Molecular Cytogenetics of Rice and Its Wild Relatives

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1 Introduction

The genus *Oryza* is divided into 24 species based on morphological, cytological, biochemical, and molecular evidence including two cultivated species, *O. sativa*, Asian cultivated rice, and *O. glaberrima*, African cultivated rice, and 22 wild species represented by 10 genome types [1] (Table 6.1). Rice (*O. sativa*) is one of the most important staple crops in the world, and thus a number of cytogenetic studies have been done in rice. In recent decades, molecular cytogenetics, which combines molecular techniques and cytogenetics, has contributed to our understanding of chromosome and genome structure, phylogeny, and genome evolution in the *Oryza* species. Fluorescence in situ hybridization (FISH) has been an essential tool in molecular cytogenetics to visualize unique and repetitive DNA sequences on chromosomes using epifluorescence microscopy. FISH uses labeled nucleotides incorporated into DNA sequences, called a probe, to hybridize to complementary chromosomal DNA sequences. The probe is detected as a signal by a fluorescent microscope. FISH targets include interphase nuclei, somatic chromosomes, meiotic chromosomes, and extended DNA fibers. Recent advances in FISH allow one to distinguish and identify chromosomes, integrate genetic maps with specific chromosomes, compare physical and genetic distances, analyze distribution of repetitive DNA throughout a genome, associate genes or specific DNA sequences to chromosomal positions on chromosomes (Fig. 6.1), and determine genome origins in hybrids.

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2 Identification and Cytological Analysis of Individual Chromosomes of Rice

Distinguishing individual chromosomes of rice is very difficult due to their small size, similar morphology, and the lack of reliable banding patterns [2]. However, identification of rice chromosomes was achieved by quantitative analysis of uneven condensation patterns (CP) that appear on mitotic prometaphase chromosomes. Based on CPs and centromeric positions, 332 (92.2 %) out of 360 chromosomes were automatically identified, indicating that CP is a reliable and useful approach for chromosome identification [3]. A quantitative chromosome map of 12 rice chromosomes was developed using a chromosome image analyzing system (CHIAS), showing condensation patterns, total chromosomal length, and arm ratio [4, 5].

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Table 6.1 Summary of 24 species of the genus *Oryza*

Species	Chromosome number	Genome type	Genome size (Mb)	Reference
O. sativa	24	AA	389	[50]
O. nivara	24	AA	448	[51]
O. rufipogon	24	AA	439	
O. glaberrima	24	AA	357	[51]
O. longistaminata	24	AA	734	[52]
O. barthii	24	AA	611	[52]
O. meridionalis	24	AA	489	[53]
O. glumaepatula	24	AA	489	[53]
O. punctata	24	BB	425	[51]
O. officinalis	24	CC	651	[51]
O. rhizomatis	24	CC		
O. eichingeri	24	CC	562	[54]
O. minuta	48	BBCC	1,124	[51]
O. malapuzhaensis	48	BBCC		
O. latifolia	48	CCDD	1,125	[54]
O. alta	48	CCDD	1,008	[51]
O. grandiglumis	48	CCDD		
O. australiensis	24	EE	965	[51]
O. ridleyi	48	HHJJ	1,283	[51]
O. longiglumis	48	HHJJ		
O. granulata	24	GG	882	[51]
O. meyeriana	24	GG		
O. brachyantha	24	FF	362	[51]
O. coarctata	48	HHKK		

