Molecular cytological analyses toward the clarification of the sex
determination and differentiation mechanisms in a dioecious liverwort,
Marchantia polymorpha L.

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Contents

Abbreviations

2,4-D	2,4-dichlorophenoxyacetic acid	LSU	large subunit
45S	18S-5.8S-26S	Mb	megabase pair
A	autosome	MS	Murashige-Skoog (medium)
B	blue light	N.D.	not determined
BAC	bacterial artificial chromosome	P	probability
bp	base pair	PAC	P1-derived artificial chromosome
BSA	bovine serum albumin	PBS	phosphate-buffered saline
c	copy	PCR	polymerase chain reaction
CCD	charge-coupled device	PEG	polyethylene glycol
CHEF	clamped homogeneous electrical field	PI	propidium iodide
CTAB	cetyltrimetylammonium bromide	PMSF	phenylmethylsulfonyl fluoride
cv.	cultivar	R	correlation coefficient
DABCO	1,4-diazabicyclo-[2.2.2]octane	RAPD	randomly amplified polymorphic DNA
DAPI	4',6-diamidin-2-phenilindol-dihydrochlorid	rDNA	ribosomal deoxyribonucleic acid
dATP	2'-deoxyadenosine 5'-triphosphate	RNA	ribonucleic acid
dCTP	2'-deoxycytidine 5'-triphosphate	RNase	ribonuclease
dGTP	2'-deoxyguanosine 5'-triphosphate	rRNA	ribosomal ribonucleic acid
DIG	digoxigenin	S.D.	standard deviation
DMSO	dimethyl sulfoxide	SDS	sodium dodecyl sulfate
DNA	deoxyribonucleic acid	SINE	short interspersed element
DNase	deoxyribonuclease	SSC	sodium chloride / sodium citrate (buffer)
dNTP	deoxynucleoside triphosphate	SSU	small subunit
dTTP	2'-deoxythymidine 5'-triphosphate	STS	sequence tagged site
dUTP	2'-deoxyuridine 5'-triphosphate	TAE	Tris / acetate / EDTA (buffer)
EDTA	ethylenediaminetetraacetic acid	TBE	Tris / borate / EDTA (buffer)
FISH	fluorescence *in situ* hybridization	TE	Tris / EDTA (buffer)
FITC	fluorescein isothiocyanate	TIS	transcription initiation site
G	green light	Tris	tris(hydroxymethyl)-aminomethane
HMW	high molecular weight	TTS	transcription termination site
IGS	intergenic spacer	UV	ultraviolet
IPTG	isopropyl-1-thio-b-*D*-galactoside	X	X chromosome
kb	kilobase pair	Y	Y chromosome
LB	Luria broth (medium)	YAC	yeast artificial chromosome

Introduction

During four billion years of the evolution, organisms on the earth have developed sexual reproduction system to resort and redistribute genetic variation. Most animal species are unisexual, with male and female gametes produced in different individuals. By contrast, sexual expressions of flowering plants are divided in three classes. Firstly, the majority of flowering plants are hermaphrodite, that is they produce bisexual flowers such as *Arabidopsis thaliana* and rice (*Oryza sativa*). These flowers are composed with male organs (stamens) and female organs (carpels). In hermaphrodites, self-fertilization that is unfavorable to maintain genetic variation cannot be excluded. Therefore, some hermaphrodites have developed mechanisms of a self-incompatibility (Nasrullah and Nasrullah, 1993, Newbigin, et al., 1993) or a dichogamy, that stamens and carpels get matured in different time (Bertin and Newman, 1993). Secondly, some others are monoecy, that is unisexual staminate or pistillate flowers are produced on the same plant, as cucumber (*Cucumis sativus*) and maize (*Zea mays*). In hermaphrodite and monoecious plants, the sex of an organ is determined organ specifically by expression of hypothetical sex determining genes *a posteriori*. For example, in maize, *TASSELSEED1* (*TS1*) and *TASSELSEED2* (*TS2*) are expressed in the capel meristems (DeLong, et al., 1993). Thirdly, a few species are dioecy, that is unisexual flowers are produced on different individuals, as hemp (*Cannabis sativa*) and asparagus (*Asparagus officinalis*). In dioecious plants, the sex of an individual is determined genetically by existence of hypothetical sex determining genes *a priori*. Occasionally, dioecious plants have morphologically heterogeneous chromosomes between female and male. Such chromosomes are called sex chromosomes and considered to encode hypothetical sex determining genes.

The first report about sex chromosome in plant was in a dioecious liverwort, *Sphaerocarpos donnellii* (Allen, 1917). A large X chromosome was found in female gametophytes and much smaller Y in the male gametophytes. Sporophytes, therefore, are all XY, and the segregation of X and Y determine sex differentiation at meiosis. Heteromorphic sex chromosomes were reported in angiosperms a few years later in

sorrel (*Rumex acetosa*) (Kihara and Ono, 1923) and red campion (*Silene dioica*) (Blackburn, 1923).

The mechanisms of sex determination by sex chromosomes have been studied for some angiosperms (Fig. 1). The sex determining systems of angiosperm could be divided in two types, active Y system similar to that in mammals, and *Drosophila*-type dosage systems. The former include *Silene* species (female: 2n=22+XX; male: 2n=22+XY) (Westergaard, 1940, Winge, 1931), the Y has dominant male leading factor (Fig. 1A). The latter include *Rumex* species (female: 2n=12+XX, male: 2n=12+XY$_1$Y$_2$) (Kihara and Ono, 1923), the sex is determined by X:A ratio (Fig. 1B). A few species have no heteromorphic sex chromosome, as kiwifruit (*Actinidia deliciosa*) and annual mercury (*Mercurialis annua*). These dioecious plants may have functional sex chromosome but not heteromorphic.

In contrast to angiosperms, bryophytes produce not flowers but archegonia (female sexual organs) and/or antheridia (male sexual organs) on haploid gametophytes. Commonly bryophytes, especially liverworts are dioecy. However, many mosses and some liverworts are monoecious haploid. In addition, pteridophytes also differentiate sexual organs on haploid gametophytes, are mainly monoecy. It is still open to argument whether the ancestor of bryophytes was monoecy or dioecy.

In monoecious bryophytes, haploid gametophytes usually have one sex chromosome specific to each sex. The female sex chromosome and male sex chromosome are called X and Y, respectively. In contrast to flowering plants, both or one of the sex chromosomes encode candidates of sex determining genes (Fig. 1C).

The existence of sex chromosomes indicates the existence of genetic sex determination mechanisms. In fact, in mammals, sex-determining region on Y *(SRY)* is located on Y sex chromosome (Sinclair, et al., 1990). However, the mechanisms of sex determination in dioecious plant are still unknown both in flowering plants and in bryophytes.

On the other hand, organ specific sex determination of hermaphrodites has been summarized as region specific expression of MADS-box genes (Coen and Meyerowitz, 1991). The sex determination of monoecious plants are described as competitive expression of two genes, *TASSELSEED2* and *SILKLESS1* in maize (*Zea mays*) (Dellaporta and Calderon-Urrea, 1993). When we turned our eyes to more primitive plants, *Chlamydomonas reinhardtii*, the unicellular green algae produce two kinds of differentiated gametes (mt+, mt-) from haploid vegetative cell directly (Ferris, et al., 1997). The sex determination is done by *mid* gene on is located in the rearranged (R) domain of the mt- locus. The gene dominantly leads the minus phenotype. These genes could be an analog of the sex determination gene of deoecious plants. Some researchers aim to isolate sex determining genes from this respect. MADS –box genes were isolated from some dioecious plants, *Rumex acetosa* (Ainsworth, et al., 1995), *Silene latifolia* (Hardenack, et al., 1994) and Homologs of *TASSELSEED2* gene were isolated from *Silene latifolia* (Lebel-Hardenack, et al., 1997). Although their expression patterns were sexual organ specific as expected, it cannot be concluded that they are the concrete genes for sex determination from these results. On the other hand, it is possible that the concrete genes for sex determination is on the sex chromosomes of these dioecious plants. Thus some other researchers aim to isolate sex chromosome specific sequences in order to obtain sex determining genes. Four Y-chromosome-specific sequences were isolated from *Silene latifolia* by the genomic subtraction method representational difference analysis (RDA) (Donnison, et al., 1996). Using randomly amplified polymorphic DNA (RAPD) method, 729-bp a male specific RAPD-PCR fragment was subcloned in another dioecious plant, *Cannabis sativa* (female: 2n=18+XX, male: 2n=18+XY) (Sakamoto, et al., 1995). Isolation of sex chromosome itself has been also tried especially in *Silene latifolia*. Isolation DNA fragments by micro-dissection from X chromosome (Buzek, et al., 1997), both X and Y chromosome (Scutt, et al., 1997), Y chromosome (Matsunaga, et al., 1999) were tried, but non of the resulting DNA fragments was specific to Y chromosome. An approach to obtain sex chromosome directly by a flow sorting method has been also tried but isolation of sex chromosome specific DNA fragment has not been achieved (Veuskens, et al., 1995).

A dioecious liverwort, *Marchantia polymorpha* has unusually small sex chromosomes. In addition, the total genome size of *M. polymorpha* is also small. Therefore *M.*

