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Two new genomes in the *Oryza* complex identified on the basis of molecular divergence analysis using total genomic DNA hybridization

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Abstract The genus *Oryza* to which cultivated rice belongs has 24 species ($2n$ = 24 or 48), representing seven genomes (*AA*, *BB*, *CC*, *EE*, *FF*, *BBCC* and *CCDD*). The genomic constitution of five of these species is unknown. These five species have been grouped into two species complexes, the tetraploid *ridleyi* complex (*O. ridleyi*, *O. longiglumis*) and the diploid *meyeriana* complex (*O. granulata*, *O. meyeriana*, *O. indandamanica*). To evaluate the genomic structure of these species in terms of divergence at the molecular level vis-à-vis other known genomes of *Oryza*, we used the total genomic DNA hybridization approach. Total genomic DNA (after restriction digestion) of 79 accessions of 23 *Oryza* species, 6 related genera, 5 outgroup taxa (2 monocots, 3 dicots) and 6 F_1s and BC_1s derived from crosses of *O. sativa* with wild species were hybridized individually with ^{32}P-labeled total genomic DNA from 12 *Oryza* species: *O. ridleyi*, *O. longiglumis*, *O. granulata*, *O. meyeriana*, *O. brachyantha*, *O. punctata*, *O. officinalis*, *O. eichingeri*, *O. alta*, *O. latifolia*, *O. australiensis*, and *O. sativa*. The labeled genomic DNAs representing the *ridleyi* and *meyeriana* complexes cross-hybridized best to all the accessions of their respective species, less to those representing other genomes of *Oryza* and related genera, and least to outgroup taxa. In general, the hybridization differential measured in terms of signal intensities was >50-fold under conditions that permit detection of 70–75% homologous sequences, both in the presence and in the absence of *O. sativa* DNA as competitor. In contrast, when total DNAs representing other *Oryza* genomes were used as probes, species of the *O. ridleyi* and *O. meyeriana* complexes did not show any significant cross-hybridization (<5%). These results demonstrate that the genome(s) of both of these complexes are highly diverged and distinct from all other known genomes of *Oryza*. We, therefore, propose new genomic designations for these two species complexes: *GG* for the diploid *O. meyeriana* complex and *HHJJ* for the allotetraploid *O. ridleyi* complex. The results also suggest that the uniqueness of these genomes is not restricted to species-specific highly repetitive DNA sequences, but also applies to dispersed sequences present in single or low to moderate copy numbers. Furthermore these appear to share relatively more genome-specific repeat sequences between themselves than with other genomes of rice. The study also demonstrates the potential of total genomic DNA hybridization as a simple but powerful tool, complementary to existing approaches, for ascertaining the genomic makeup of an organism.

Key words *Oryza* · Species complex · Genomic constitution · Molecular divergence · Total genomic DNA hybridization

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Introduction

The genus *Oryza* to which cultivated rice (*O. sativa*, $2n$ = 24, *AA*) belongs has 22 wild species with $2n$ chromosome numbers of 24 or 48. Seventeen of these species represent the *AA*, *BB*, *CC*, *BBCC*, *CCDD*, *EE* and *FF* genomes. The genomic constitution of five species is unknown. These latter species have been grouped into two species complexes: the diploid *O. meyeriana* complex (*O. granulata*, *O. meyeriana*, *O. indandamanica*), and the tetraploid *O. ridleyi* complex (*O. ridleyi*, *O. longiglumis*) (Vaughan 1989). Cytological studies reveal that both *O. ridleyi* and *O. longiglumis* are allotetraploid and have similar genomes (Sitch et al. 1991; Katayama 1992), whereas the *O. granulata* genome is similar to that of *O. meyeriana* (our unpublished results) and *O. indandamanica* (Khush et al. 1990).

The conventional approaches used for assigning new genome(s) are based on morphological, physiological, and biochemical differences: crossing relationships, chromosome number, and chromosome pairing behavior of F_1 hybrids from crosses between species with known and unknown genomic constitution (the latter being the most important criterion for classifying rice genomes; Morinaga 1964). However, in most cases production of F_1 hybrids between cultivated and distantly related species is difficult, mainly owing to strong hybridization barriers. In fact, this has been the major reason for not assigning genome(s) to the species of the *O. ridleyi* and *O. meyeriana* complexes. Under these circumstances, where intersectional hybrids are difficult to produce, homology per se at the DNA sequence level, being central to chromosome pairing, can serve as a direct, simple index of the genomic makeup of a species. Therefore, we analyzed various *Oryza* species for homology/divergence at the DNA level to identify and, if possible, classify the genomes of the two species complexes.

Several molecular approaches are now available to determine genomic relationships among species. Some of these are based on thermal denaturation profiles of DNA (Sibley and Ahlquist 1986; King and Ingrouille 1987), restriction fragment length polymorphisms (RFLPs) of nuclear sequences (Wang et al. 1992), multilocus mini- and microsatellite-specific repeat sequences (Rayburn and Gill 1987; Aggarwal et al. 1994), and total genomic DNA hybridization both on Southern blots and in situ (Anamthawat-Jonsson et al. 1990). Among these, RFLPs and multilocus probes provide useful information, but these involve considerable effort in isolating and cloning genome-specific sequences. Further, such sequences might be localized to specific region(s) of the genome, and/or might evolve at different rates (Flavell 1986), and thus might not be true indicators of overall genomic differentiation among species. Similarly, the DNA renaturation approach involving DNA-DNA solution hybridization of single to low copy sequences, although informative, is cumbersome and time consuming. In comparison, the total genomic DNA hybridization approach provides a simple, semiquantitative assay to study similarities or dissimilarities spread over whole genomes in a given set of species. The technique has been widely used in both plants and animals to characterize wide hybrids, identify alien chromosome additions and translocations, and understand the genomic relationships among related group of species (Brandriff et al. 1991; Bennett et al. 1992; Orgaard and Heslop-Harrison 1994). However, the potential of this technique to ascertain and define the genomes of species with unknown genomic constitution has remained unexplored.

In the present investigation, we demonstrate that the genomic constitution of species can be inferred based on the novel approach of evaluating divergence at the molecular level through total genomic DNA hybridization. We provide evidence that the genomes of species either the *O. meyeriana* or *O. ridleyi* complex are quite similar but each complex is highly diverged from other species of *Oryza*, thus warranting new genomic assignments.

Materials and methods

Plant materials

The material used comprised 79 rice accessions belonging to 23 *Oryza* species, 6 related genera, 2 cultivated monocots (maize and sugarcane), 3 dicot (soybean cultivars) and 6 F_1s and BC_1s derived from interspecific crosses involving cultivated and wild species of *Oryza* (Table 1). Green leaves of these plant samples were obtained from the Genetic Resource Center (GRC) and the Plant Breeding, Genetics, and Biochemistry division (PBGB), International Rice Research Institute. The F_1 and BC_1 progenies from interspecific crosses were available from PBGB.

DNA extraction, digestion, and Southern blotting

Total genomic DNA was isolated from green leaves (approximately 10 g) according to the method of Dellaporta et al. (1983) with minor modifications. The quality and quantity of DNA were determined both spectrophotometrically as well as visually by ethidium bromide staining on agarose gels.

For Southern blotting, 6–7 μg DNA from each sample was digested with *Dra*I and electrophoresed overnight in a submerged horizontal agarose gel (0.9%) at 30 V in 1 × TAE buffer (40 mM TRIS-acetate, 1 mM EDTA, pH 8.0). In some cases, *Xba*I, *Eco*RV and *Sca*I were used in place of *Dra*I for DNA digestion. After electrophoresis, the gels showing approximately equal concentrations of digested DNA for all samples were used for Southern blotting onto Hybond N+ nylon membrane following the alkaline transfer protocol as detailed by the manufacturer (Amersham, USA). Five identical sets of Southern blots, each representing all the 96 plant samples, were made and each set of blots was reprobed 3–4 times after stripping.

