there are two *Hin*dIII sites located outside the coding region, resulting in a 15 kb fragment. As shown in Fig. 4, comparison between *Eco*RI and *Hin*dIII digestions revealed that the *wx* flanking regions were more diversified than inside of the coding region. Only a Javanica line had unique fragments in the *wx* coding region, but

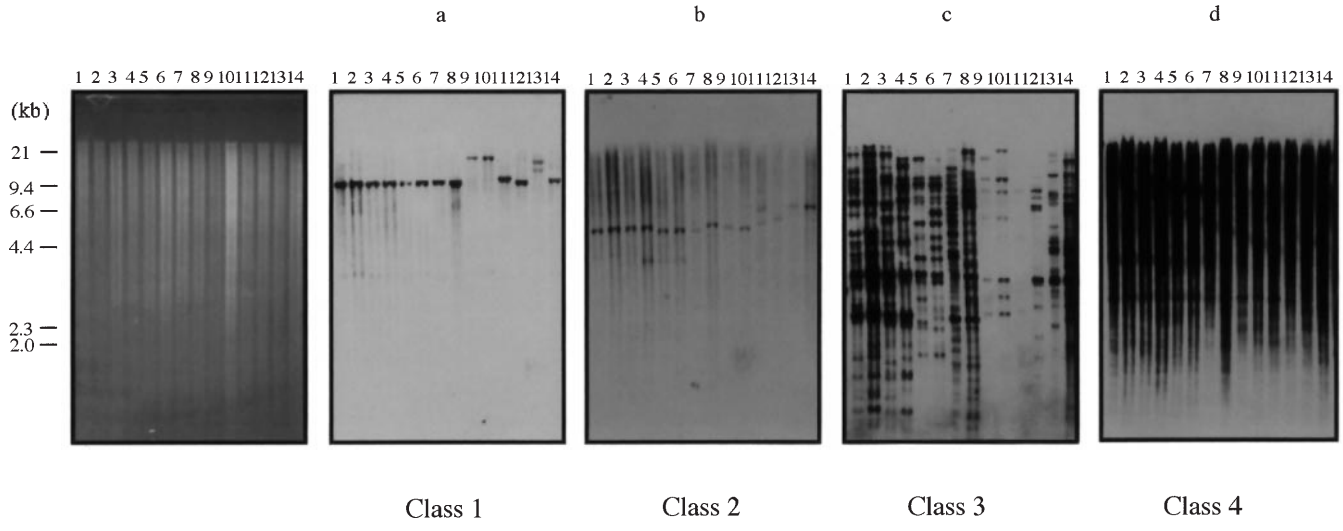

Fig. 3a–d Representative patterns for each of the four classes resulted from genomic hybrdizations in the 14 lines. **a** The class-1 pattern, giving a single fragment or a few discrete fragments without a smear background, **b** class 2, which shows a single fragment or a few discrete fragments with a background smear pattern, **c** class 3, giving multi-discrete fragments without a background, **d** class 4, giving a strongly smeared pattern. The hybridization patterns observed in **panels a–d** were obtained by following probes: **a** fragment no. 47, **b** no. 17, **c** no. 20, **d** no. 5

Fig. 4a, b Southern blotting patterns for the genomic DNAs from the 14 *Oryza* lines probing with 6 kb fragment containing the *wx* gene. Genomic DNAs were digested with *Hin*dIII (**a**) and *Eco*RI (**b**) and loaded onto 0.8% gel. The *numbers above* both panels correspond with the numbers of the materials shown in **Table 1**