polymorpha can be a potential model plant for the research of the sex determination systems, because the genes responsible for the sex determination would be located on these small sex chromosomes.   Although the X chromosome of *C. elegans* was recently sequenced completely, it had no counter part chromosome (Ainscough, et al., 1998).   If the complete sequence of the pair of the X and Y sex chromosomes was determined in *M. polymorpha*, the knowledge drawn from them gives tremendous information to the research area of plant molecular biology.
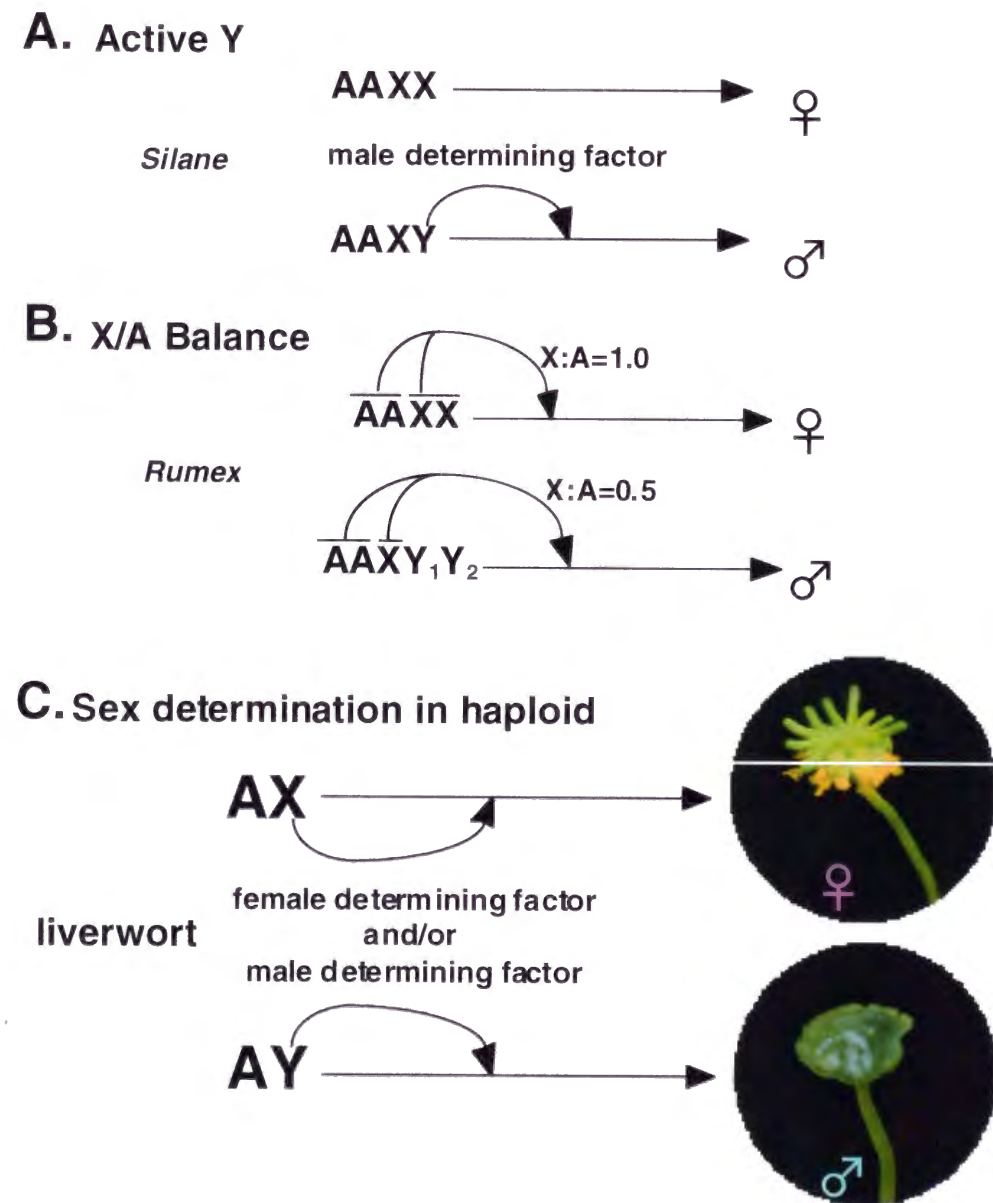

A. Active Y

AAXX ⟶ ♀

Silane   male determining factor

AAXY ⟶ ♂

B. X/A Balance

X:A=1.0

AAXX ⟶ ♀

Rumex

X:A=0.5

AAXY₁Y₂ ⟶ ♂

C. Sex determination in haploid

AX ⟶

female determining factor
liverwort   and/or
male determining factor

AY ⟶

Fig, 1.   Sex determining systems in plants.

Chapter I. Cytological analyses of a deoecious liverwort, *Marchantia polymorpha*: genome sizes and karyotypes.

Introduction

A liverwort, *Marchantia polymorpha* L. is dioecious and haploid (n=9) during most of its life cycle (Heitz, 1927).   In addition, each female and male has relatively small sex chromosome, which is called X and Y respectively (female: n=8+X, male: n=8+Y) (Haupt, 1932).   Bischler has studied several species of *Marchantia* by Giemsa C-banding and reported that the X chromosome is 1.5-1.7 times larger than the Y chromosome in length (Bischler, 1986).   However, quantitative studies based on sizes of the nuclear genomes and the sex chromosomes have not been reported ever since. One of reasons for it may be due to the difficulty of the constant chromosome observation at metaphase from thalli.

On the other hand, *M. polymorpha* has stable suspension culture systems (Ono, 1973).   Ono has reported occurrence of abnormal karyotype using a female suspension cultured cell line and restored thalli from the cell line during subculture (Ono, 1976). The female suspension cultured cell line has been subcultured for more than two decades since then.   Using the cell line, the complete genomic sequences of both chloroplast (Ohyama, et al., 1986) and mitochondrial (Oda, et al., 1992) has been determined.

In this study, we have analyzed the genome size by using a flow cytometry and the relative sizes of chromosomes by using a simple photon counting method. From these value the approximate sizes of the X and Y sex chromosomes were calculated.   In addition, the occurrences of the genomic recombination in the cell lines were demonstrated by FISH analyses using rDNA as a probe.

Materials & Methods

Plant materials

The plant materials used are summarized in Table 1-1.   Two female (Fc, FDc) and two male (Mc, MEc) cell lines of suspension culture and a pair of thallus lines (MEt, FEt)

were used. The old female and male cell lines, Mc and Fc will be described in chapter 2. The female and male thallus lines, FEt and MEt were collected in the yard of Kyoto International Community House. New cell lines were generated by callus generation on MS medium containing 2% glucose and 1% gellan gum (Katoh, 1981) for several months followed by continuous subculture in 1M51C medium. The new female cell line, FDc was derived from a female gemma (Takemura 1994, unpublished data) which was collected in the campus of Kyoto University. The new male cell line, MEc was derived from a gemma of MEt. The cell lines were maintained in 1M51C medium (Ohyama, et al., 1988). The thallus lines were maintained on 0M51C-agar medium, which is free of 2,4-D from 1M51C.

Table 1-1. List of plant materials.

Line	Thallus		Cell			
	FEt	MEt	Fc	Mc	FDc	MEc
Sex	female	male	female	male	female	male
Collected place Origins	Kyoto this paper	Kyoto this paper	Hiroshima Ono, 1973	Shiga Katoh, 1992, unpublished	Kyoto Takemura, 1994, unpublished	Kyoto this paper

Flow cytometry

For the estimation of nuclear DNA content, samples of *M. polymorpha* were prepared in the solution of fluorescence staining, measured the value of fluorescence by the flow cytometer, PA (Partec GmbH, Moster, Germany). The results were calculated for the DNA content according to the standard curve.

The thalli (FEt and MEt) at 4 weeks after seeding from gemmae and suspension cultured cell (Fc, Mc, FDc and MEc) at 3 days after subculture to new medium were used for analysis of nuclear DNA content. One g of each tissue sample was placed on plastic petri dishes. Four hundred μl of solution A (nuclei isolation buffer) of a high resolution DNA kit type P (Partec GmbH, Moster, Germany) was dropped onto the tissue and sliced the tissue using a sharp scalpel. Sliced sample was left for 2 min in the dish at room temperature. Solution A was transferred from the dish to a sample tube through 50 μm nylon mesh. Two ml of solution B (DAPI staining buffer) was added into the sample tube, after staining, this sample tube was set in the flow cytometer. At

least three different isolations from each sample were analyzed. The young leaf of *Arabidopsis thaliana* L. (Heynh.) strain Columbia (2c = 0.15 pg) (Bennett and Smith, 1991) and *Oryza sativa* cv. Nipponbare (2c = 0.75 pg) (Bennett and Smith, 1991) were used for drawing the standard curve as the marker of known DNA c values of flow cytometry. *A. thaliana* was grown for 2 weeks at incubator, *O. sativa* was grown at paddy field. The c values for nuclear content of *M. polymorpha* samples were estimated by using the standard curve.

Preparations of pollen and spore nuclei

This procedure was followed mainly the previous report on kiwifruit (Matsunaga, et al., 1996). Mature spores of *M. polymorpha* were collected from sporophytes formed on the archegoniophores of FEt. Pollen of *A. thaliana* c.v. Columbia was kindly provided by Dr. Ishiguro, Faculty of Science, Kyoto University. Pollen of *Oryza sativa* c.v. Nipponbare was collected at paddy field of Hokuriku National Agricultural Experiment Station. Each grain were gathered into a micro tube and suspended into fixative (3: 1 = ethanol: glacial acetic acid). Five μl of the fixative containing 200~2,000 grains were dropped onto the same position of a slide one after another. Then, after the fixative solution was dried, 10 μl of 1 μg/ml DAPI solution was supplied onto the slide and they were covered with a cover slip. Then grains were squashed with thumb and incubated in dark for at least 1 h until the nuclei were completely stained with 1 μg/ml DAPI solution.

Chromosome preparation

Preparation of chromosomes from the suspension-cultured cell was performed as described in chapter 2. The condition of cell synchronization was examined using 1mg/ml colchicine and 10 μg/ml aphidicholin. The preparations were stained with 1 μg/ml DAPI solution or 2% Giemsa's solution.

Simple photon counting

The preparations of nuclei or chromosomes was observed under a fluorescent

microscope, Axiophot (Zeiss) using an UV excitation filter, No. 01 (Zeiss).   The fine nuclei images of the three species were photographed with a chilled CCD camera, PXL1400 (Photometrics). The fluorescence intensity of each nucleus or chromosome was measured with the image analyzing software, IPLab Spectrum 3.1 (Signal Analytics Corporation).   Nucleus or chromosome areas were segmented and their "sum" (sum of brightness), "area" (area of each chromosomal region) and "mean" (mean of brightness in the area) were measured. The other area was selected as background and its "mean" was measured. The "sum" value of each area was re-calculated by subtracting background "mean".   Their relative fluorescence intensity was calculated by new "sum".

rDNA probe

A clone (lMPrDNA2B4) contains more than a single repeat unit of the 18S-5.8S-26S rRNA gene (45S rDNA) was used for the probe(see chapter 2).

Dot-blot hybridization

Total genomic DNAs and the probe DNA were prepared from each lines of thallus and cell by modification of CTAB methods (Strauss, 1994).   And, sonicated by a sonicator for 30 sec.   The amount of DNAs were quantified by ethidium bromide staining. They were diluted to 1 µg/ml (1 ng/ml for the probe DNA) and a dilution series were prepared. The DNAs were denatured by boiling at 95 °C for 5 min and rapid cooling.   Then, they were blotted to a filter membrane by a vacuum blotter.   The filter was hybridized with ^{32}P labeled probe DNA in modified Church's buffer (0.5 M $NaPO_4$ pH 7.2, 7% SDS, 50% formamide) (Church and Gilbert, 1984) for 16 h.   The filter was washed with 0.5 x SSC containing 0.1% SDS at 55 °C for 1 h.   Then, it was exposed with BAS Imaging Plate (Fujifilm) for overnight.   The radioactivity of each blot was measured by the bio imaging analyzer (BAS2000, Fujifilm).   A standard curve was drawn from the dilution series of the probe DNA.   The relative content of the sequence used for probe DNA in the genome was calculated from the radioactivity of the sample genomic DNA.   The proportion of nuclear DNA per total genomic DNA was set as 0.7 from the proportion

of organella DNA clones in the total genome library.

Fluorescence in situ hybridization (FISH)

FISH procedure was performed as described in chapter 2. lMPrDNA2B4 was biotinylated by using Biotin-High Prime labeling kit (Boehringer).   The prepared glass slides were supplied with anti-fading solution (12.5 mg/ml 1,4-diazabicyclo[2.2.2]octane (DABCO), 90% glycerol, 0.1 x PBS, pH 8.7) and 1 µg/ml propidium iodide (PI) and covered with cover slips.   The slides were observed under the fluorescence microscope and photographed with the chilled CCD camera.