Probe DNA and labeling

The total genomic DNA of 12 different *Oryza* species (Table 2) was used as probe in separate hybridization experiments. In each case, approximately 1 μg *Hin*fI-digested DNA was labeled with ^{32}P-αdCTP using the nick translation protocol (Amersham, USA). The labeled DNA was denatured by keeping it in a heating block at 100° C for 7–8 min, and then plunging it into ice for 10 min before adding to the hybridization solution. The *Hin*dIII-digested λDNA, used as marker on the gels, was labeled along with the genomic DNA.

In some experiments involving total DNA of *O. granulata*, *O. brachyantha, O. punctata*, and *O. ridleyi* as probe, the effect of genomic blocking on hybridization was also evaluated using unlabeled DNA from rice cultivar IR31917-45-3-2 as specific competitor. For this purpose, ~1 μg DNA of the latter was sheared to 200–500 bp fragments by autoclaving for 10 min, denatured similar to the probe DNA, and then added in 10-, 20-, 50- and 100-fold excess to labeled probe at the time of hybridization.

Southern hybridization

The membranes were prehybridized for 6–8 h in 5 × SSPE, 5 × Denhardt's, 0.5% SDS (Sambrook et al. 1989) along with sheared denatured salmon sperm DNA (100 μg/ml hybridization mixture) as a nonspecific blocking agent at 64° C. The ^{32}P-labeled probe was added at 20 ng/ml ($1–5 \times 10^6$ cpm/ml) to the same prehybridization solution. The membranes were allowed to hybridize with the probe DNA for 16–18 h with gentle shaking at 64°–65° C.

Table 1 Plant materials used in the study. (? Unknown genome, *Lane no.* number of lanes on the autoradiograph, *CRRI* Central Rice Research Institute, Cuttack, India, *PBGB*, *IRRI* Plant breeding, Genetics and Biochemistry Division, International Rice Research Institute)

Lane no.	Species	Genome	Accession	Origin	Lane no.	Species	Genome	Accession	Origin
1	*Oryza sativa*	*AA*	IR-31917	PBGB, IRRI	49			103787	Colombia
2			IR-56	PBGB, IRRI	50	*O. grandiglumis*	*CCDD*	105155	Brazil
3			IR-64	PBGB, IRRI	51			105157	Brazil
4	*O. rufipogon*	*AA*	105908	Thailand	52			105560	Brazil
5			105909	Thailand	53			105669	Brazil
6			105910	Thailand	54	*O. malamphuzhaensis*	*BBCC* ?	105223	India
7			106412	Vietnam	55			105328	India
8			106423	Vietnam	56			105329	India
9	*O. longistaminata*	*AA*	103886	Tanzania	57	*O. ridleyi*	Unknown	100820	Thailand
10			103890	Senegal	58			100821	Thailand
11			103902	Tanzania	59			106028	Thailand
12	*O. barthii*	*AA*	101937	Senegal	60			105973	Indonesia
13	*O. nivara*	*AA*	103407	Sri Lanka	61	*O. longiglumis*	Unknown	105146	Indonesia
14			105721	Cambodia	62			105147	Indonesia
15			106185	India	63			105148	Indonesia
16	*O. glumaepatula*	*AA*	100969	Suriname	64			105562	Indonesia
17	*O. perennis*	*AA*	104823	Thailand	65	*O. australiensis*	*EE*	100882	via CRRI, India
18	*O. punctata*	*BB*	103896	Tanzania	66			103318	Australia
19			104064	Nigeria	67			105269	Australia
20			105690	Kenya	68			105272	Australia
21			105980	Cameroon	69	*O. brachyantha*	*FF*	101232	Sierra Leone
22		*BBCC*	100884	India	70			94-10482	via CRRI, India
23			101409	Ghana	71	*O. granulata*	Unknown	100879	India
24			104975	Kenya	72			102118	Thailand
25	*O. officinalis*	*CC*	100896	Thailand	73			104503	Malaysia
26			101116	Philippines	74			106449	India
27			101399	Vietnam	75			104986	via CRRI, India
28			105100	Brunei	76	*O. meyeriana*	Unknown	wsp-90-5	??
29			105220	Indonesia	77			106473	Philippines
30			100176	via CRRI, India	78			106474	Philippines
31	*O. rhizomatis*	*CC*	103421	Sri Lanka	79	*O. indandamanica*	Unknown	105694	India
32			105448	Sri Lanka	80	*Porteresia coarctata*	·	104502	Bangladesh
33			105449	Sri Lanka	81	*Leersia tisseranti*	·	101384	New Guenia
34	*O. eichingeri*	*CC*	101424	Uganda	82	*Leersia perrieri*	·	105164	Madagascar
35			105181	Uganda	83	*Rhynchoryza subulata*	·	100913	Argentina
36			105182	Uganda	84	*Hygroryza aristata*	·	105457	Sri Lanka
37			105408	Sri Lanka	85	*Chikusichloa aquatica*	·	106186	Japan
38			105413	Sri Lanka	86	Maize	·	Local cultivar	Philippines
39	*O. minuta*	*BBCC*	101089	Philippines	87	Sugarcane	·	Local cultivar	Philippines
40			101141	Philippines	88	Soybean	·	cv. UPSY-4	Philippines
41			103876	Philippines	89	Soybean	·	cv. Clark	Philippines
42			105253	Philippines	90	Soybean	·	Local cultivar	Philippines
43	*O. alta*	*CCDD*	100888	via CRRI, India	91[a]	BC_1	*AAE*	WHD-1145-1	PBGB, IRRI
44			100952	via CRRI, India	92[b]	F_1	*AF*	WHD-457-1	PBGB, IRRI
45			100967	Suriname	93[c]	BC_1	*AAF*	WHD-188-1	PBGB, IRRI
46			105143	Guyana	94[d]	F_1	*A*?	WHD-614-1	PBGB, IRRI
47	*O. latifolia*	*CCDD*	100914	Mexico	95[e]	BC_1	*AA*?		PBGB, IRRI
48			100955	??	96[f]	F_1	*AC*		PBGB, IRRI

[a] 91, (*O. sativa* IR31917-45-3-2 × *O. australiensis* Acc. 100882) × IR31917-45-3-2
[b] 92, *O. sativa* IR56 × *O. brachyantha* Acc. 101232
[c] 93, (*O. sativa* IR56 × *O. brachyantha* Acc. 101232) × IR56
[d] 94, *O. sativa* IR31917-45-3-2 × *O. granulata* Acc. 100879
[e] 95, (*O. sativa* IR31917-45-3-2 × *O. granulata* Acc. 100879) × IR31917-45-3-2
[f] 96, *O. sativa* cv. UPR 63 × *O. officinalis* Acc. 101116

Posthybridization washings and signal detection

The membranes were monitored for hybridization signal following two different washing regimes. All the post hybridization washings were carried out at 60°–64° C for 30 min each. First, membranes were washed in 3 × SSC (20 × SSC is 0.3 M NaCl, 0.03 M sodium citrate, pH 7.0), 0.1% SDS, to remove weakly hybridized and unhybridized probe. Subsequently, the membranes were washed twice in 2 × SSC, 0.1% SDS, followed by a rinse in 2 × SSC at room temperature. The wet membranes were wrapped in Saran wrap and exposed for autoradiography for 30 min, 60 min, 3 h, or 14 h. The next day, the same membranes were further washed once each in 1 × SSC, 0.1% SDS and 0.5 × SSC, 0.1% SDS, and twice in 0.1 × SSC, 0.1% SDS followed by a final rinse in 2 × SSC at room temperature. The membranes were again autoradiographed for 30 min, 60 min, 90 min, 3 h, 4.5 h, 14 h, or 28 h. Afterward, the

Table 2 *Oryza* species from which total genomic DNA was used in hybridization

Species	Accession	Genome
O. sativa	IR-64	*AA*
O. punctata	105980	*BB*
O. officinalis	101116	*CC*
O. eichingeri	105413	*CC*
O. australiensis	100882	*EE*
O. brachyantha	101232	*FF*
O. alta	100967	*CCDD*
O. latifolia	100914	*CCDD*
O. ridleyi	100821	Unknown
O. longiglumis	105669	Unknown
O. granulata	100879	Unknown
O. meyeriana	106473	Unknown

membranes were stripped of the bound radioactive probe before reprobing.