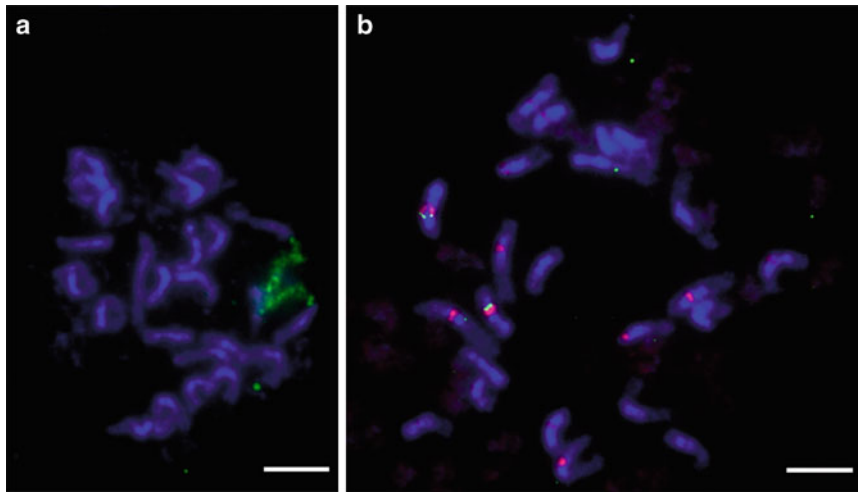

Fig. 6.1 Use of FISH to detect ribosomal DNA on mitotic prometaphase chromosomes of *O. sativa* L. ssp. *japonica* cv. Nipponbare. Chromosomes were counterstained with DAPI (*blue*). (**a**) 45S rDNA is detected on chromosome 9 as *green* signals. (**b**) 5S rDNA and BAC B1109A06 containing CentO repeat are detected on chromosome 11 as *green* and *red* signals, respectively. Bars represent 5 μm

FISH using chromosome-specific bacterial artificial chromosome (BAC) clones is also a powerful tool to identify chromosomes. Cheng et al. [6] used 24 chromosomal arm-specific BAC clones as probes for FISH to identify chromosomes at the meiotic pachytene stage. Pachytene chromosomes have higher resolution and more distinct cytological characteristics such as heterochromatin, euchromatin, and centromeres. The heterochromatin distribution pattern is highly conserved between *japonica* and *indica* subspecies, and an ideogram of pachytene chromosomes with the distribution of heterochromatin was developed based on the staining pattern of 4′,6-diamidino-2-phenylindole (DAPI) in *japonica* subspecies [6].

3 Physical Mapping of Low- or Single-Copy Sequences on Rice Chromosomes

Physical mapping of low- or single-copy sequences using FISH is an effective way to know the precise location of the sequences on chromosomes and their relation to chromosomal cytological features. In plants, the minimum length of detectable probes is around 1–3 kb [7–9]. However, the use of small probes has issues with low frequency of detection (e.g., distinguishing hybridization signals from background fluorescence noise) as well as inconsistency of the results [10]. To overcome this obstacle, large-insert genomic clones containing small target DNA sequences have been used successfully to map low- or single-copy DNA sequences in many plant genomes. For example, in rice, several BAC clones closely linked to a specific gene such as *Pi-b*, *Xa-21*, and *Pi-ta* [2] were successfully mapped onto mitotic chromosomes using FISH [7, 11, 12].

The integration of genetic and cytological maps is essential to understand chromosome structure and recombination frequency along chromosomes and to determine the precise location of target DNA sequences. Genetic maps do not reflect the actual physical distance across chromosomes because recombination is a nonrandom process across each chromosome. FISH mapping using large DNA clones containing genetic markers is useful to integrate cytogenetic and genetic maps. The rice genetic and cytogenetic maps have been integrated via 24 chromosomal arm-specific BAC clones containing RFLP markers [6]. Eighteen BAC/PAC clones containing RFLP markers were physically mapped onto rice pachytene chromosomes 10 and 5, respectively, to show the precise localization of each BAC clone on pachytene chromosomes. The comparison between genetic and FISH-based cytological maps illustrated the uneven distribution of genetic recombination along entire chromosomes [13, 14].

4 Repetitive Sequences Maintaining Chromosomal Structure and Function in the Genus *Oryza*

Plant genomes are composed of a significant fraction of repetitive sequences including tandem and dispersed repeats. Some of these repeats are responsible for maintaining chromosomal structure and function such as centromeres, telomeres, and other heterochromatic regions. Transposable elements—DNA transposons and RNA transposons (i.e., retrotransposons)—exist in all species of the genus *Oryza*. Among these elements, long terminal repeat (LTR) retrotransposons are the most abundant. LTR retrotransposons can increase the genome size of an organism in a relatively short time period because of their replicative mode of transposition and large element size. It is known that recent amplifications of LTR retrotransposons have contributed to the genome expansion of both *O. australiensis* (EE) and *O. granulata* (GG) resulting in genome sizes more than twice the size as all the AA genome species, including *O. sativa* [15]. The abundance and distribution patterns of LTR retrotransposons differ among families; some retrotransposons are dispersed genome wide, whereas others are concentrated in heterochromatic regions, such as centromeres, pericentromeres, or telomeres [16].