Results & Discussions

Nuclear genome sizes of M. polymorpha

Fluorescence values of *A. thaliana* and *O. sativa* were proportional to known DNA values with a high correlation coefficient (R>0.9).   Then, the standard curve was drawn each time when the sample was measured.   The genome sizes of the thallus lines (FEt and MEt) were 280±10 Mb each (Table 1-2).   Estimation by a simple photon counting method of DAPI staining images of liverwort spore nuclei also suggested similar size (Fig. 1).   On the other hand, in the cultured cell lines, however, variations were observed between 270±19 to 640±11 (Table 1-2).

Table 2.   Estimation of DNA content by flow cytometry. The DNA content is calculated from the standard line are shown by pg and Mb with standard deviations.

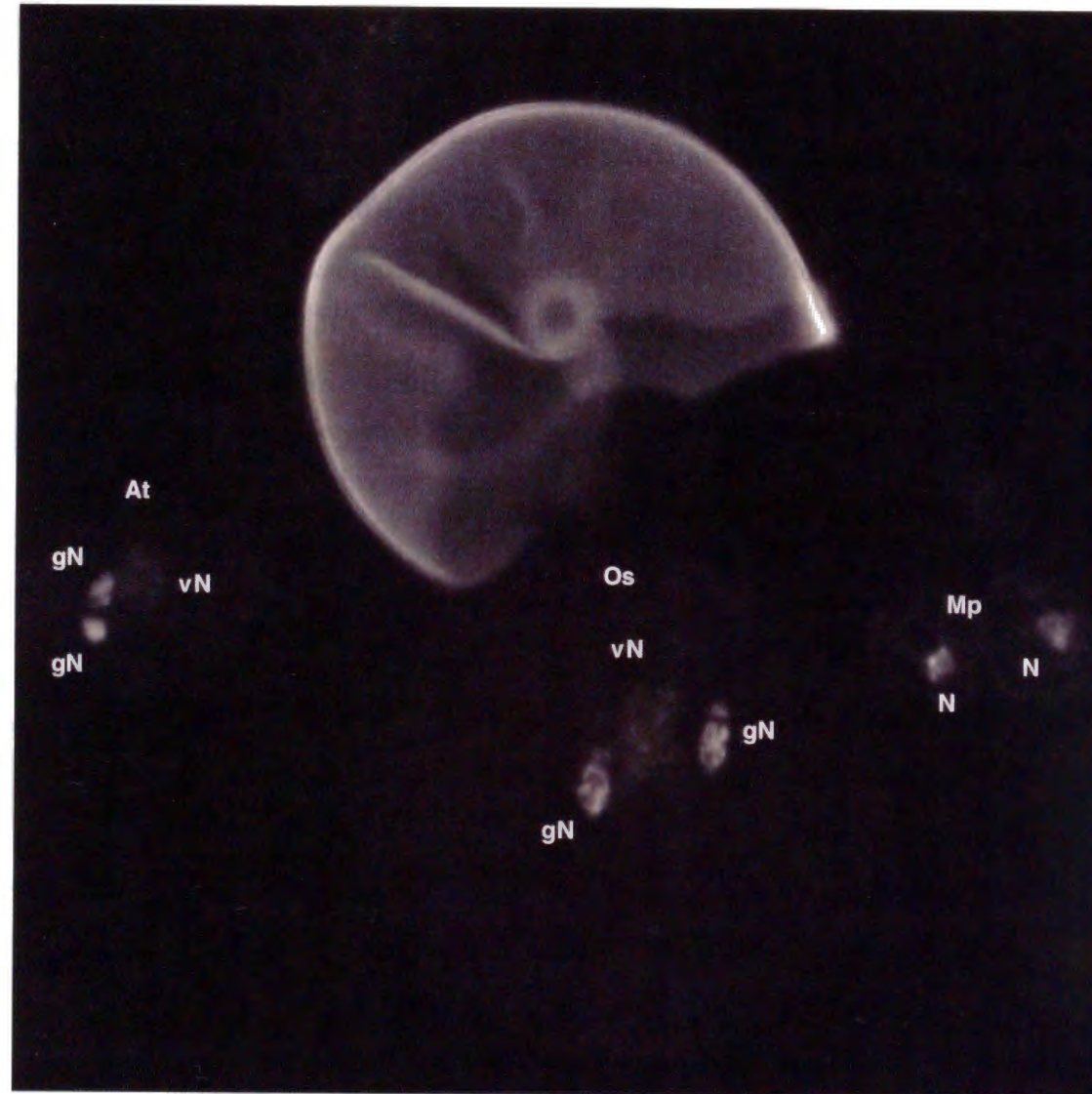| Line	Thallus			Cell		
	FEt	MEt	Fc	Mc	FDc	MEc
Karyotype	n=8+Y	n=8+X	2n=17+2X	n=8+Y	n=8+X	2n=14~20+2Y n=7~11+Y,
pg±S.D.	0.29±0.01	0.29±0.01	0.66±0.01	0.34 ±0.01	0.28±0.02	0.64±0.03
pg ±S.D./haploid			0.33 ±0.01			0.32±0.01
Mb±S.D.	280±10	280±10	640±11	320±18	270±19	620±25
Mb±S.D./haploid			320±6			310±13
Number of analysis	13	13	4	7	3	4

Fig. 1-1. Comparison of nuclear genome sizes. At: Pollen nuclei of *Arabidopsis thaliana* (100 Mb/n). Os: Pollen nuclei of Oryza sativa (400 Mb/n). Mp: Spore nuclei of *Marchantia polymorpha* was then calculated as 250±50 Mb/n. gN: generative nucleus, vN: vegitative nucleus, N: spore nucleus.

Correlation of the genome sizes and the karyotypes

The genome size variation was appeared to be polyploidy and aneuploidy of the cell lines. The highest nuclear DNA amount (640±11 Mb) was observed in the oldest female cell line (Fc) which appeared to be diploidal aneuploid (2n=17+2X) (Fig. 2A). Although Fc was a diploidal aneuploid, three cell lines cultured for a long period of time

(Fc, Mc snd FDc) were mostly stable for their chromosome numbers (Fig. 2A, B and C), while the new male cell line (MEc) was very unstable. The genome size of MEc was 620±25 as the chromosome number of MEc varied from n=7+Y to 2n=20+2Y (Fig. 2D). The reason that standard deviation for the genome size of MEc was the largest (±25) may be reflection of the unstability. The chromosomes from female and male thallus were difficult to obtain because they were not easy to synchronize cell cycle at metaphase. It was achieved to 7.0% in the suspension-cultured cells by 4 h treatment with 1 mg/ml colchicine without the pre-treatment of aphidicolin.
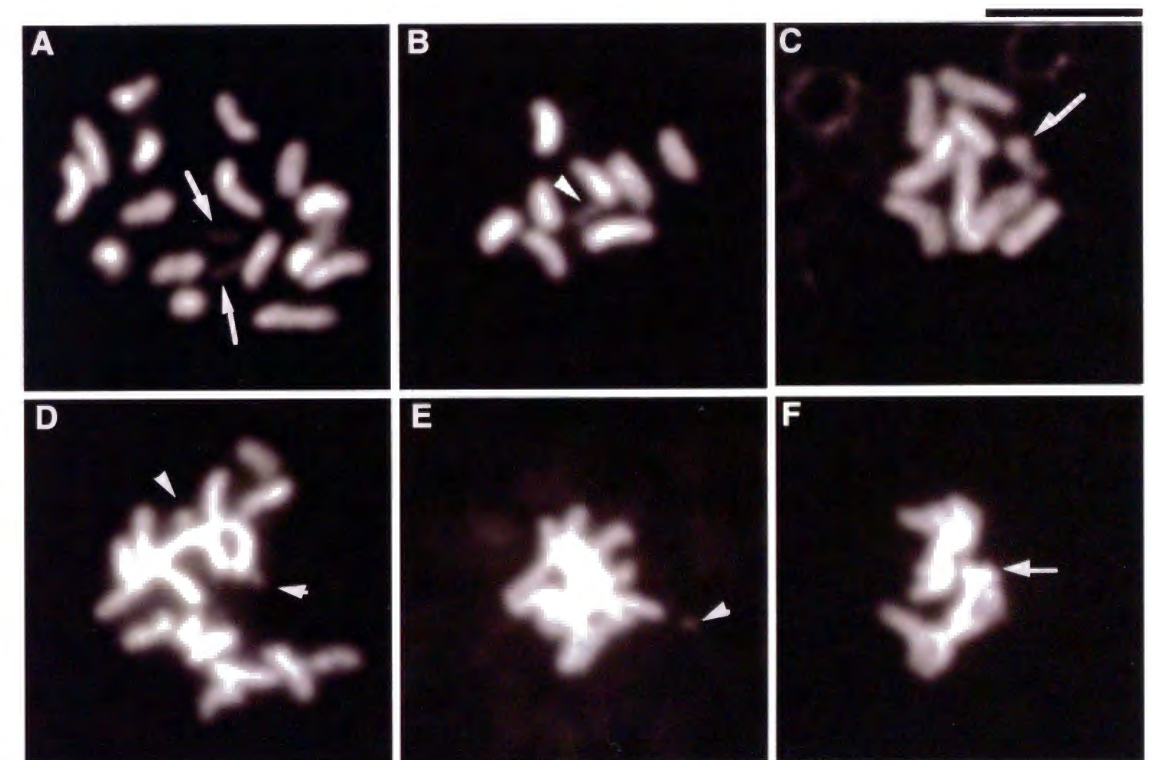
Fig. 1-2. Karyotype of various lines of *M. polymorpha*. A. Chromosomes from the old female suspension cultured cell (Fc). 2n=17+2X. B. Chromosomes from the old male suspension cultured cell (Mc). n=8+Y. C. The new female suspension cultured cell (FDc). n=8+X. D. The new male suspension cultured cell (MEc). 2n=17+2Y. E. Chromosomes from the male thallus line (MEt). (n=8+Y). F. The female thallus line. (n=8+X). The arrows indicate X chromosomes. The arrowheads indicate Y chromosomes. The black bar indicates 5 μm.

Table 1-3. The correlations of the rDNA repeat copy number and the genome sizes.

Sex	Thallus		Cell	
	MEt	FEt	Mc	FDc
Karyotype	n=8+Y	n=8+X	n=8+Y	n=8+X
Genome size (Mb)	280±10	280±10	320±18	270±19
rDNA content (%)	6.0±1.7	7.3±2.6	9.3±2.4	3.6±0.3
Copy number	1000±300	1300±500	1900±500	600±100

Fig. 1-3. Dot-blot analysis using rRNA repeat unit as probe. Using the signal length of the probe (P) as standard, relative amount of rDNA sequence in the total genomic DNA was calculated.
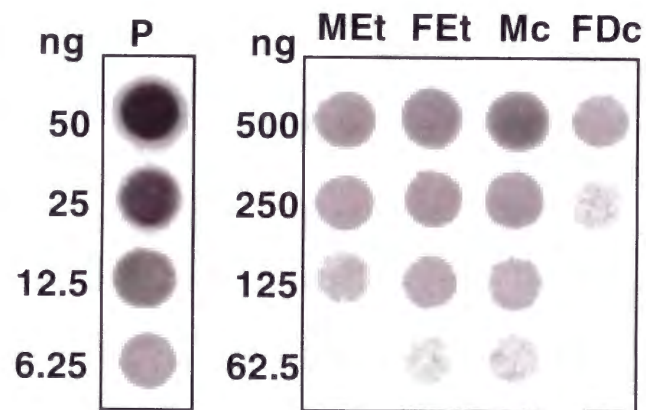
Another cause of genome size variation was estimated to be the changes of the copy number of the repeated sequences. It has been reported that the most significant contributor to the genome size among the repeated sequence was rDNA repeat in the fission yeast, *Saccharomyces cerevisiae* (Dujon, 1996). Thus, the copy numbers of the rDNA repeat were investigated for the cell lines and the thallus lines (Fig. 1-3, Table 1-3). For example, normal haploid line , it had the largest rDNA copy number (1900±500 copy) and the largest haploid genome size (320±18 Mb). It has been reported that various strain of *Oryza sativa* contained different copy number of repeated sequences, correspond to their genome sizes (Uozu, et al., 1997).