Stringency of hybridization and washing

The conditions for hybridization and washings were optimized based on standard filter hybridization kinetics (Anderson and Young 1985) and known parameters for DNA hybrid duplex stability (Meinkoth and Wahl 1984), effect of mismatched base pairs on melting temperature of the hybrid DNA duplex (Bonner et al. 1973), and taking the average %G + C content of cereals to be 45.5% (Swanson and Webster 1975). Accordingly, the hybridization conditions used were permissive for stable hybridization of DNA sequences with 70–75% homology, following a first-order kinetics of hybridization for double-stranded DNA probes to excess target sequences on the blots. Further, the stringency of the two posthybridization washing regimes (2 × SSC and 0.1 × SSC) favored detection of either partially related sequences (70–75%) or almost identical sequences (>95% matched) on the autoradiographs, respectively.

Signal quantification

Differences in the hybridization signal obtained on autoradiographs were visually analyzed and also measured semiquantitatively on a microcomputer-based image-digitizing system, the Computing Laser Densitometer, Model 300A ImageQuant 3-22 (Molecular Dynamics, USA). The total amount of hybridization intensity per lane on the autoradiographs, was calculated in optical density units in volume integration mode after subtracting the background value, which was determined from reading an unhybridized lane. Subsequently, the values were averaged over each species and normalized relative to the hybridization intensity of lanes representing the genomic probe (lanes showing maximum hybridization). For estimates of hybridization differential, the autoradiographs with shorter exposure times (60, 90 min, 3 h) were found to be best because the most intense signals did not saturate the detector response.

Results

The Southern profiles *Dra*I-digested DNA of *Oryza* species and other outgroup taxa, obtained after hybridization with ^{32}P-labeled total genomic DNA under different washing stringencies, are shown in Figs. 1–6. In each case, striking differences in hybridization pattern and signal strength were evident across the species tested. Each genomic probe revealed a highly specific pattern of hybridization, suggesting the presence of specific genomic complexes among the taxa analyzed. These differences were most pronounced in the case of specific genomic probing with the *O. meyeriana* and *O. ridleyi* complexes, followed by that of *O. brachyantha*, wherein the hybridization differential was of the order of 40- to 50-fold. In comparison, it varied only 10- to 20-fold across all the taxa, when other genomes (*AA*, *BB*, *CC*, *EE*, *FF*, *BBCC*, *CCDD*, Table 2) were used as probes. The quantification of hybridization intensity profiles, obtained after probing with six genomes of *Oryza* (Table 3), confirmed the observations made visually on the autoradiographs.

The hybridization patterns in general were characterized by conspicuous bands overlying the smears of hybridization, representing families of repetitive DNA sequences, specific to the probe (genomic DNA) used. In contrast, the strong smear that constituted ~80% of the total signal present in all lanes represents the hybridization of dispersed sequences of low to moderate or single copy numbers available in the hybridizing genomic DNA. The hybridization of blots prepared with restriction enzymes *Sca*I, *Xba*I or *Eco*RV revealed different families of repeat specific DNA fragments but without any apparent effect on the overall hybridization differential (data not shown).

Presence of plant genome-specific DNA repeat sequences

Hybridization profiles revealed the presence of a few families of DNA repeat sequences that were common to all the species tested, irrespective of the genomic DNA used as probe. One of the most conspicuous of these repeat families was visible as a strongly hybridizing polymorphic band in the range of ~8.0–9.5 kb across all the *Oryza* species as well as all other taxa (Figs. 1–6). The role of such conserved repeat sequence families in the structure and function of the plant genome needs further investigation.

Effect of washing stringency and blocking DNA

In general, increasing the stringency of washing from 2 × SSC (0.3 M Na^+) to 0.1 × SSC (0.015 M Na^+), led to an overall reduction in the hybridization intensity on the

Fig. 1, 2 Southern blots of *Dra*I-digested DNA of 96 representative *Oryza* species and other taxa (see Table 1) hybridized with ^{32}P-labeled total genomic DNA of *O. granulata* Acc. 100879: **1** 14 h exposure after 2 × SSC low stringency wash; **2** 14 h exposure after 0.1 × SSC high stringency wash. Note the differences in hybridization intensities across lanes 71–79 and 94–95 in comparison with the rest of the lanes. *Small arrowheads* indicate repeat sequence bands specific to taxa of the *O. ridleyi* complex (lanes 57–64)