Centromeres are critical sites for sister chromatid cohesion and kinetochore assembly. Centromeres become visible as primary constrictions on mitotic and meiotic chromosomes.

Centromeres in plant genomes are often organized into megabase (Mb)-sized blocks, consisting mostly of repetitive sequences, such as satellite tandem repeats and retrotransposons. Because of the density of repetitive sequences, centromere organization is still poorly understood even in sequenced higher organisms. Rice is one of the few species where centromere organization is understood due to the use of cytogenetics to assist in the sequencing of several rice centromeres.

Rice centromeres primarily consist of two types of repetitive sequences, centromeric retrotransposons in rice (CRR), and centromere-specific tandem repeats (CentO [17–19]). CRR elements belong to the Ty3-gypsy superfamily, and FISH confirmed that they are enriched in centromeric regions. Unlike most plant LTR retrotransposons, CRRs are highly conserved in the genus *Oryza* and across the *Poaceae* family [17]. Southern blot results indicated that CRRs were present in almost all species of the genus *Oryza*, including the most distant relative of rice, *O. granulata* [20]. One exception is *O. brachyantha* (FF) where CRR elements were absent and their role has likely been replaced by another LTR retrotransposon named FRetro3 [20].

In contrast to centromeric retrotransposons, centromere-specific tandem repeats are variable within and between species of the genus *Oryza*. CentO is present in the AA, BB, CC, BBCC, CCDD, and EE genomes but not in the FF genome [19, 21, 22]. In *O. officinalis* (CC), the amount of CentO is quite low and another satellite repeat, CentO-C, is the dominant tandem repeat. FISH results showed that CentO exists at centromeres only in one pair of chromosomes in *O. officinalis* (CC), and the other ten centromeres contain CentO-C [23]. In *O. brachyantha* (FF), CentO has been completely replaced by CentO-F at all 12 centromeres and has no sequence similarity with CentO [21].

The size and intensity of FISH signals of repetitive sequences are correlated with the relative abundance of the repetitive sequences at their positions. Based on FISH signals of CentO in rice pachytene chromosomes and fiber-FISH results, the amount of CentO in the 12 chromosomes was estimated to range from 60 kb to 2 Mb [19] in size. Centromere 8 is the smallest and was the first completely sequenced centromere from any multicellular eukaryote [24, 25]. The amount of CentO dramatically changes from species to species, even in orthologous chromosomes (Fig. 6.2).