Do the rDNA loci decrease during subculture?

Fluorescence *in situ* hybridization (FISH) revealed that not only the copy number but the loci of the rDNA repeat were also varied from cell line to cell line. In the oldest female cell line (Fc), rDNA loci was detected on two autosome. Fc was diploid so it has only one rDNA locus per haploid (1A/n) (Fig. 1-4 Fc). In the haploid male cell line (Mc), a large accumulation of rDNA was on the long arm of a large autosome and minor rDNA cluster was at the telomeric region of another autosome (2A/n) (Fig. 1-4 Mc). However, in the haploid female cell line (FDc), rDNA clusters were at four autosomes and X chromosome in addition (4A+X/n) (Fig. 1-4 FDc). In the unstable male line, rDNA loci was detected on both telomeric regions of two autosome and single telomeric region of four autosomes (6A/n) (Fig. 1-4 MEc). In the male thallus line, rDNA loci was detected on almost all chromosomes but except one autosome and and Y chromosome (7A/n) (Fig. 1-4 MEt).
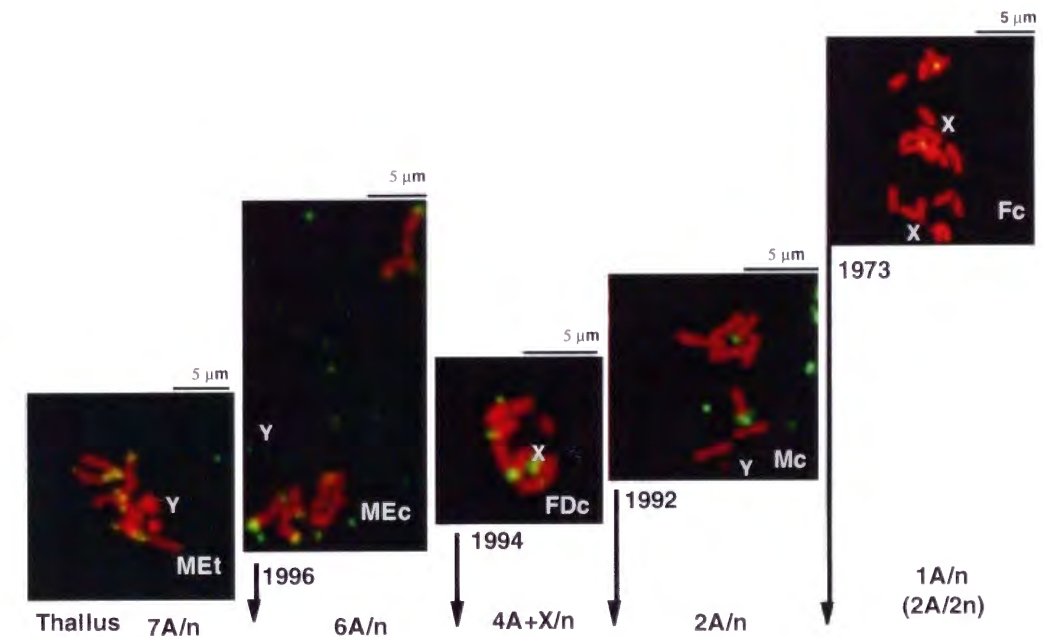

Fig. 1-4. The number of the rDNA loci and the established date of callus formation of each cell lines and a thallus line. The yellow region on red chromosomes are the rDNA loci detected by FITC. The chromosomes are counter stained by PI. The numbers followed by letter A indicates the numbers of autosome with rDNA loci. In FDc line, rDNA loci were observed on the X chromosome.

These results indicate the occurrence of re-organization of rDNA clusters during the subculture and/or the existence of ecotypical variations of rDNA clusters in *M. polymorpha*. Such drastic difference of rDNA loci in a single species has been first observed in the liverwort *M. polymorpha*.

Estimation of the size of the X and Y sex chromosomes

The DNA size of each sex chromosome (X, Y) were estimated to be approx. 10 Mb and 5 Mb respectively by a simple photon counting (Fig. 1-5). Although complete X chromosome (18 Mb) of *C. elegans* was recently sequenced (Ainscough, et al., 1998), its size was found to be larger than that in liverwort X and Y chromosmes together in size. The sex chromosomes of *M. polymorpha* appeared to be unusually small amoung plant sex chromosomes. In addition, the total genome size of *M. polymorpha* is also small. Therefore, *M. polymorpha* can be a potential model plant for the research of the sex determination systems in the bryophytes, because the genes responsible for the sex determination would be possible to locate on these small sex chromosomes.
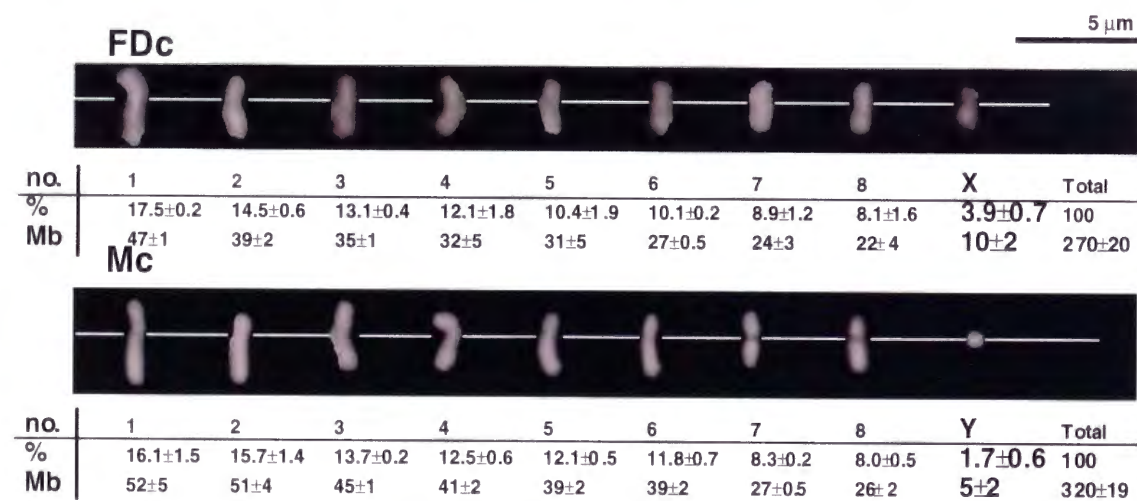

| FDc | | | | | | | | | 5 μm |
no.	1	2	3	4	5	6	7	8	X	Total
%	17.5±0.2	14.5±0.6	13.1±0.4	12.1±1.8	10.4±1.9	10.1±0.2	8.9±1.2	8.1±1.6	**3.9±0.7**	100
Mb	47±1	39±2	35±1	32±5	31±5	27±0.5	24±3	22±4	**10±2**	270±20

| Mc | | | | | | | | | |
no.	1	2	3	4	5	6	7	8	Y	Total
%	16.1±1.5	15.7±1.4	13.7±0.2	12.5±0.6	12.1±0.5	11.8±0.7	8.3±0.2	8.0±0.5	**1.7±0.6**	100
Mb	52±5	51±4	45±1	41±2	39±2	39±2	27±0.5	26±2	**5±2**	320±19

Fig. 1-5. Karyotype had been analyzed for the stable two cell lines using image analyzing software, IPLab Spectrum 3.1 (Signal Analytics Corporation). For each chromosome in gray images of a cell, its region was selected its region and determined their order in size by the sum of brightness and then numbered. The genome size of each chromosome was estimated from the sum of brightness.

Chapter II. Identification of the localization of 18S-5.8S-26S and 5S rDNA of the liverwort by fluorescence *in situ* hybridization (FISH).

Introduction

In prokaryotes, ribosomal RNAs (rRNAs) consist of a small subunit (SSU; typically 16S), a large subunit (LSU; typically 23S) and a 5S rRNA, and their genes are scattered in the genomes as multiple operons. The three rRNAs are transcribed in the order of 16S, 23S and 5S by prokaryotic RNA polymerases. On the other hand, rRNAs of the most eukaryotes consist of a SSU (typically 18S), a 5.8S, a LSU (typically 26S) and a 5S rRNA. The 18S, 5.8S and 26S rRNA genes (45S rDNA) are clustered in tandem repeat units and transcribed as one cistron (typically 45S rRNA) by RNA polymerase I, while the 5S rRNA genes (5S rDNA) are located in separate regions of the genomes and transcribed by RNA polymerase III.

In most eukaryotes, the 5S rDNA and 45S rDNA repeat units occupy the different loci except for certain fungi and protozoa (Srivastava and Schlessinger, 1991). Although in plants numerous angiosperms and some gymnosperms have been analyzed for the organization of the rDNA repeat unit to date, existence of the 5S rDNA in the 45S rDNA repeat unit has not been reported (Beech and Strobeck, 1993, Rogers and Bendich, 1987). Fluorescent *in situ* hybridization (FISH) has been widely used to locate the 5S and the 45S rDNAs, and the loci of the 45S and the 5S rDNAs were shown to exist in close proximity in some cases, such as in the oilseed rape, *Brassica napus* (Kamisugi, et al., 1998). However, to our knowledge coexistence of the 45S and the 5S rDNAs has not been reported in those plants.

In this paper, we present data on the chromosomal location of the 45S and 5S rDNAs on the genome of a liverwort, *Marchantia polymorpha*, by Southern blot and FISH. Based on the complete nucleotide sequence of a rDNA repeat unit of *M. polymorpha*, we discuss the evolution of the rDNA organization in plants.

Materials & Methods

Plant materials

Female suspension-cultured cells of *M. polymorpha* (Fc) are originally derived from a female thallus (Ono, 1973) and maintained in 1-M51C medium (Ohyama, et al., 1988). Male suspension-cultured cells of *M. polymorpha* (Mc) derived from a male thallus were kindly provided by Dr. Kenji Kato and they are also maintained in the same culture medium.