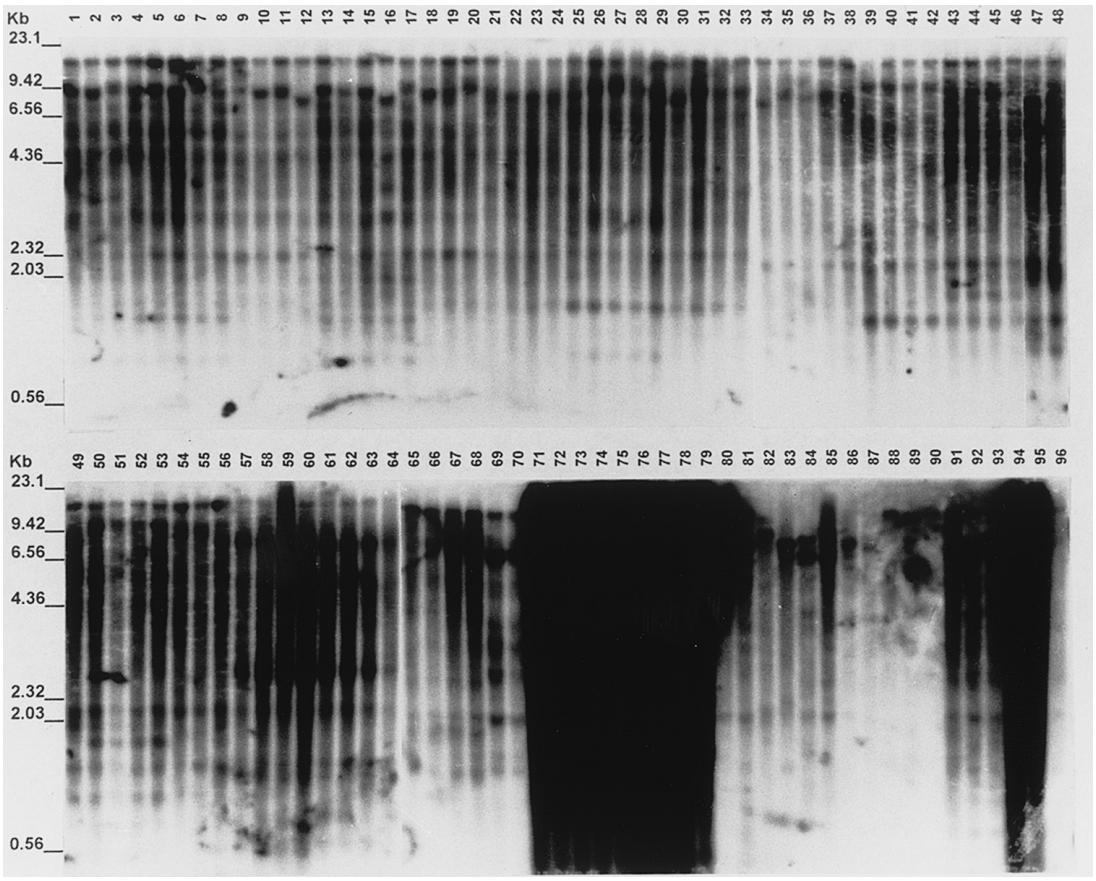

Fig. 1 for legend see p.4

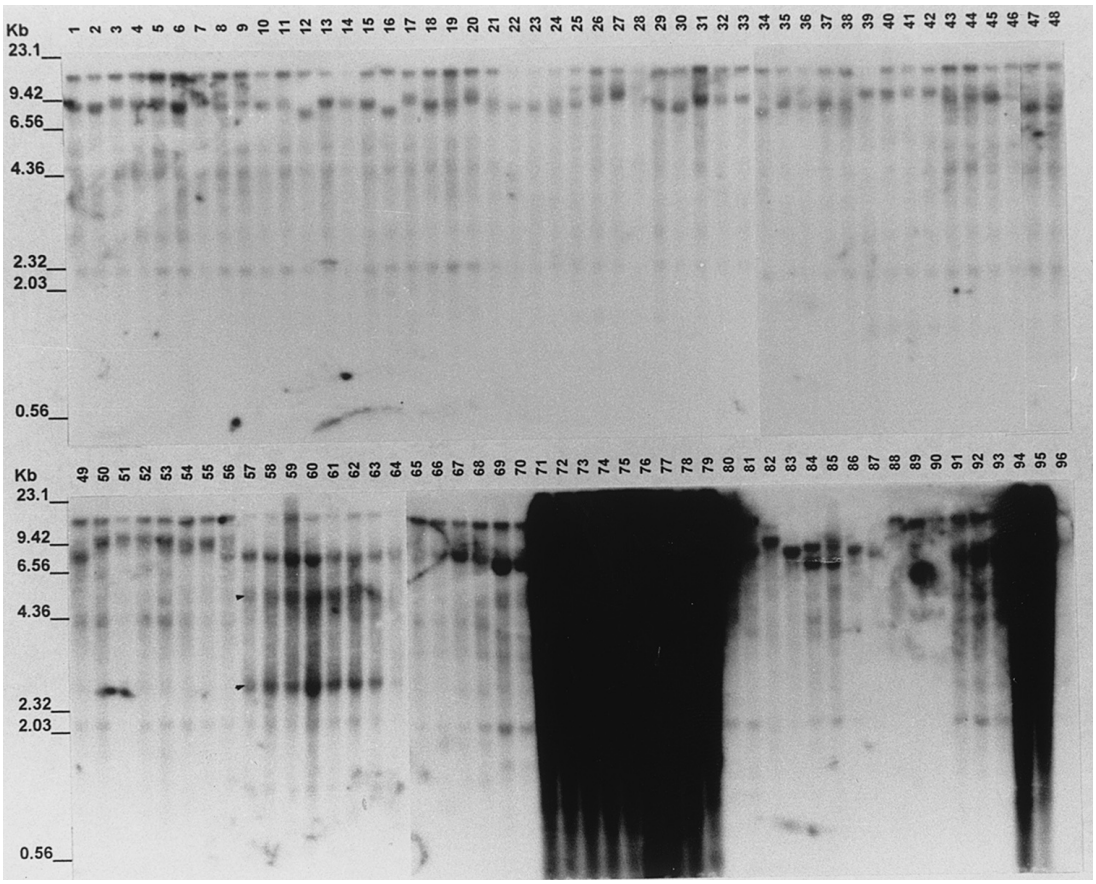

Fig. 2 for legend see p.4

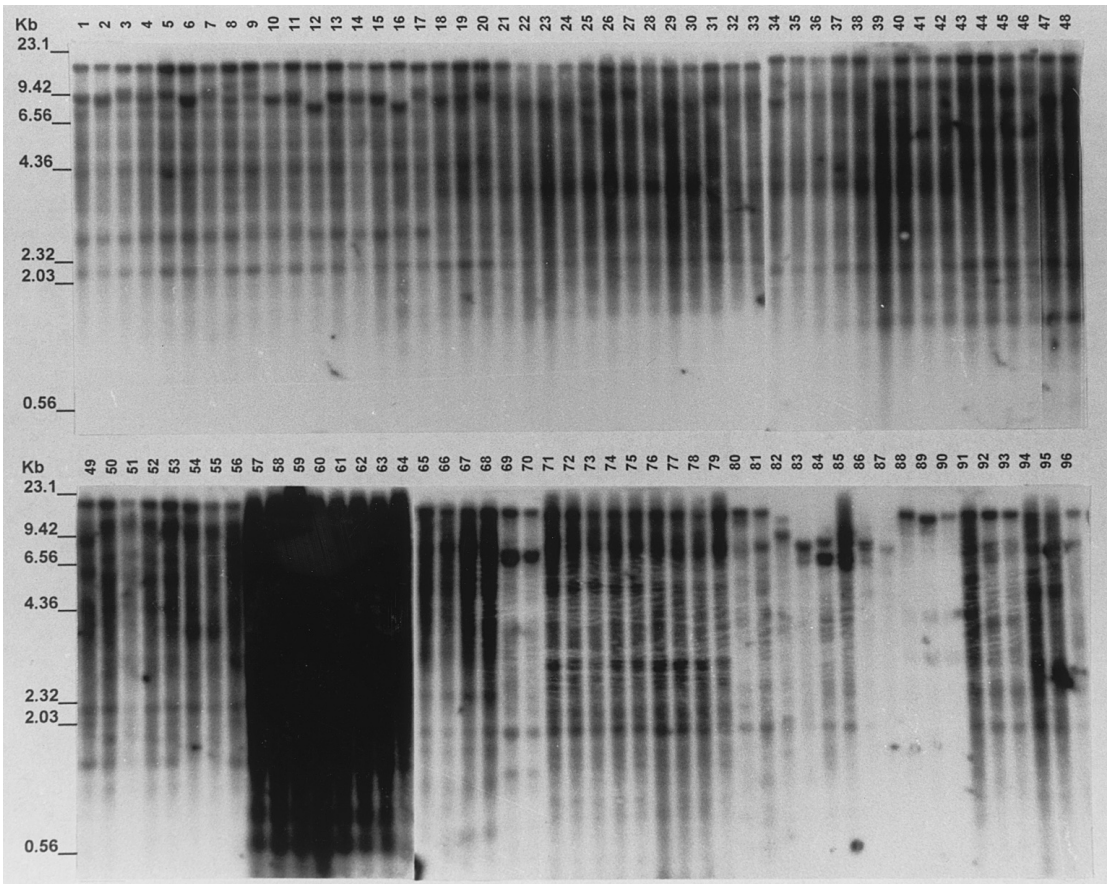

Fig. 3 for legend see p.7

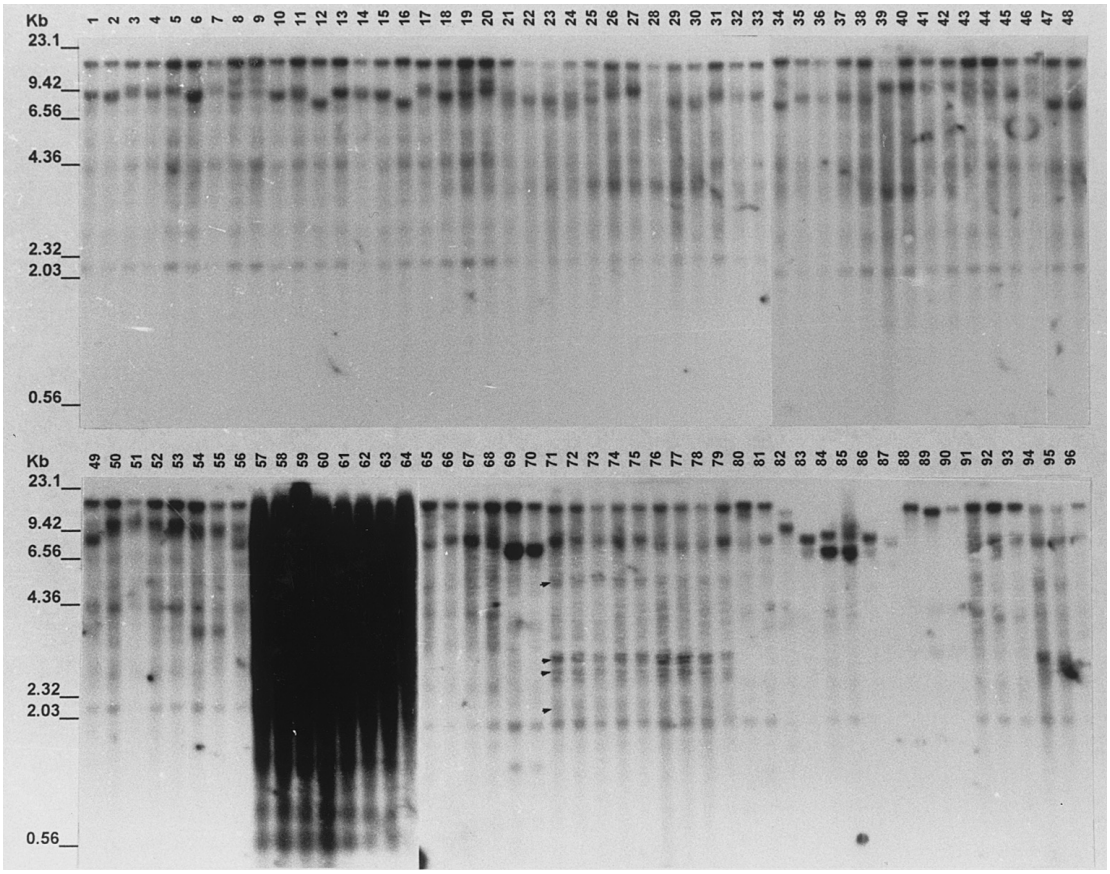

Fig. 4 for legend see p.7

blots. This effect was more pronounced for DNA from taxa other than those belonging to the probe DNA complex, thereby further increasing the hybridization differential considerably across the species (see Table 3; compare lanes, Figs. 1, 2; and 3, 4). Moreover, bands representing repetitive DNA sequences were more easily detectable after high stringency washing with 0.1 × SSC.

The effect of genomic blocking on hybridization was negligible. Inclusion of *O. sativa* DNA as specific competitor (block) in various proportions (blocking DNA to probe = 10:1, 20:1, 50:1, and 100:1) had no effect on the hybridization differential across the taxa. However, similar to the effect of high stringency washing inclusion of competitor DNA also increased the prominence of genome-specific repetitive bands in all cases.

Probing with genomic DNA representing the *O. meyeriana* complex

The *Dra*I fragments of all the *Oryza* species and other taxa that hybridized with genomic DNA of *O. granulata* (Acc. 100879), at two of washing stringencies are shown in Figs. 1 and 2 (Fig. 1, 14 h exposure after 2 × SSC wash to discriminate 70–75% homologous sequences; Fig. 2, 14 h exposure after 0.1 × SSC wash to display >95% matched DNA sequences). Overall appraisal of the hybridization signal, evident from autoradiographs at different exposure times, revealed that the probe DNA hybridized almost exclusively to the species of its own complex, i.e., *O. granulata*, *O. indandamanica*, and *O. meyeriana* (lanes 71–79, Figs. 1, 2), and the F_1 and BC_1 (lanes 94, 95) derived from a cross of *O. granulata* with *O. sativa* (see Table 1). In comparison, the intensity of hybridization to all the remaining *Oryza* species and other taxa was almost negligible (less than 5%), irrespective of the level of stringency used (see Fig. 1. Table 3). In separate experiments, *O. granulata*-derived monosomic alien addition lines could easily be identified following a similar genomic hybridization procedure independent of the stringency level and blocking DNA used (unpublished results).