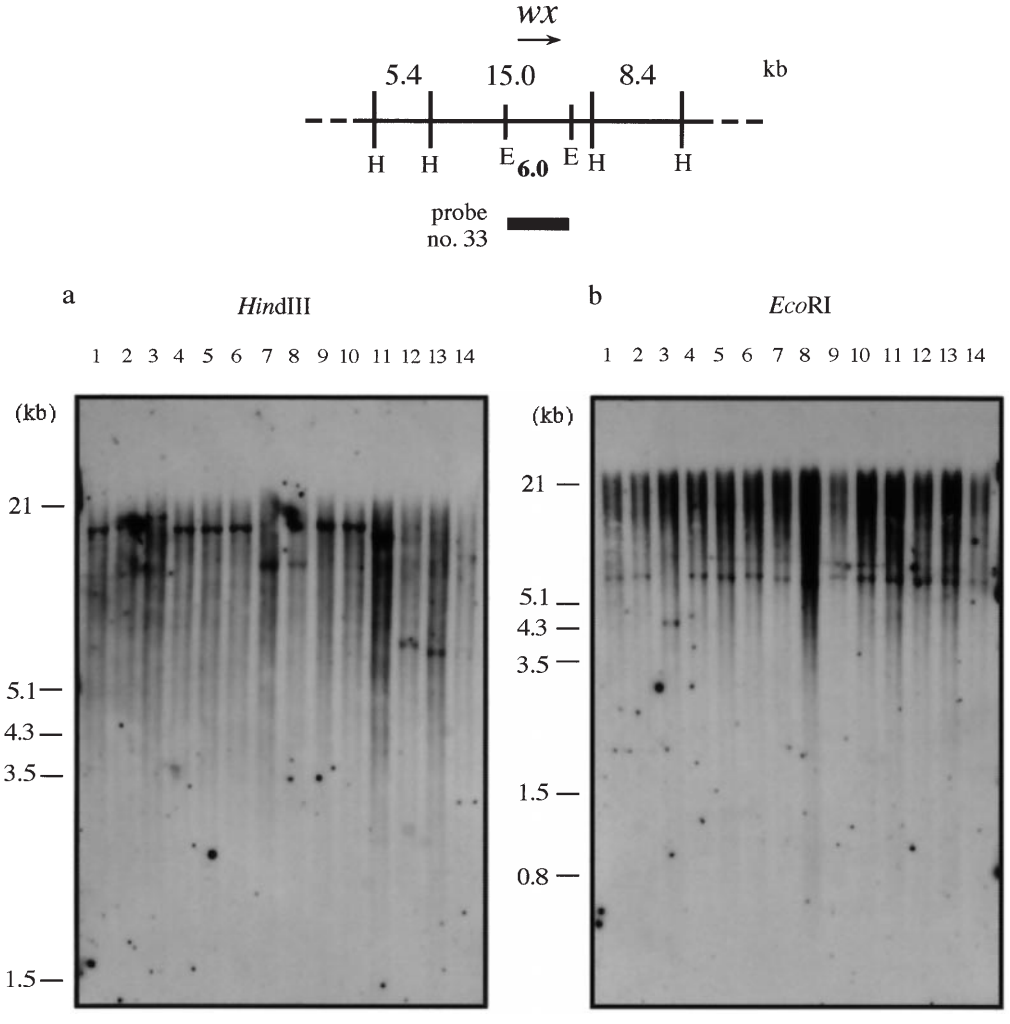

Table 3 Sizes of the fragments (kb) resulting from genomic hybridization of the segments belonging to classes 1 and 2[a]

Sample[b]	Fragment[c]														
	1	3	4	15	16	17	22	23	24	25	33	47	49	50	56	59
1	2.1	3.3	1.8	2.7	4.5/2.75/2.2	4.5	5.2	3.2	3.5	19.0	15.0	8.4	5.8/3.0	5.8	8.8	6.4
2	2.1	3.3	1.8	2.7	4.5/2.6/2.2	4.5	5.2	3.2	3.5	19.0	16.0	8.4	5.8/3.0	5.8	8.8	6.4
3	2.1	3.3	1.8	2.7	4.5/3.2/2.6/2.2	4.5	5.2	3.2	3.5	19.0	20.0	8.4	5.8/3.0	5.8	8.8	6.4
4	2.1	3.3	1.8	2.7	4.5/3.2/2.6/2.2	4.5	5.2	3.2	3.5	7.2	15.0	8.4	5.8/3.0	5.8/8.8	8.8	6.0
5	2.1	3.3	1.8	2.7	4.4/3.2/2.6/2.15	4.4	5.2	3.2	3.4	15.5	15.0	8.4	5.8/3.0	5.8/12.0	8.8	6.0
6	2.1	3.3	1.8	2.7	4.4/3.2/2.6/2.15	4.4	5.2	3.2	3.4	15.5	15.0	8.4	5.8/3.0	5.8/12.0	8.8	6.0
7	2.1	3.3	1.8	2.7	4.4/3.6/3.5/2.6	4.4	5.2	3.2	3.5	7.2	10.5/4.6	8.4	5.8/3.0	5.8	11.0	5.6
8	2.1	3.3	1.8	2.7	4.5/3.6/3.5/2.6/2.2	4.6	5.2	3.2	3.5	7.2	8.2	8.4	6.0/3.0	6.0	11.0	6.4
9	2.1	3.3	1.8	2.7	4.45/2.6/2.2	4.5	5.2	3.2	3.5	7.2	15.0	1.4	6.8	6.8	11.0	13.0
10	2.1	3.3	1.8	2.7	4.4/2.6/2.2	4.4	5.2	3.2	3.5	7.2	15.0	1.4	6.8	6.8/7.8	11.0/8.4	4.9
11	2.1	3.3	1.8	2.65	7.4/6.8/4.5/2.75	4.6/5.6	5.2	3.2	3.5	7.2	14.0/5.6	8.6	8.8/6.8	8.5/5.3	10.5	6.0
12	2.1	3.3	1.9	2.6	7.4/7.2/4.5/4.3/3.0	4.9	6.2	3.2	3.5	6.6	6.2	8.4	8.8/6.0	6.2	11.0	13.0
13	2.1	3.3/3.4	1.9	2.9/2.65	5.5/2.8/2.2	5.8	5.2	3.2	3.5	3.9	6.0	12.5/10.5	6.8	6.6/6.4	7.4	5.8
14	2.0/2.1	3.3/3.4	1.9	2.9/2.65	5.5/3.6/2.9/2.65/2.2	5.8	5.2	3.2	3.5	3.9	6.0	8.6	6.0	6.5/6.3	13.0	5.6