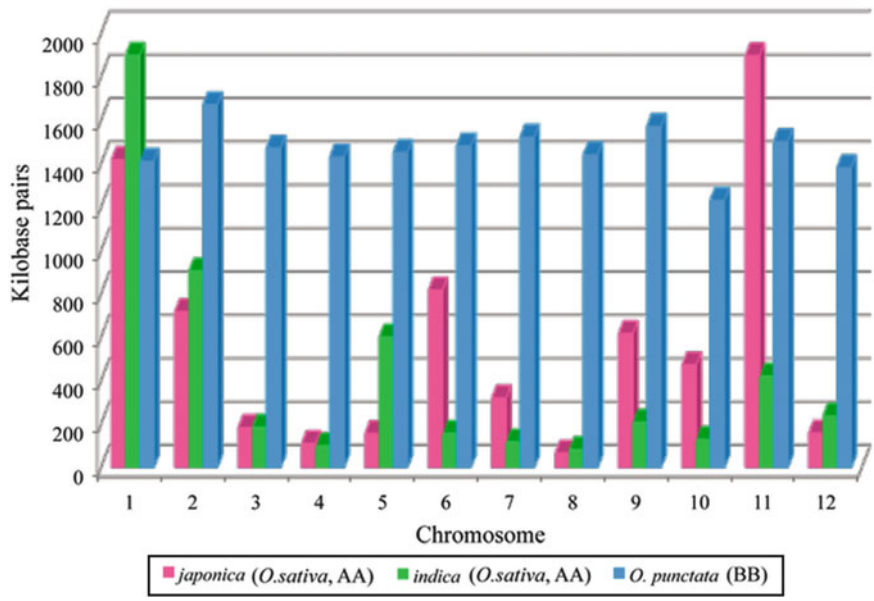

Fig. 6.2 The amount of CentO repeats in three genomes from two subtypes of *O. sativa* (AA) and *O. punctata* (BB) [19, 22]

A question arises as to why the amount of CentO is so different between orthologous *Oryza* chromosomes. Recently, 87 kb of CentO sequence in centromere 8 from the Kasalath variety (*indica* subspecies) was fully sequenced, and the result suggested that CentO was likely amplified in Kasalath after the divergence of subspecies *japonica* and *indica* [26]. However, the difference in size of CentO repeats can be more than fivefold between orthologous centromeres of *japonica* and *indica*. The simple sequence amplification model may not fully explain such a large difference after the divergence of these two subspecies from their common ancestor. Deletions of the CentO sequence in one species may have accompanied expansion in the other.

Telomeres serve as physical caps to protect the ends of chromosomes from degradation and fusion with each other. So far, telomere sequences from more than 120 eukaryotes have been identified and are included in the Telomere Database [27]. The telomeric repeat, TTAGGG, is highly conserved in vertebrates, invertebrates, plants, and some fungi. The TTTAGGG motif is abundant in telomeric regions of rice and other species of the genus *Oryza* [28]. The amount of telomeric repeats is variable among different chromosomes. Mizuno et al. [28] estimated that rice telomeres ranged in size from 5.1 kb in chromosome 7L to 10.8 kb in chromosome 6L by using a combination of the terminal restriction fragment (TRF) southern blot and fiber-FISH methods. In addition to telomeric repeats, 355-bp TrsA tandem repeats were identified in subtelomeric regions of rice and other *Oryza* species. Not all subtelomeric regions contain TrsA sequence and the TrsA-block sizes vary among rice varieties [29]. The function and origin of subtelomeric repeats are not clear, but it was proposed that they may act as buffer sequence to block the spread of gene silencing at telomeric positions [30]. Recent FISH mapping study of subtelomeric repeats in *Oryza* species provided interesting insight into the origins of centromeric and subtelomeric repeats. TrsC repeats, which are exclusive to the CC genome, were detected at multiple subtelomeric regions of *O. officinalis* (CC); however, the same repeats had multiple subtelomeric and centromeric loci in *O. rhizomatis* [23] (CC).

5 Applications of Extended DNA Fiber FISH

Extended DNA fiber FISH (fiber or EDF-FISH) is an improvement of FISH mapping in terms of spatial resolution down to a few kilobases and detection sensitivity up to 700 bp [31–33]. EDF-FISH has multiple applications including estimation of physical length and copy numbers of repetitive sequence and physical gaps between adjacent sequences (e.g., Fig. 6.3). EDF-FISH using telomere sequences and TrsA revealed the structure of the ends of chromosomes 6 and 12 of rice including the distance between telomere sequence and TrsA, their total lengths, and copy numbers [28, 34, 35]. EDF-FISH also revealed the intermingled structure between CentO and CRR [17, 19]. In the rice sequencing project,