Analysis of autoradiographs from longer exposures, i.e., a 14 h exposure at a lower washing stringency of 2 × SSC (Fig. 1), and 14 h (Fig. 2) and 28 h exposures after higher stringency washes of 0.1 × SSC, revealed a family of repeat sequence fragments at ~1.2, 2.4, and 4.8 kb specific to taxa of the *O. ridleyi* complex (lanes 57–64, Fig. 2). Secondly, a ~5- to 10-fold differential was visible in the hybridization intensities of rice entries other than those of the *O. meyeriana* complex (lanes 1–70, Fig. 2) vis-à-vis related genera (lanes 80–85). This differential was even more pronounced in the case of the outgroup taxa, maize, sugarcane, and soybean (lanes 86–90, Fig. 2), wherein no hybridization signal was observed except for the plant-specific repeat sequences detailed above.

Inspection of the hybridized lanes from shorter exposure times (30, 60 min), revealed the presence of a ladder of restriction fragments starting at ~1.0 kb (1.0, 2.0, 3.0, 4.0 kb) that were visible in all the species of the *O. meyeriana* complex and one band of ~6.0 kb that was specific only to the *O. granulata* accessions. This ~6.0 kb repeat sequence band, specific to *O. granulata* could also be identified in its hybrid and backcross derivative. The pattern of such repeat DNA-specific bands was found to vary with different restriction enzymes. The *Eco*RV digested DNA in particular, exhibited the most complex pattern (data not shown).

The results obtained from hybridization with genomic DNA from *O. meyeriana* Acc. 106473 were essentially similar to those obtained with *O. granulata*, except that it did not reveal the 6.0 kb repeat sequence band specific to *O. granulata* genomic DNA (see above). Instead, it showed two bands of ~4.8 and 6.2 kb, specific only to the *O. meyeriana* and *O. indandamanica* genomes.