Polymerase chain reaction (PCR) for the 5S rDNA fragment

Two sets of primers for PCR were designed from the published sequence of *M. polymorpha* 5S rRNA (GenBank accession no. X01617) (Katoh, et al., 1983). The first set,

5'MPrrn5PstI: 5'CCCCTGCAGTTAAGCGCCCTT3'

3'MPrrn5PstI: 5'GGGCTGCAGAGTTCTGATGGG3'

, were to amplify a single 5S-rDNA repeat unit from the *Pst*I site within a 5S rDNA to the next, assuming the presence of tandemly repeated 5S-rDNAs repeats. The second set,

5'MPrrn5: 5'GGATGCGGTCATACCAGG3'

3'MPrrn5: 5'TGGATGCAGCACCGGGAC3'

, were to amplify the coding region of the 5S rDNA. PCR was performed in the following condition: two cycles of 1 min at 94 °C, 1.5 min at 56 °C and 2 min at 72 °C, then 35 cycles of 30 sec at 94 °C, 1.5 min at 58 °C and 2 min at 72 °C using 10 ng of total genomic DNA of Fc cells as template.

Hybridizations

A wheat 45S rDNA clone, pTa71 (Gerlach and Bedbrook, 1979), was labeled with [α-^{32}P]-dCTP using Megaprime[TM] DNA Labeling System (Amersham) for screening of the λ phage genomic library of Fc (Akashi, et al., 1996) by plaque hybridization.

Ten μg genomic DNAs from Fc cells were hybridized with the pTa71 or the 5S rDNA PCR fragment, each labeled with ^{32}P by a random primer method at 42 °C for 18 h in a buffer containing 0.5 M sodium phosphate (pH 7.2), 7% SDS, 50% formamide (Church and Gilbert, 1984). The membranes were washed with 0.1% SDS, 0.2 x SSC at 55 °C for 1 h.

Fluorescent in situ hybridization (FISH)

Probes for 45S rDNA were labeled with biotin-16-dUTP or digoxigenin-11-dUTP using hexameric random primers. Probes for 5S rDNA were labeled by a direct PCR labeling method using the inward primer set (5'MPrrn5 and 3'MPrrn5) and 10 ng total genomic DNA of Fc as PCR template.

Three-days-old cultured cells were cultured in a fresh culture medium (1-M51C) for 12 h at 25 °C with shaking at 160 rpm, and then 1 mg/ml colchicine were added to the cells. Four hours later, cells were fixed with four changes of a fixative (3:1 ethanol: glacial acetic acid), then rinsed twice with sterilized water. The fixed cells were treated with an enzyme solution containing 1% (w/v) Pectolyase Y-23 (Seishin), 0.4% (w/v) cellulase Onozuka RS (Yakult) and 75 mM KCl for 30 min to remove cell wall. Resulting protoplasts were washed by sterilized water twice, and further fixation with two changes of the fixative. Fixed cells were stored in the fixative at -30 °C for later use. Chromosome samples were spread onto glass slides and air-dried. Chromosomes on the slides were stained with 1 μg/ml 4',6-diamidin-2-phenylindol-dihydrochlorid (DAPI, Boehringer) or 2% Giemsa's solution (Merck) and then observed microscopically. FISH was carried out according to the previously described method (Ohmido, et al., 1998) with a slight modification. A hybridization mixture containing 10 ng/μl of biotin- and digoxigenin-labeled DNA probes, 50% formamide, 5% dextran sulfate, 2 x SSC, 500 ng/μl autoclaved salmon sperm DNA and 100 ng/μl sonicated total DNA of Fc cells was applied to pre-hybridized slides. The slides were heated at 80°C for 5 min on a hot plate, and incubated at 37°C over night for hybridization. After hybridization, those slides were rinsed by a solution containing 50% formamide and 2 x SSC at 42 °C for 10 min twice, by 2 x SSC at 42 °C for 10 min, by 0.1 x SSC at 60 °C for 10 min twice, and by 4 x SSC at 42°C for 10 min. The slides were incubated with a

BT-buffer containing 0.1 M sodium hydrogen carbonate and 0.05% Tween-20 (pH 8.3) for 5 min, with 5% BSA dissolved in the BT-buffer at 37 °C for 1 h, and then rinsed by the BT-buffer three times for 5 min. The first detection buffer contained 10 ng/µl avidin-fluorescein isothiocyanate (avidin-FITC, Boehringer) and 1% BSA in BT-buffer. The second detection buffer contained 10 ng/µl biotinylated anti-avidin (Vector), 20 ng/µl anti-digoxigenin-rhodamin (Boehringer) and 1% BSA in BT-buffer. The third detection buffer contained 10 ng/µl avidin-FITC, 15 ng/µl Texas red anti-sheep IgG (Vector) and 1% BSA in BT-buffer. Chromosomes were counter-stained with 1 µg/ml DAPI. Slides were observed under a fluorescent microscope using an UV excitation filter, and metaphase nuclear plates were searched. Each metaphase plate was photographed with a CCD camera (PXL1400, Photometrics) using a green excitation filter for rhodamin/Texas red images, a blue excitation filter for FITC images and the UV excitation filter for DAPI images. The three images were pseudo-colored and overlaid with each other by an image analyzing software (IPLab Spectrum 3.1, Signal Analytics).

DNA sequencing

Nucleotide sequence was determined by a shotgun method using M13 mp18 as a cloning vector, Big Dye [TM] Primer Cycle Sequencing Kit or Big Dye [TM] Terminator Cycle Sequencing Kit (Perkin Elmer) and a DNA sequencer (ABI PRISM[TM] 377, Perkin Elmer). Sequence data were assembled by a software (Sequencher[TM], Gene codes) and coding regions were predicted by homology search against known sequences using BLAST or BLAST2 algorithms (Altschul, et al., 1990). Repeated sequences were detected by LFASTA program (Pearson and Lipman, 1988).

Results
Isolation of 5S and 45S rDNA

Since 5S rDNAs usually form a tandem repeat of 300 bp to 1 kb in higher plants, the outward primer set of 5'MPrrn5PstI and 3'MPrrn5PstI was used to amplify a 5S-rDNA repeat unit by assuming the presence of tandemly repeated 5S rDNAs. But no significant amplification was observed (data not shown). This suggested that there is

no short tandemly repeated 5S rDNAs in *M. polymorpha*. On the other hand, a 119-bp DNA fragment was amplified from the total DNA of Fc cells with the inward primer set (5'MPrrn5 and 3'MPrrn5) as expected (Fig. 2-1 lane1). The 119-bp DNA fragment was directly sequenced and its sequence was confirmed to encode previously identified 5S rRNA (X01617) (Katoh, et al., 1983).
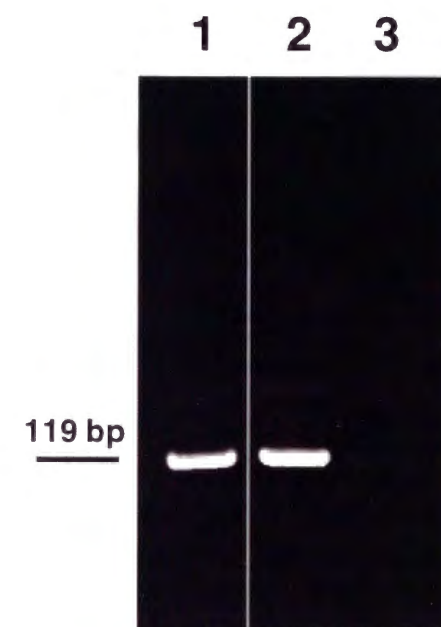
Fig. 1. Polyacrylamide gel electrophoresis of the PCR fragments amplified by the inward primer sets, 5'MPrrn5 and 3'MPrrn5 and DNA templates of the total DNA of Fc cells (lane 1), the λ phage DNA, lMPrDNA2B4 (lane 2). Lane 3 is the negative control without template DNA.

Eleven λ phage clones were isolated from the Fc λ phage genomic library by using the wheat 45S rDNA probe (pTa71). One of the clones, lMPrDNA2B4, with the largest insert of 17.5 kb in length was used for further analyses. Using the λ phage DNA of lMPrDNA2B4 as template, a 119-bp PCR product was also detected as shown in Fig. 1 (lane 2).

Linkages of 5S and 45S rDNA in Southern blot analyses

In order to know the organization of the rDNAs in *M. polymorpha*, restriction-digested total DNA samples were probed by the ^{32}P-labeled pTa71 DNA encoding wheat 45S rDNA and the 119-bp PCR fragment of the 5S rDNA, respectively (Fig. 1-2). Both the 45S and the 5S rDNA probes hybridized with 16-kb *Hin*dIII fragments (Fig. 1-2, lane 1 and 4) and 7.5-kb *Xho*I fragments (Fig. 1-2, lane 3 and 6). This suggests coexistence of

the 45S and the 5S rDNAs on the same chromosomal locations. The minimal length of the rDNA repeat unit was estimated to be 16 kb, because both probes hybridized with single 16-kb *Hin*dIII bands (Fig. 1-2 lane 1 and 4).

Fig. 2. Genomic Southern blot analyses of the 45S rDNA and the 5S rDNA. lanes 1-3, hybridization with ^{32}P-labeled pTa71 encoding wheat 45S rDNA. lanes 4-6, hybridization with ^{32}P-labeled 119-bp PCR fragment encoding *M. polymorpha* 5S rDNA. The total DNA ssamples digested with *Hin*dIII (lanes 1 and 4), with *Pst*I (lanes 2 and 5), and with *Xho*I (lanes 3 and 6) are electrophoresed in 0.7% agarose gel. Molecular sizes are indicated in kb.
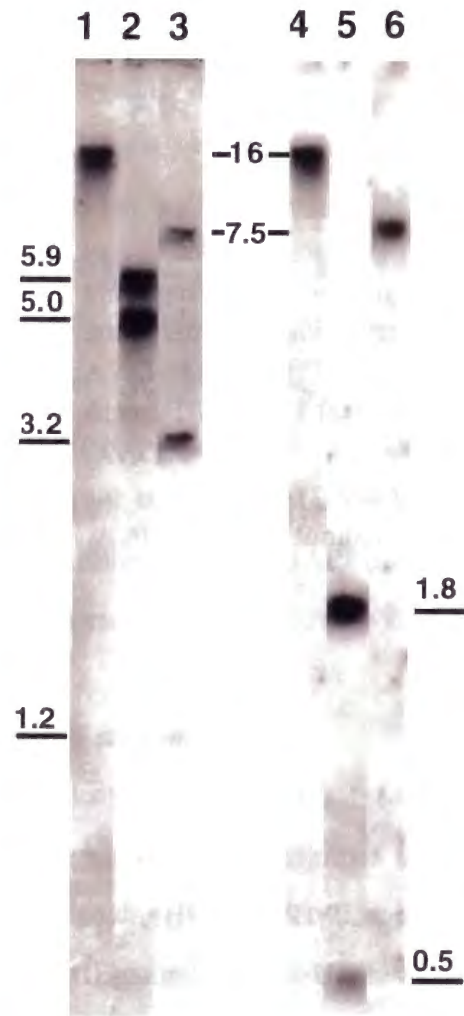
The organization of the rDNA repeat unit