[a] Genomic hybridizations were carried out with *Hin*dIII-digested DNAs from the 14 lines
[b] The sample numbers are listed in Table 1
[c] The segments which showed patterns corresponding to classes 1 and 2 are listed, and the segment numbers refer to the numbers in Fig. 3

the other lines commonly indicated the 6 kb fragment. A *Hin*dIII fragment from T65*wx*, Japonica line, whose grains indicate the *waxy* mutated phenotype, was different from that of the normal Japonica line.

Three successive fragments in the class-3 site were remarkably diverse in copy numbers among the 14 lines (Fig. 5). This region might differentiate during the speciation of *Oryza* species carrying the AA genome. The lines belonging to *O. sativa*, *O. rufipogon*, *O. meridionalis*, and *O. longistaminata* possess high-copy numbers more than 50 of its homologous fragments, whereas only four bands were found to be hybridized in the lines from *O. glaberrima*, *O. barthii*, and *O. glumaepatula* (Fig. 5). Such dramatic change of the copy numbers in this site is of interesting feature for analyzing the differentiation of the AA-genome species. On the other hands, we could not detect particular differences in the patterns sorted into the class-4 in the 14 genomes (data not shown). This fact might indicate that the copy number and kinds of repetitive sequences were less variable through the AA-genome species.

Discussion

Using the fine restriction map of rice BAC clones carrying the *wx* locus, we compared the region around *wx* among 14 *Oryza* lines with the AA genome. A method in which 60 overlapping fragments in the 260-kb of the *wx* vicinity were employed as probes could reveal structural characteristics in terms of RFLP and copy numbers. The results obtained by this method could compensate data from sequencing analyses.

All of the 60 fragments employed as probes from the 260-kb contig hybridized with the DNAs from the examined AA-genome species as similar copy numbers except for those fragments sorted into class 3. Taking colinearity in the closely related genomes into account (Gale and Devos 1998), we believe these fragments to be present parallel in the AA-genome species. Despite of the parallel sequence order, the conserved regions and variable regions coexisted in the 260-kb segment. In comparison of the restriction patterns in the 14 lines, we found three regions being markedly polymorphic; first region resides in the class 3 site along with its left side (fragments nos. 16–20), second corresponds to the *wx* locus (fragment no. 33), and third is located near the right end of the contig (fragment nos. 50 and 59). We are currently investigating these 60 fragments on the other types of the *Oryza* species. So far, these three regions found to be also variable in other types of the *Oryza* genomes (Unpublished data Nagano et al.).

The region corresponding with the fragment nos. 16–20 appeared to be particularly variable among the 60 fragments. This region comprises two parts belonging to class 2 and 3. The probe of no. 16 fragment, which was hybridized with three *Hin*dIII fragments being in the BAC contig, 4.5, 2.75, and 2.2 kb, resulted in highly diverged patterns for the RFLP. In addition, the adjacent