Probing with genomic DNA representing the *O. ridleyi* complex

The genomic DNA of *O. ridleyi* Acc. 100821 and *O. longiglumis* Acc. 105669 were used as probes. Figures 3 and 4 show representative autoradiographs from hybridization with *O. ridleyi* genomic DNA as probe at two of stringency levels. Similar to the case of genomic probing specific to the *O. meyeriana* complex, here also the probes hybridized almost exclusively to lanes containing DNA of the probe complex and all the remaining taxa studied showed negligible signal (see 57–64, Figs. 3, 4; Table 3). The hybridization differential in signal intensity between species of the *O. ridleyi* complex vis-à-vis all the other species was >50-fold even at the lower stringency of washing, but was visibly less than that observed in the case of genomic probing with the *O. meyeriana* complex (Table 3). A closer inspection of the autoradiographs developed after 14 or 24 h at both stringencies of washing revealed: (a) the presence of repeat sequence restriction fragments at ~2.4, 2.6, 2.8 and 4.8 kb, specific only to the lanes of *O. meyeriana* complex species (see bands marked with arrowheads, lanes 71–79, Fig. 4); and (b) ~5- to 10-fold differential in hybridization signal intensity between the rest of the *Oryza* species (lanes 1–56, 65–79) as compared with the related genera (lanes 80–85) and 10- to 20-fold compared with other outgroup taxa (lanes 86–90, Figs. 3, 4). Further, the autoradiographs developed after 30 and 60 min at

Fig. 3, 4 Southern blots of *Dra*I-digested DNA of 96 representative *Oryza* species and other taxa (see Table 1) hybridized with ^{32}P-labeled total genomic DNA of *O. ridleyi* Acc. 100821: **3** 14 h exposure after 2 × SSC low stringency wash; **4** 14 h exposure after 0.1 × SSC high stringency wash. Note the differences in hybridization intensities across lanes 57–64 in comparison with the rest of the lanes. *Small arrowheads* indicate repeat sequence bands specific to taxa of the *O. meyeriana* complex (lanes 71–79)

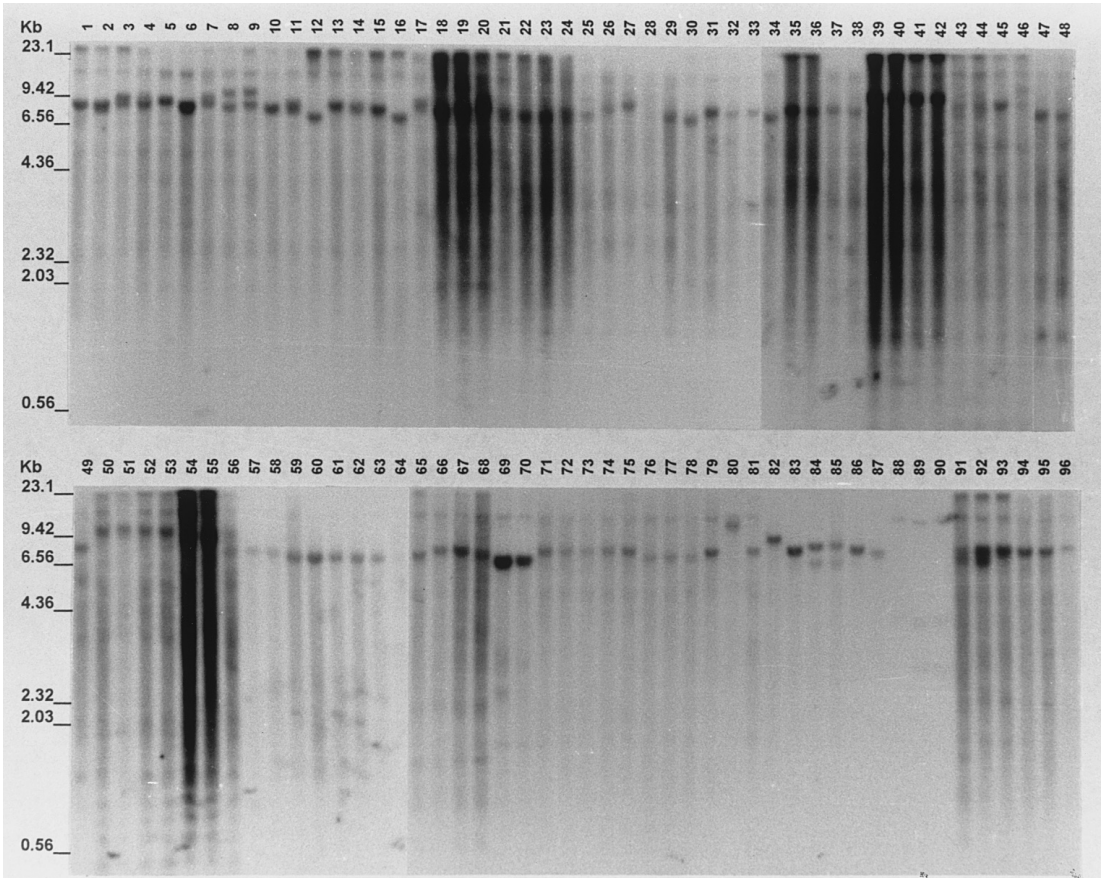

Fig. 5 for legend see p.9

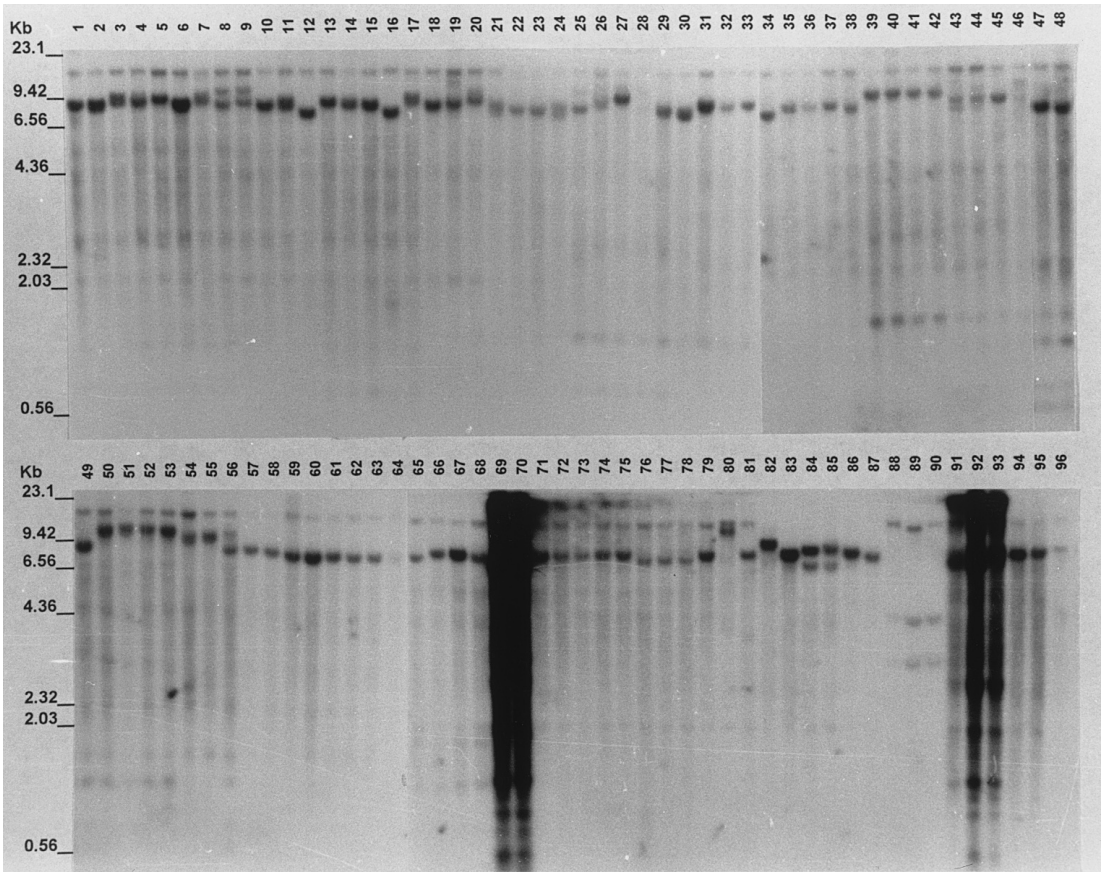

Fig. 6 for legend see p.9

high washing stringency, revealed the presence of a repeat sequence family specific to the *O. ridleyi* complex starting at ~6.0 kb (0.9, 1.2, 1.8, 2.4, 3.0, 3.6, and 4.8 kb). Similar results were obtained when *O. longiglumis* DNA was used as the genomic probe.