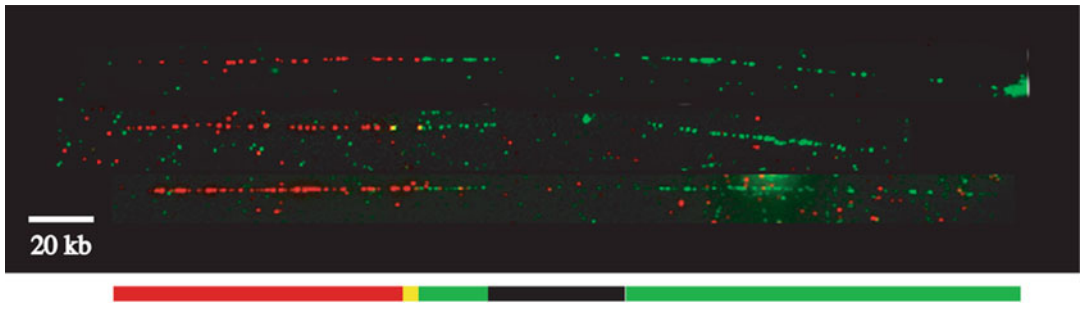

Fig. 6.3 Fiber-FISH of BAC clones 23I19 (*green*) and 92N12 (*red*) in *O. sativa* L. ssp. *japonica* cv. Nipponbare. *Yellow* fluorescence results from overlapping signal. A gap approximately 47.2 ± 14.9 kb in size consistently appears in the hybridization pattern of 23I19

EDF-FISH was successfully used to determine the size of unsequenced gaps for chromosomes 1, 4, and 10 [36–38].

6 GISH in *Oryza* Species

Genomic in situ hybridization (GISH) uses a total genomic DNA from one species as a probe to hybridize with target chromosomal DNA. The extent of hybridization depends on sequence identity or divergence between probe DNA and target chromosomal DNA. GISH allows one to identify the genomic contribution in interspecific hybrids and polyploids, alien chromosomes, introgressed chromosomes and chromosomal regions, and the cytogenetic relationship among related genomes. Table 6.2 summarizes the reports of GISH experiments in a variety of *Oryza* species. These studies show successful GISH experiments in polyploid *Oryza* species, indicating that their genomes are sufficiently diverged to be distinguished using GISH. The extent of GISH hybridization on *O. latifolia* (CCDD) and *O. minuta* (BBCC), using *O. officinalis* (CC) as a probe, indicates that the C and D genomes are more closely related to each other than the B and C genomes [39].

GISH can also be useful for breeding. GISH has been used to reveal the genomic constitution, including introgressed or translocated regions of chromosomes of interspecific hybrids and derived lines [40–44].

Table 6.2 GISH studies in the *Oryza* species

Target chromosomal DNA	Probe DNA	Reference
O. latifolia (CCDD)	*O. officinalis* (CC)	[39]
O. minuta (BBCC)	*O. officinalis* (CC)	[39]
Somatic hybrid of *O. sativa* (AA) × *O. punctata* (BBCC)	*O. sativa* (AA), *O. officinalis*(CC)	[39]
F1 hybrid of *O. sativa* (AA) × *O. eichingeri* (CC)	*O. eichingeri* (CC)	[41]
F1 hybrid of *O. sativa* (AA) × *O. meyeriana* (GG)	*O. sativa* (AA), *O. meyeriana* (GG)	[44]
Monosomic alien addition line (MAAL) of *O. officinalis* to *O. sativa* and its backcross progenies	*O. officinalis* (CC)	[42, 43]

7 Detection of Epigenetic Modification on Chromosomes

Heterochromatin plays a significant role in the suppression of genes and transposable elements as well as the maintenance of chromosome structure. The epigenetic networks of molecular interactions, including DNA methylation, histone modification, and recruitment of protein complexes, are associated with heterochromatin formation and maintenance. Several studies have shown the association of particular epigenetic modifications with gene silencing and heterochromatin. For example, in *Arabidopsis*, dimethylated histone H3K9 has been shown to be a critical mark for DNA methylation and gene silencing [45]. Both methylated DNA and dimethylated histone H3K9 are also prominent epigenetic marks for heterochromatic regions, indicating their potential roles in heterochromatin formation and maintenance [46]. Rice has the patterns of epigenetic modifications consistent with *Arabidopsis*. Methylcytosine immunoprecipitation (mCIP) and immunostaining using an anti-5-methylcytosine antibody revealed that the chromosomal distribution of DNA methylation is coincident with heterochromatin on rice meiotic chromosomes [47]. Immunostaining using anti-dimethylated H3K9 showed that dimethylated H3K9 signals were detected along rice pachytene chromosomes, especially at pericentromeric regions, consistent with the DNA methylation pattern on rice pachytene chromosomes (Fig. 6.4) [48]. Figure 6.4d shows a pachytene chromosome where the signal of dimethylated H3K9 is enriched at condensed regions within each chromomere.