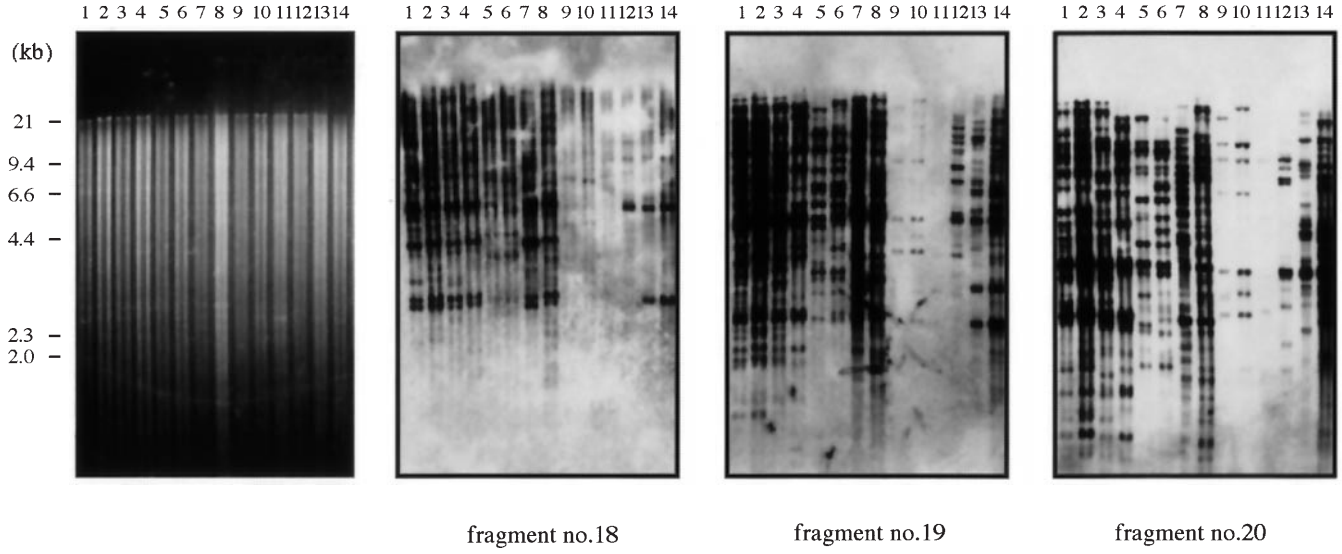

Fig. 5 Southern blotting patterns for the genomic DNAs from the 14 *Oryza* lines probing with three fragments (no. 18 to 20) present in the class-3 site. Genomic DNAs were digested with *Hin*dIII and loaded onto 0.8% gel (the *left panel*). The *numbers above* both the panels correspond with the numbers of the materials shown in **Table 1**. These three hybridization patterns commonly showed that the material nos. 9, 10, and 11 (*O. glaberrima*, *O. barthii*, and *O. glumaepatula*) had remarkably less fragments hybridized in comparison with those in the other lanes

three fragments in the class-3 site gave distinctly different hybridization patterns among the AA-genome species (Fig. 5). Like transposable elements, these hybridization patterns showed dramatic changes in the copy numbers (Fischer et al. 1995). Different copy number of a specific DNA sequence among closely related species is also analogous to rapid amplification of a retrotransposon subfamily in the mouse genome (DeBerardinis et al. 1998). If a degree of specific sequence amplification differs among the *Oryza* genomes, its sequence becomes clue to approach a mechanism giving rise to genome divergence. Interestingly, the right-side fragments (fragment no. 22–24) of the class-3 site are a conserved region, indicating less variations of RFLP. We are now investigating sequences in these regions and comparing them to aim at understanding a cause of the polymorphisms and change of the copy number among the rice species.

We focused on the vicinity of the *wx* locus in this study, since *wx* might have differentiated during domestication of the rice cultivars spread in Asian and African continents (Nakagawara et al. 1986). Our result demonstrated that the *wx* region was one of diverged regions within the 260-kb contig, there were detectable polymorphisms even intra-species comparisons of *O. sativa* and *O. rufipogon* (Table. 2 and Fig. 4). Since the 15-kb *wx* fragment found in *O. sativa* was shared with the lines from *O. rufipogon*, *O. glaberrima*, and *O. barthii*, this 15-kb was predicted as a authentic fragment for these lines and the other sized-fragments in *wx* might be variants of the 15-kb fragment. Previously, Okagaki and Wessler (1988) reported that the *wx* regions of 16 rice strains were not as variable as those of maize strains. As they suggested, mutations within the *wx* coding regions might be less detectable, whereas it was conceivable that proximal regions of the *wx* gene have accumulated the gross changes. It is of interesting to address whether this region is a hot spot for mutations or the mutations were preferentially selected during the domestications. Unfortunately, our present data could not answer this question, because the materials are limited number, and structural alterations around the *wx* locus were unknown due to repetitive sequences encompassing the 15 kb *wx* fragment.

We failed to discriminate the 14 lines underlying the copy numbers of the repetitive segments with smear hybridization patterns. This implies that these species share similar intergenic components such as retrotransposons (SanMiguel et al. 1996, 1998). Quantitative data of the nuclear DNA contents also shows an invariable in AA-genome species (Uozu et al. 1997). Taking together with these results, the AA-genome species were suggested to have a common genomic organization, while there are structural differences detected in comparisons of the *wx* vicinity among the *Oryza* species with the same genome.

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