The complete nucleotide sequence of the clone lMPrDNA2B4 was determined (GenBank accession no. AB021684) and the deduced organization of the rRNA genes and the restriction map are shown in Fig. 2-3. The sequence data revealed the following features of the *M. polymorpha* rDNA.
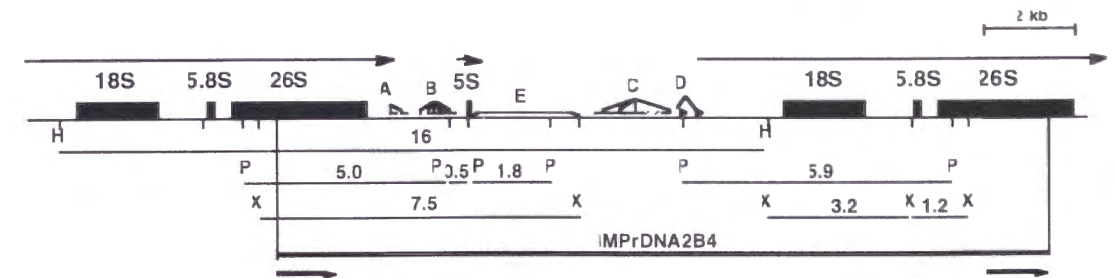

Fig. 2-3. The organization of the rDNA repeat unit of *M. polymorpha*. The 17,477-bp insert of lMPrDNA2B4 indicated by the thick bar at the bottom is including the *Mbo*I sites at both ends. Two arrows under the thick bar indicate completely identical regions of 1,374 bp in the insert DNA. The filled boxes indicate coding regions of the rRNA genes. Arrows over the boxes indicate putative transcribed region with their transcriptional orientations. The restriction sites of *Hin*dIII, *Pst*I, and *Xho*I are indicated by H, P and X, respectively. Numbers on lines indicate molecular sizes in kb of the DNA fragments that were detected by genomic Southern blots shown in Fig. 2-2. Subrepeat sequences detected in the IGSs are shown by the short horizontal lines with letters A, B, C and D. A/T-rich regions are indicated by the letter E.

First, the size of a single repeat unit containing 45S rDNA was 16,103 bp in length, because the nucleotide sequence of the 5'-end 1,374-bp region and that of the 3'-end 1,374-bp region in the 17,477-bp insert was completely identical each other. Existence of the 16-kb *Hin*dIII fragments which hybridized with both pTa71 and the 119-bp PCR fragment coincide the fact that the λ phage contains a single *Hin*dIII site, only one in a repeating unit (Fig. 2-2 lane 1 and 4, Fig. 2-3 H). All other bands (Fig. 2-2) correspond to the restriction map (Fig. 2-3). These results strongly suggest that the majority of the rDNA repeat units in *M. polymorpha* have tandemly repeated organizations.

Second, the 45S and the 5S rDNAs are encoded within the same repeat unit.

Third, both of the intergenic spacers, IGS1 between the 26S and the 5S rDNAs, and IGS2 between the 5S and the 18S rDNAs were appeared to contain several repeated sequences. The IGS1 contained two groups of repeated sequence (Fig. 2-3A and B): subrepeat-A of 122-127 bp in length, repeated twice and subrepeat-B of 76-79 bp in length repeated 8 times. The IGS2 also contains two groups of repeated sequences (Fig. 2-3C, D). Subrepeat C of 366-371 bp in length repeated 6 times and subrepeat D of 86 bp in length repeated twice. IGS2 also contains 2,657 bp of A/T rich region, the

A/T content of which is 70% (Fig. 2-3E).

Both the subrepeats B and C (Fig. 2-3B, C) contained a sequence GACTTGCC, which correspond to previously reported GACTTGC-box found at the transcription termination site (TTS) of *Xenopus* (Labhart and Reeder, 1986). Similar GACTCGC sequences were also found in subrepeat A. Nucleotide sequences, such as TATATAAGGG and TATATGAGGG, found in the subrepeat D are similar to the TATA(R)TA(N)GGG-motif which has been reported to be conserved among the transcription initiation sites (TIS) of RNA polymerase I in many plants, e.g. *Arabidopsis thaliana* (Doelling, et al., 1993). Oligomeric T-residues which is reported to function as a transcription termination site for RNA polymerase III (Campbell and Setzer, 1992) were found in the A/T-rich region between the 3' end of the 5S rDNA and the repeat C.

Identical physical location of 5S and 45S rDNAs was detected by FISH

In order to know weather other 45S rDNA cluster without the 5S rDNA existed or not, FISH analysis was carried out using the cloned DNA fragment, lMPrDNA2B4 and a PCR fragment of the 5S rDNA as probes. Since the Fc cells were aneuploids (2n = 17+2X), the male suspension cultured cells, Mc cells, with normal haploids (n = 8+Y) were used for FISH analyses (Fig 2-4). Fluorescent signals for the probes were detected at two loci on the chromosomes. In addition to a long arm of a large autosome, a telomeric region of a shorter autosome was painted by the probe (B, C, E, and F in Fig. 2-4). The similar images were observed when the lMPrDNA2B4 was labeled by biotin (Fig. 2-4B) and the 5S was labeled by digoxigenin (Fig. 2-4C). Moreover, when those probes were labeled in counter stained, the similar images were detected (Fig. 2-4E and -F). Independent signals of the 45S rDNA without 5S rDNA signals were not detected. This result suggests that most 5S rDNAs are included in the 45S rDNA repeat units.
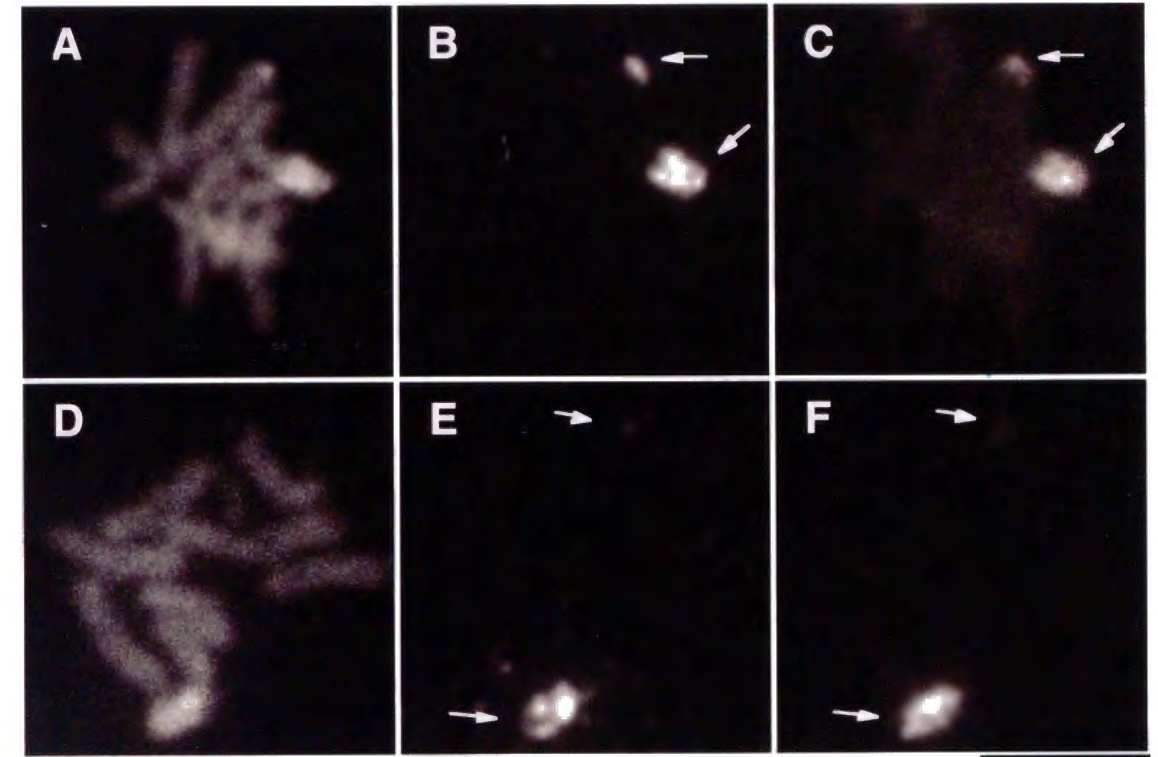
Fig. 2-4. FISH images of the Mc metaphase chromosomes (n = 8 + Y) probed with rDNAs. A and D. Chromosomes stained with DAPI. B. A λ phage DNA lMPrDNA2B4 containing 45S and 5S rDNAs labeled with biotin was detected by avidin-FITC on the same chromosomes shown in A. C. The 5S rDNA probe labeled with digoxigenin was detected by anti-digoxigenin-rhodamin on the same chromosomes shown in A. E. The 5S rDNA probe labeled with biotin on the same chromosomes shown in D. F. A λ phage DNA lMPrDNA2B4 labeled with digoxigenin on the same chromosome shown in D. The FISH signals are indicated by white arrows. The bar at the bottom indicates 5 μm.

Discussions

Coexisitence of the 45S and 5S rDNA on the M. polymorpha genome

Genomic Southern blot analysis (Fig. 2-2) indicated that major popularity of the 5S rDNA is linked to the 45S rDNA repeat in *M. polymorpha*. The complete sequence of the 45S rDNA repeat unit clearly showed that 5S rDNA was encoded in the 45S rDNA repeat unit as in the cases of some lower eukaryotes such as *S. cerevisiae* and some *Pythium* species (Belkhiri, et al., 1992, Bell, et al., 1977). On the other hand, existence of independent tandemly repeated 5S rDNAs has been described in angiosperms. In

the genomic Southern blot analysis using 5S rDNA as probe, discrete bands such as the 16-kb HindIII band (Fig 2-2) are detected rather than ladder-like bands resulted from partial digestion of the 5S rDNA repeat unit. Such ladder-like bands have been observed in many other plants possessing tandemly repeated 5S rDNAs which is located at the other loci from 45S rDNA (McIntyre, et al., 1992). This is also consistent with the fact that no amplification was observed with the outward primer sets (5'MPrrn5PstI and 3'MPrrn5PstI). From the result of FISH, existence of independent 45S rDNAs without the 5S rDNA was not observed (Fig. 2-4). This type of rDNA organization has not been reported in higher land plants to our knowledge.

Interestingly, the rDNA sequence of *M. polymorpha* shared a high level of similarity with that of the 45S rDNA repeat unit from a moss, *Funaria hygrometrica* (GenBank accession no. X80212) (Capesius, 1997). It turned out that the 11,132-bp rDNA repeat unit of *F. hygrometrica* also included the 5S rDNA in its IGS between 26S and 18S rRNA genes as in the case of *M. polymorpha*.

The difference in the length of the rDNA repeat unit between *M. polymorpha and F. hygrometrica* is caused by the difference in the length of IGS1 and IGS2. They are 2,178 bp and 6,914 bp, respectively in *M. polymorpha*, whereas they are only 504 bp and 4525 bp, respectively in *F. hygrometrica*. Although the putative promoter sequences in *F. hygrometrica*, TATGTGGGGG and GATAGGGGG, are slightly different from the conserved TATA(R)TA(N)GGG motif seen in the angiosperms (Capesius, 1997), they are conserved between the angiosperms and *M. polymorpha*. Although group I introns which have been found in the 18S or 26S rDNAs of some fungi (Hibbett, 1996) and protozoa (Aimi, et al., 1994, Aimi, et al., 1994) also exist in the 26S rDNAs of *F. hygrometrica* and another moss, *Physcomitrella patens* (GenBank accession no. X98013), they are not found in the rDNA of *M. polymorpha*.