Probing with genomic DNA representing *AA*, *BB*, *CC*, *CCDD*, *EE* and *FF* genomes of the *Oryza* complex

The Southern blots of *Dra*I-digested DNA of the *Oryza* species and other taxa were also hybridized with labeled total genomic DNA from other rice species representing *AA*, *BB*, *CC*, *CCDD*, *EE*, and *FF* genomes (Table 2), separately. Figures 5 and 6 show the representative hybridization patterns obtained after probing with *O. punctata* and *O. brachyantha* genomic DNA, respectively. In all the experiments, the genomic probe tested cross-hybridized more strongly and specifically to lanes representing its own genome and at a low to moderate level to the rest of the taxa studied. In each case, the overall level of hybridization was observed to be minimal for species of the *O. meyeriana* and *O. ridleyi* complexes, followed only by the outgroup taxa (Table 3, also compare, lanes 57–64 and 71–79 with lanes 80–90 and the remaining ones, Fig. 5). Moreover, except when probing with the *FF* genome, the hybridization differential observed across various taxa was 2 to 3 orders of magnitude less than that observed with genomic probing specific to the *O. meyeriana* and *O. ridleyi* complexes (Table 3). In contrast, probing with *O. brachyantha* DNA (*FF* genome) exhibited a hybridization differential as striking as that obtained with *O. granulata* genomic probing. Furthermore, probing with different genomic DNA probes revealed a highly variable pattern of repeat sequence-specific bands among the taxa studied.

Discussion

In the last two decades, a number of studies have demonstrated the utility and potential of various molecular tools to solve many of the problems and controversies of evolution and biosystematics that have remained unsolved in spite of best efforts made through conventional approaches (Avise 1994). In the *Oryza* complex, the genomic constitution of species belonging to the *O. ridleyi* and *O. meyeriana* complexes remains unknown. This has mainly been due to limited success in obtaining intersectional hybrids to analyze their meiotic behavior. To circumvent this problem, we analyzed the genomic constitution of these two complexes directly at the molecular level using a total genomic DNA hybridization approach.

In cereals, about 55–70% of the genomic DNA consists of highly repetitive interspersed and tandem repeats (Flavell 1986). The estimate for similar DNA sequences in rice, which has the smallest genome among the cereals, is around 52% (Deshpande and Ranjekar 1980). Total genomic DNA hybridization mainly makes use of the genetic variation that resides in these repetitive DNA sequences distributed throughout the genome (Anamthawat-Jonsson et al. 1990). Hence, it has the potential of providing reliable estimates of genetic variation at the DNA level, which is central to genome evolution and differentiation. In the present study, genetic variation was quantified (using total genomic DNA as probe) through Southern hybridization, which provides precise indices of DNA homology/divergence. DNA sequences with varying degrees of homology or mismatch can be detected just by modulating the hybridization and washing conditions. The distantly related sequences with high degrees of mismatch (divergence) are analyzed under relaxed conditions of hybridization, which favor a relatively higher discrimination ratio (Anderson and Young 1985). In contrast, closely related DNA sequences can be discriminated by the inclusion of excess unlabeled blocking DNA as specific competitor to the hybridizing probe (Anamthawat-Jonsson et al. 1990). The inability of the blocking DNA to exert its effect as a specific competitor further indicates that the hybridizing DNA sequences are highly diverged.

Divergence of *O. ridleyi* and *O. meyeriana* complex species

The results show that the differences in the hybridization profiles were genome specific but not species specific. Thus, different species carrying similar genomes were indistinguishable from each other on genomic probing. A similar situation was observed for species/accessions belonging to the *O. ridleyi* and *O. meyeriana* complexes, indicating that the genomes of species in each of the two complexes are identical. Southern hybridization profiles and quantification of their signal intensities revealed that species belonging to the *O. ridleyi* and *O. meyeriana* complexes are highly diverged from all other *Oryza* species at the molecular level.

Probing with DNA representing the two species complexes revealed a 40- to 50-fold differential in the hybridization intensities at levels of stringency that are permissive for the detection of 70–75% homologous (25–30% divergent) DNA sequences. This hybridization differential was not affected when washing stringency was raised to 0.1 × SSC, or upon inclusion of *O. sativa* (*AA* genome) DNA as the competitor. These results indicate that the two species complexes have diverged much more than 25–30% from all other genomes of

Fig. 5, 6 Southern blots of *Dra*I-digested DNA of 96 representative *Oryza* species and other taxa (see Table 1) hybridized with ^{32}P-labeled total genomic DNA from: **5** *O. punctata* Acc. 105980: **6** *O. brachyantha* Acc. 101232. Washing stringency, 2 × SSC; 3h exposure. Note that hybridization differential across lanes is considerably less in the case of probing with a *BB* genome (**Fig. 5**) compared with that of an *FF* genome (**Fig. 6**)

Table 3 Per cent hybridization intensities obtained after probing with genomic DNA of six rice species, quantified from autoradiographs developed after 3 h exposure at two different washing stringencies

Lanes[a]	Genome	% Hybridization signal (relative to rice species representing the probe DNA)											
		BB(*O. punctata*)		*CC*(*O. officinalis*)		*EE*(*O. australiensis*)		*FF*(*O. brachyantha*)		Unknown(*O. ridleyi*)		Unknown(*O. granulata*)	
		2 × SSC	0.1 × SSC	2 × SSC	0.1 × SSC	2 × SSC	0.1 × SSC	2 × SSC	0.1 × SSC	2 × SSC	0.1 × SSC	2 × SSC	0.1 × SSC
1–17	*AA*	16.4	13.0	5.0	3.5	9.8	9.4	7.5	4.4	2.6	1.5	2.9	1.2
18–20	*BB*	100.0	100.0	9.8	5.3	16.8	10.4	5.6	4.1	3.5	2.7	2.7	1.0
21–24	*BBCC*	56.1	45.3	24.0	12.1	15.5	7.4	3.1	1.7	3.4	1.6	2.5	0.9
25–30	*CC*	12.1	7.9	100.0	100.0	20.7	8.4	3.4	1.9	3.3	2.8	4.7	1.2
31–33	*CC*	8.5	7.7	90.2	78.4	18.6 E_{max}	8.1	3.2	2.1	3.6	2.2	4.2	1.0
34–38	*CC*	33.2	32.4	24.0	18.0	14.1	9.7	3.6	3.0	3.1	1.8	2.3	0.8
39–42	*BBCC*	55.5	63.4	41.5	29.3	38.3	54.4	4.9	3.5	4.6	2.5	3.1	1.0
43–46	*CCDD*	18.9	13.6	21.8	9.1	29.2	13.1	5.2	3.4	3.7	2.5	5.9	1.3
47–49	*CCDD*	17.9	11.4	23.0	9.0	27.6	13.5	7.7	4.3	4.1	2.7	5.4	1.7
50–53	*CCDD*	18.1	12.6	21.4	9.4	23.3	12.7	6.2	4.3	4.1	1.8	6.0	1.2
54–56	*BBCC*	42.6	60.5	36.5	25.6	25.6	12.4	5.8	2.9	3.7	1.8	2.0	0.6
57–64	Unknown[b]	6.1	5.3	3.9	2.7	9.2	6.2	2.3	1.6	100.0	100.0	11.5	4.3
65–68	*EE*	15.8	3.6	22.2	9.1	100.0	100.0	4.4	1.6	4.3	3.1	3.1	1.2
69–70	*FF*	11.6	8.6	4.8	3.4	3.1	2.4	100.0	100.0	1.1	4.4	5.3	2.8
71–79	Unknown[c]	5.6	1.4	5.0	1.5	3.8	1.2	7.2	3.7	3.5	2.4	100.0	100.0
80–85	Related genera	3.0	1.1	0.6	0.0	0.4	0.3	4.5	3.5	0.5	0.0	2.0	0.6
86–90	Outgroup taxa	0.3	0.2	0.2	0.0	0.4	0.0	1.7	1.0	0.1	0.0	0.3	0.2
91	*AAE* (BC_1)	14.7	7.1	11.7	4.8	69.2	73.1	8.1	4.9	2.9	2.5	5.4	1.7
92	*AF* (F_1)	15.6	10.2	4.9	6.3	5.5	1.8	69.0	62.7 E_{max}	1.8	2.7	4.1	2.3
93	*AAF* (BC_1)	17.2	8.1	3.8	4.0	5.0	2.1	49.4 E_{max}	36.2	2.1	2.2	3.5	2.2
94	*AU*[d] (F_1)	14.2	4.8	4.1	3.1	4.1	1.4	7.6	4.1	4.1	2.9	75.4	68.9
95	*AAU*[d] (BC_1)	15.5	4.3	4.4	2.4	4.1	1.8	4.4	4.7	4.9	2.3	62.4	55.6
96	*AC* (F_1)	11.5	4.8	60.0	43.5	5.1	1.7	4.2	3.6	2.3	2.7	2.9	1.2