8 Conclusion and Prospectives

Rice has been and continues to be a model system for genetic, genomic, and cytogenetic studies. With the availability of genetic resources

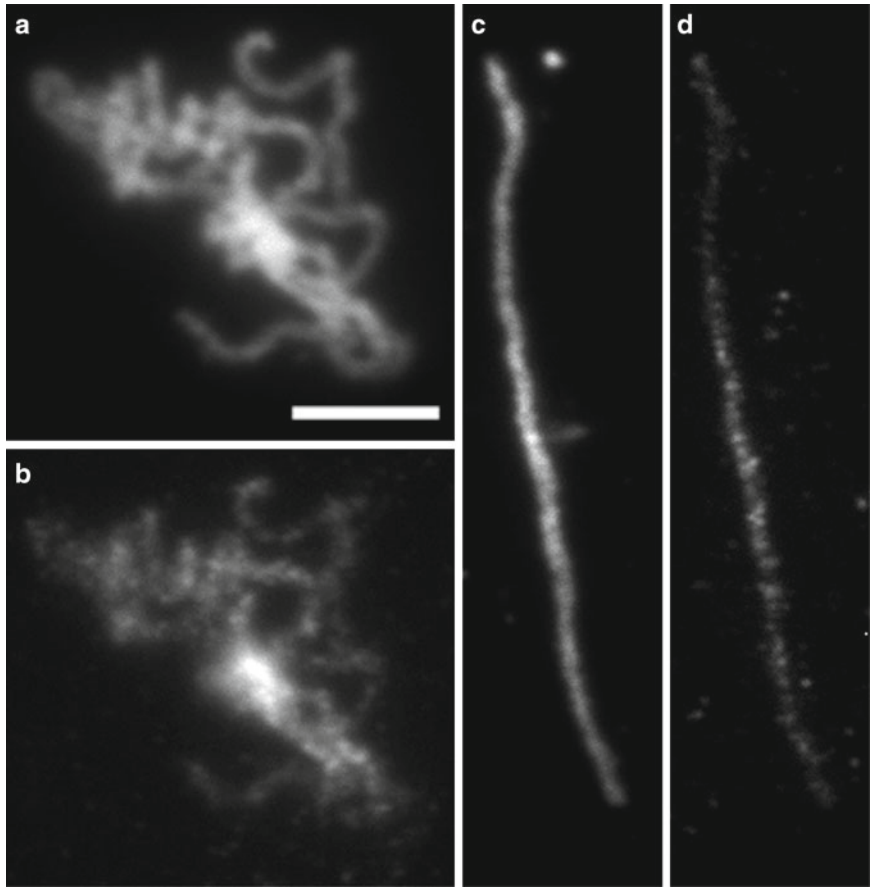

Fig. 6.4 Immunostaining results of histone dimethylated H3K9 [48]. This image is modified from Iwata et al. (**a**) Meiotic chromosomes at early pachytene stage counterstained with DAPI. (**b**, **d**) Signals of dimethylated H3K9. (**c**) A pachytene chromosome counterstained with DAPI. Bar represents 5 μm

spanning the entire genus [49], studies have led to greater knowledge of chromosomal structure and behavior, positions of interesting genes, centromere structure and function, epigenetic modifications, and chromosomal evolution in the genus *Oryza*. The development of FISH techniques and their applications have clearly broadened our fundamental knowledge of chromosomal biology. The combination of molecular cytogenetics and high-throughput sequencing techniques allows one to explore new facets of chromosome behavior and the effect of chromosome structure and positioning on gene function and patterns of epigenetic modifications, associated with chromosomal structures. Molecular cytogenetics research in the wild relatives of rice, spanning 15–20 MY of evolution, will illuminate the effects of evolutionary pressures on chromosome structure and behavior. We expect that the primary component of plant genomes, retrotransposons, will be a major modeling component of chromosomes, but it remains to be seen either at a chromosomal level or even at a genome level how these genomic "parasites" affect chromosomal and genetic adaptation and evolution.

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