Despite the above differences, the sequence of the coding region of each rDNA and the organization of the rDNAs in *M. polymorpha* and *F. hygrometrica* were quite similar. The orientation of the 45S and 5S rDNAs in *F. hygrometrica* were the same as that in *M. polymorpha*. The IGS1 of *F. hygrometrica* contained short direct repeats,

which were not described by the author. The IGS2 also contained several subrepeat families (Capesius, 1997). Both *M. polymorpha* and *F. hygrometrica* commonly contained GACTCCGC sequences or GACTCGC sequences that resemble the GACTCCGC-box of *Xenopus* (Labhart and Reeder, 1986) in some of their subrepeat sequences. Although there was not A/T rich region correspond to that of *M. polymorpha* in the 3'end of 5S rDNA of *F. hygrometrica*, oligo T_4, oligo T_6 and oligo A_{25} sequences were existed. As 45S rDNA and the 5S rDNA in the same repeat were found in closely related species the liverwort and the moss, the distribution of these structure in plant kingdom were the next concerns.

Evolution in the organization of the 5S rDNAs with the 45S rDNA repeat in plants

Other examples of coexisitence of the 5S rDNA with 45S rDNA in plant have been found in 4 species of cryptomonad algae, *Rhinomonas pauca*, *Storeatula major*, *Komma caudata*, and isolate Cs 134 (Gilson, et al., 1995). Cryptomonad algae contain two kind of eukaryotic genomes: a nucleus of the host and a nucleomorph, which appears to be the vestigial nucleus of an eukaryotic algal endosymbiont (Douglas, et al., 1991). The 5S rDNA linked to the 45S rDNA is derived from the nucleus. On the other hand, A numerous angiosperms and some gymnosperms have been analyzed for the structure of the rDNA repeat unit to date, but a coexistence of the 5S rDNA with the 45S rDNA has not been reported (Beech and Strobeck, 1993, Rogers and Bendich, 1987). Linkages of the 5S and the 45S rDNAs were denied in a green algae, *Chlamydomonas* sp. (Marco and Rochaix, 1980), and an euglenophyte, *Euglena gracilis* (Schnare, et al., 1990). In order to know their relationship better, phylogenetic tree deduced from the sequence of representing species' 18S rRNA gene sequences are shown in Fig. 2-5. From the tree, it was difficult to say that the common origin of the plant possessed the 5S and 45S in the same repeat and they were lost in the green algae and the spermatophytes. Of course, more information for the other bryophytes, the charophytes and the pteridophytes is necessary to give a conclusion.
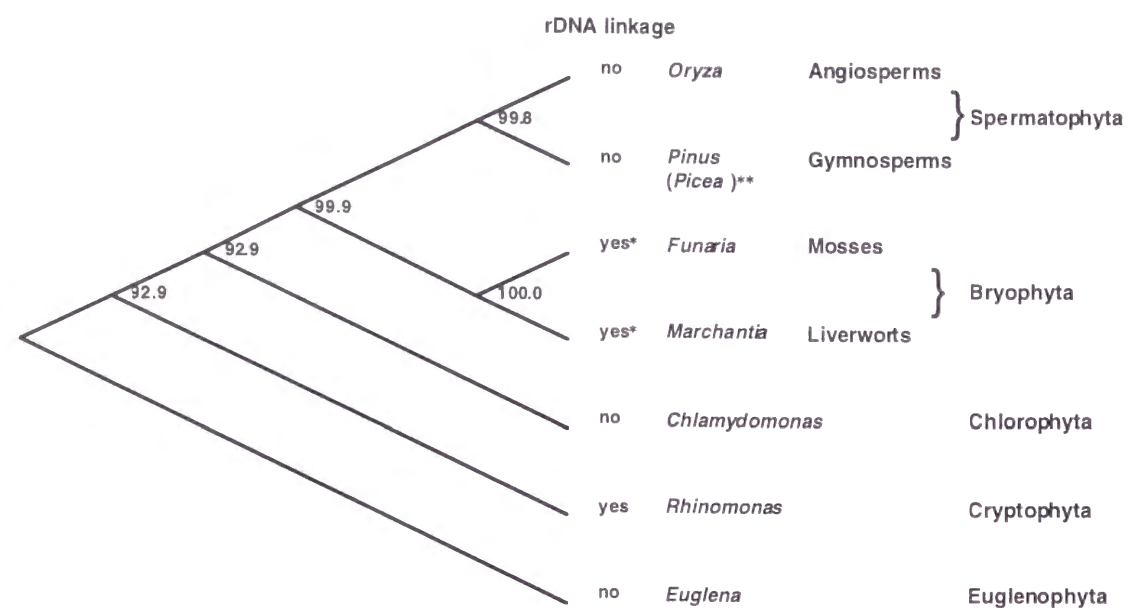
Fig. 2-5. Phylogenetic distribution of the linkage of the 45S and the 5S rDNAs in plants. The phylogenetic tree is an adaptation of the original tree of Kranz et al. (1995), which was deduced from SSU rDNA sequences. Only organisms that were studied for rDNA linkage are shown, and Euglenophyta and Cryptophyta were added. Distances were corrected by Kimura's 2 parameter model. Resulting data were visualized as a phylogram rooted with Euglenophyta by a phylogenetic tree drawing software (TreeView 1.5.3) (Page, 1996). The values at the nodes indicate the percentage of bootstrap analysis with 1,000 re-samplings and with random number generator seed as 111 by Neighbor-joining method (Saitou and Nei, 1987). References of the SSU rDNA sequences and the rDNA linkages: *Oryza sativa* (GenBank accession no. X00755, X54194), *Pinus wallichiana* (X75080), *Picea glauca* (Beech and Strobeck, 1993), *Funaria hygrometrica* (X74114, X80212), *Marchantia polymorpha* (X75521, AB021684), *Chlamydomonas reinhardtii* (M32703), *Rhinomonas pauca* (U53132), *Euglena gracilis* (M12677). *: Results of this paper. **: Although the gymnosperms were represented by *Pinus wallichiana* in the tree, the information of the rDNA linkage was known only in *Picea glauca*.

There has been a hypothesis that the co-localization of the 5S and the 45S rDNAs in same repeat unit found in some lower eukaryotes might represent a transitional status from the prokaryotic to the higher eukaryotic arrangement of the rDNAs (Belkhiri, et al., 1992, Srivastava and Schlessinger, 1991).

However, it has been found that not only the lower eukaryotes, but some animals also have the linked 5S rDNAs. The nematode *Meloidogyne arenaria* (Vahidi, et al., 1988) and some crustaceans *Calanus* sp., *Temora longicornis*, *Balanus balanoides*, *Thysanoessa raschi* (Drouin, et al., 1987, Drouin, et al., 1992), the spider *Artemia* sp.

(Drouin, et al., 1992) are also known to possess the 5S rDNAs in their major rDNA repeating units. In some organisms, the 5S rDNA found to linked to other kind of tandemly repeated multigene family. For example, the nematode *Caenorhabditis elegans* and the protozoa *Trypanosoma rangeli* incorporate their 5S rDNA in the repeat unit of trans-spliced leader (Aksoy, et al., 1992, Stratford and Shields, 1994) and in the crustacean *Artemia salina*, the 5S rDNA are found in the histone repeat units (Andrews, et al., 1987). Drouin and Moniz de Sa constructed phylogenetic trees of the animals which possessed the linked 5S rDNA and pointed out the distribution of the linked 5S rDNAs in such a wide taxa. Thus, they proposed another hypothesis that the 5S DNAs had been inserted into the 45S rDNA repeat unit by DNA-mediated or RNA-mediated transposition in certain species (Drouin and Moniz de Sa, 1995).

Nucleotide sequnece analysis of the support the RNA-mediated insertion of the 5S rDNA. Both in *M. polymorpha* and *F. hygrometrica*, a poly(A)-like sequence were found at 3' end of the 5S rDNA. An $A_{13}TA_{13}$ sequence was found in the A/T rich region of *M. polymorpha* and an oligo A_{25} sequence was found in *F. hygrometrica* as already mentioned. The presence of the poly(A)-like sequence suggests a similarity to Alu-like short interspersed elements (SINEs). A similar poly(A)-like sequence has been found at 3'-end of the 5S rDNA located in the trans-spliced leader repeat of *Trypanosoma vivax* (Roditi, 1992). The 5S rDNA might be inserted into the repeat sequence of these organisms by retrotransposon-like integration.

The sequence of coding regions of rDNA has been researched in a numerous species because the sequences were useful in phylogenetical analyses (Kranz, et al., 1995), while the sequence of IGS has been analyzed in many higher plant because the rapid evolutional ratio of IGS sequences are useful for phylogenetical analyses in small taxa (King, et al., 1993). However, the sequence of IGS has not been researched enough in lower plants. More information for the other bryophytes, the charophytes and the pteridophytes are necessary in order to know whether our finding of the coexistence of 5S rDNA and 45S rDNA are exception or common feature in lower plants.

Chapter III. Isolation and analysis of a sex-chromosome-specific clone from male PAC genomic library of *Marchantia polymorpha*

Introduction

Dioecism, production of male and female gametes by separate individuals, is a minority reproductive strategy among flowering plants. Approximately 4% of angiosperm species are dioecious (Yampolski and Yampolski, 1922). A few of these species are known to possess morphologically distinguishable sex chromosomes, among which two functionally distinct types of sex determination systems exist. In active Y system, possession of an Y chromosome leads to male sex expression, whereas in X/A balance systems, the X chromosome to autosome ratio controls sexuality of individuals. These are parallel with the sex determination systems in animals such as mammalian and *Drosophila*. The examples of both systems are *Silene latifolia* (female: 2n=22+XX, male: 2n=22+XY) (Blackburn, 1923) and *Rumex acetosa* (female: 2n=12+XX, male: 2n=12+XY$_1$Y$_2$) (Kihara and Ono, 1923), respectively. Sex expressions of the angiosperms occur in the diploid sporophytic generation.

In contrast to the angiosperms, the bryophytes express sexuality in the haploid gametophytic generation. In the bryophytes, especially in the liverworts, dioecism is a major reproductive strategy. In the dioecious species, each female and male contains a set of autosomes and usually one sex chromosome. For example, the first report of sex chromosomes in the plant was a liverwort *Sphaerocarpos donnellii* (Allen, 1917). A female gametophyte of *S. donnellii* has a large X chromosome in addition to 7 autosomes (n=7+X), while a male gametophyte has a small Y chromosome (n=7+Y). Therefore, the sex determination systems of the bryophytes may be different from the case of above diploid organisms. Among them, a dioecious liverwort, *M. polymorpha* has unusually small sex chromosomes (Haupt, 1932); the size of the X and Y sex chromosomes are approximately 10 Mb and 5 Mb, respectively (chapter 1). These are smaller in size than the X chromosome of *C. elegans,* which was recently sequenced completely. (Ainscough, et al., 1998) In addition, the total genome size of *M. polymorpha* is also small as approximately

280 Mb (chapter 1). Therefore *M. polymorpha* can be a potential model plant for the research of the sex determination systems. The genes responsible for the sex determination would be located on these small sex chromosomes. Therefore, the author aimed to isolate and sequence the sex chromosome of the liverwort in this study.