[a] Lanes on the autoradiographs; for details, see Table 1
[b] Unknown genome of *O. ridleyi* complex
[c] Unknown genome of *O. meyeriana* complex
[d] Haploid set of unknown genome of *O. granulata*

Oryza, the additional degree of divergence being directly proportional to the hybridization differential obtained at lower washing stringency. Assuming that each 3- to 4-fold hybridization differential reflects an additional DNA divergence of about 1%, one could make a conservative estimate of more than 35–40% overall divergence of the genomes in the two complexes relative to other *Oryza* species.

The presence of strong distinct signals in the hybridized lanes suggested that there are several families of DNA repeat sequences specific to the genome(s) of the two species complexes. The presence of genome-specific repeat sequences has been demonstrated for almost all the known genomes in rice (Zhao et al. 1989; Aswidinnoor et al. 1991). The relative abundance and distribution of such genome-specific repeat sequences are probably central to the observed high degree of divergence in these genomes. However, the divergence in the two complexes is seemingly a general feature of their respective genomes, also involving single copy and low to moderately repeated DNA sequences, evidenced as strong smears along any given hybridized lane, irrespective of the restriction enzyme used for digesting the DNA used on the blot. The very high level of divergence specific to single and low copy DNA sequences was also apparent in *O. granulata* (*O. meyeriana* complex) from the analysis of 52 rice cDNA and genomic RFLP probes distributed randomly over the molecular map of rice (Aggarwal et al. 1996). Wang et al. (1992), using 25 single copy RFLP probes, observed that the species belonging to these two complexes (*O. ridleyi*, *O. longiglumis*, *O. meyeriana*) were the most diverged and genetically distant from the remaining species of rice. In another experiment, we have analyzed the plant materials tested in the present study using 45 RFLP markers covering all rice chromosomes, to determine the divergence specific to unique, single and low copy genomic DNA sequences. The preliminary findings (unpublished) of the study also indicate that the *O. ridleyi* and *O. meyeriana* complexes have highly diverged and unique genomes, and that genomic differentiation in these is evenly spread over the single copy DNA sequences, as revealed by hybridization of total genomic DNA.

New genomic designations for species in the *O. ridleyi* and *O. meyeriana* complexes

The hybridization differentials observed on probing with genomic DNA of different *Oryza* species (Tables 2, 3) clearly established that the genomes in species of the *O. ridleyi* and *O. meyeriana* complexes are highly diverged and distinct from other known genomes of *Oryza*, and that these share relatively more genome-specific repeat sequences between themselves. Nevertheless, the genomes in both the complexes share relatively more similarity with other species of *Oryza* than with related genera (*Leersia*, *Rhynchoryza*, *Porteresia*, *Hygroryza*), and outgroup taxa (sugarcane, maize, and soybean). Further, the degree of divergence among the known genomes of rice, except for the *FF* genome, was much less than that observed for the two species complexes. Based on these results, we propose new genomic designations for these complexes: *GG* for the diploid *O. meyeriana* complex, and *HHJJ* for the allotetraploid *O. ridleyi* complex. The results suggest that generation of genome-specific repetitive DNA sequences has probably been central to the evolution of different genomes in the *Oryza* complex, whereas species differentiation within a genomic group has involved mainly micro-evolutionary changes like sequence divergence and chromosomal rearrangement. Involvement of repetitive DNA sequences (their type, position, amplification, deletion, turnover) in genomic evolution and speciation of various grasses and higher eukaryotes is now well accepted (Ahn et al. 1993; Moore et al. 1993).

Distinctive status warranting new genomic assignments to the above two complexes is further supported by earlier cytogenetic and morpho-physiological studies. Analysis of a few intersectional hybrids revealed that *O. ridleyi* is probably an allotetraploid having genomes different from *AA*, *BB*, *CC*, *CCDD*, *BBCC* and *EE*, and that the *O. meyeriana* genome is different from *AA* (Katayama and Onizuka 1979; Katayama et al. 1981; Katayama 1992). Similarly, analysis of F_1 hybrids also demonstrated that both *O. ridleyi* and *O. longiglumis* are allotetraploid and have similar genomes (Sitch et al. 1991) whereas the *O. granulata* genome is similar to that of *O. indandamanica*, but different from *AA* (Khush et al. 1990). Studies on seed protein profiles also suggested that species in the two complexes have genomes other than those currently recognized in the genus *Oryza* (Sarkar and Raina 1992).

The results presented here thus validate, at the molecular level, the present biosystemic status of the *O. ridleyi* and *O. meyeriana* complexes as the most diverged taxa in the genus *Oryza* in the subfamily Oryzoideae of the Poaceae (Gramineae) family. Moreover, the observation that genomes of these two complexes share relatively more genome-specific repeat sequences between themselves, and that members of these complexes overlap considerably in their habitat and geographic distribution suggest a relatively closer phylogenetic relationship between them. Our results also support the removal of *Leersia perrieri*, *L. tisseranti*, *Porteresia coarctata*, and *Rhynchoryza subulata* from the genus *Oryza*, as they showed the least similarity to any of the rice species, like other related genera (*Chikusichloa aquatica*, *Hygroryza aristata*) and outgroup taxa (maize, sugarcane, soybean).

This study demonstrates that the approach of revelation of the molecular divergence through total genomic probing can provide a reliable index of the genetic makeup of a species and is thus a powerful complementary tool for studying genomic evolution and biosystematics. It can also be used in situations where gene(s) suppressing chromosome pairing could affect conclusions regarding homology from analysis of the

meiotic behavior of chromosomes (Bennett et al. 1992). The present work provides the first evidence that genomic hybridization on Southern blots can be efficiently used in deducing the uniqueness of genome in the two species complexes of rice.

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