Materials & Methods

Plant materials

Because no inbred line of *M. polymorpha* had been established, a colony of siblings was selected from local wild populations, and female and male thalli of *M. polymorpha* were collected (FEt and MEt). Single gemma from each strain was sterilized and was maintained on 0-M51C-agar medium (Ono, 1979). A suspension cultured cell line (MEc) was generated from MEt by culturing on MS medium containing 2% glucose and 1% gellan gum (Katoh, 1981) for several months followed by continuous subculture in 1M51C medium.

Preparation of protoplasts

Gammae were transferred on a disposable petri dish containing 0-M51C solid medium and were incubated in a growth chamber at 20 °C under continuous light. After 3-4 weeks of culture, plantlets with a diameter of 5-10 mm were used for protoplast isolation. Plantlets were cut into pieces of 3-4 mm across with a scalpel. After rinse with the wash solution (0-M51C medium containing 10.5% mannitol instead of sucrose), 100 g of cut plantlets was suspended in 150-200 ml of enzyme solution containing 1% Cellulase Onozuka-RS (Yakult), 0.5% Hemicellulase (Sigma) and 0.5% Pectolyase Y-23 (Kikkoman) in the wash solution, pH 5.8-6.0. The suspension was incubated at 30 °C for 2.5-3 h and filtered through Miracloth (Calbiochem). Protoplasts were rinsed twice with the wash solution and were collected at 150 g for 5 min.

Preparation of high molecular weight (HMW) DNA

Pellet of protoplasts was equilibrated to 40 °C and was mixed with an equal volume of 1% InCert agarose (FMC Bioproducts) in wash solution, as described by Ioanou and de Jong (1996). Protoplast-gel mixture was aliquoted in plug molds (Bio-Rad) and was allowed to solidify at 4 °C. The plugs were treated with 10 volume of Proteinase K solution containing, 2% N-lauroyl sarcosine, 0.4 M EDTA, pH 8.0, 1 mg/ml Proteinase K (Boehringer), at 50 °C twice for 2 h and once for 12-14 h. The plugs were dialyzed twice against 10 mM Tris-HCl, pH 7.6, 50mM EDTA, 0.1mM phenylmethylsulfonyl fluoride (PMSF) at 4 °C for 2 h, rinsed twice with 0.5 M EDTA, pH 8.0 for 30 min and stored in 0.5 M EDTA, pH 8.0 at 4 °C. Partial digestion was performed with 0.3-0.6 units of *Mbo*I per plug, as described by Ioannou and de Jong, 1996. The plugs containing HMW DNAs were dialyzed twice in 0.5 x TBE buffer for 30 min and then were run in a 1% Seakem GTG agarose gel (FMC Bioproducts) using a clamped homogeneous electrical field (CHEF) (Chu, et al., 1986) apparatus (Bio-Rad), with 0.5x TBE buffer at 14 °C and 6 V/cm for 16 h with 0.1-5 sec pulse time and pulse directions at 120° angle. Marker lanes and both edges of the genomic DNA lane were cut off and were stained with ethidium bromide to indicate the location of the size range. Gel slices were then cut to cover the 100-200 kb range. Recoveries of the size-fractionated DNA were performed by the electroelution procedure (Osoegawa, et al., 1998).

Construction of PI-derived artificial chromosome (PAC) genomic libraries

The PAC library construction was carried out using pCYPAC2 vector, kindly provided by Dr. P. de Jong, Roswell Park Cancer Institute (Ioannou and de Jong, 1996) with modification of the original protocol reported by Osoegawa, et al., 1998. PAC vector DNA was carefully prepared as described in the protocol (Osoegawa, et al., 1998) but without purification by electrophoresis. Ligation and dialysis were performed as described by Osoegawa, et al. 1998. Twenty μl of the dialyzed ligation mixture was used to transform 40 μl ElectroMAX DH10B competent cells (Gibco BRL) by electroporation. Transformation was performed using a Gene Pulser® II (Bio-Rad) with the following conditions; cuvettes with 1 mm gap (Bio-Rad), on 1.3 kV/mm of voltage gradient, 25 μF of capacitance and 400Ω of resistance. The cell mixture was resuspended in 500 μl of SOC

medium (2% Bacto-tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl$_2$, 10 mM MgSO$_4$, 20 mM glucose) and was incubated at 37 °C for 1 h. Cells were plated on LB-agar plates containing 5% sucrose and 50 µg/mg kanamycin and were incubated 37 °C for 16 h.

Evaluation of PAC genomic libraries

Each batch of library was evaluated by randomly picking up 100 clones. Plasmid DNAs of the selected clones were isolated by a plasmid isolation machine (PI-100Σ, Kurabo) and the rate of large insert clone (above 20 kb) was checked by electrophoresis on 1 x TAE, 0.7% agarose gel, at 100 V for 1 h after BamHI digestion. For clones with large insert (above 20 kb), their insert sizes were measured by electrophoresis on 1% agarose gel using CHEF (Chu, et al., 1986) apparatus (Bio-Rad), with 0.5x TBE buffer at 14 °C and 6 V/cm for 8 h with 6-40 sec pulse time and pulse directions at 120° angle after NotI digestion. Then, average insert sizes were calculated. The separated DNA fragments were transferred to nylon membranes after treatment with the following buffers for 15 min each. Buffer I (0.5 N HCl), buffer II (0.5 N NaOH, 1.5 M NaCl), and buffer III (0.5 M Tris-HCl, pH 7.5, 1.5 M NaCl). Contamination of organellar DNAs were detected by probing the membranes with ^{32}P-labeled chloroplast and mitochondrial DNAs isolated from a female cell line (Oda, et al., 1992, Ohyama, et al., 1982). Batches that passed the quality evaluation were stored in 96 well microplate in –80 °C.

Screening by colony hybridization

Clones from 17 microplates were spotted two times each in a 6 x 6 grid pattern onto nylon membrane laid on LB-agar plates containing 50 µg/ml kanamycin and 1 mM isopropyl-1-thio-β-D-galactoside (IPTG) using a robotics workstation with a high density replicating tool (Biomek 2000, Beckman) as described by Ioannou, et al. 1994 (Ioannou, et al., 1994). Colonies were grown at 37 °C for 15 h and the DNAs were denatured and fixed to the membranes by autoclaving at 120 °C for 2 min followed by exposing to UV cross-linker. The filters were dried at 80 °C for 30 min and pre-washed in 4 x SSC containing 0.4% SDS

prior to use. Total genomic DNAs isolated from MEt and FEt by cetyltrimethylammonium bromide (CTAB) method (Strauss, 1994) were labeled with [α-^{32}P] dCTP by random prime labeling method for differential screening. Duplicated filters were hybridized either with the ^{32}P-labeled male genomic DNA or female genomic DNA. In order to remove false positives, additional screening by probing the same genomic DNAs to isolated plasmid DNAs of candidate clones digested with BamHI was performed. The hybridization was performed at 42 °C in a solution containing 6 x SSC, 0.1% SDS, 200 µg/ml sermon sperm DNA, 1 x Denhaldt's solution and 50% formamide for 16 h. Hybridized membranes were washed with 2 x SSC containing 0.1% SDS at 55 °C for 1h.

Genomic Southern blot analysis

Plasmid DNA of each clone was isolated and was labeled with [α-^{32}P] dCTP by random prime labeling method for genomic Southern blot analysis and general screening. The total genomic DNAs from MEt and FEt were digested with BamHI or EcoRI and electrophoresed on 1 x TAE, 0.7% agarose gel at 50 V for 6 h. Then separated DNA fragments were to nylon membrane as described above and were probed with the ^{32}P-labeled probe DNA. The buffers and the conditions were as described above.

Fluorescence in situ hybridization (FISH)

FISH procedure was performed following the method described in chapter 2. The PAC clone DNA was digested simultaneously with HhaI, HpaII and HaeIII following modification by Ohmido (personal communication) in prior to labeling with biotin-Highprime kit (Boehringer). The chromosomes were prepared from the cell suspension culture, MEc. The hybridization were carried out in the buffer containing 10 ng/µl of biotin- labeled DNA probes, 50% formamide, 5% dextran sulfate, 2 x SSC, 500 ng/µl autoclaved salmon sperm DNA and 100 ng/µl sonicated total genomic DNA. The probe was detected with avidin-fluorescein isothiocyanate (avidin-FITC, Boehringer) and amplification by biotinylated anti-avidin (Pierce).

Sequencing

PAC clone was prepared by ultra-centrifugation method and directly sequenced (Wang, et al., 1994). Sequence reaction was carried out using T7 and SP6 primers and Big Dye [TM] Terminator Cycle Sequencing Kit (Perkin Elmer) for direct sequencing. The sequence reaction was carried out using Big Dye [TM] Primer Cycle Sequencing Kit (Perkin Elmer) for sequencing the subclones. Raw sequence data were collected by a sequencer (ABI PRISM [TM] 377 DNA sequencer, Perkin Elmer). Raw sequence data were assembled by an assembly software (Sequencer [TM], Gene codes). Computer aided analysis of nucleotide sequences were carried out using a nucleotide sequence analyzing program (DNASIS 3.7, Hitachi Software Engineering).

Screening by polymerase chain reaction (PCR)

Primers for PCR were designed from the analyzed sequence to amplify 150 to 400-bp DNA fragments. Screenings using PCR were performed to collect adjacent clones. In order to perform screening efficiently, superpools (containing 96 x 12 or 96 x 16 clones), pools (containing 96 clones) and subpools (containing 12 or 8 clones) of the library clones were prepared as PCR template for the PCR screening (Shepherd, et al., 1994). The condition of PCR was as following; 2 min at 94 °C then 30 cycles of 1 min at 94 °C, 1 min at 59 °C, 1 min at 72 °C. The products were electrophoresed on 1 x TAE, 1.2% agarose gel to analyze the right products.

Results & Discussions

Construction of PAC genomic libraries with large insert size of DNA fragments

At first, several vectors were used before constructing the libraries. A yeast artificial chromosome (YAC), pYAC4 (Burke, et al., 1987), a bacterial artificial chromosome (BAC), pBeloBAC11 (Shizuya, et al., 1992), another BAC, pBACe3.6 (Osoegawa, et al., 1998), and a P1-derived artificial chromosome (PAC) (Ioannou, et al., 1994), pCYPAC2 were tried as a cloning vector. pCYPAC2 was chosen as a cloning vector because it had following advantages. Stable propagation of large inserts, ease of separating the recombinant DNA from host DNA by alkaline extraction procedures, the ability to create

libraries with low levels of chimeric clones, simple selection system with *SacBII* gene and ability to increase copy number by induction with IPTG.

Several key points on the library construction have already been described in the previous protocols (Ioannou and de Jong, 1996, Osoegawa, et al., 1998). Some other points in the case of the liverwort genomic library constructions were as following; (1) Preparation of fresh protoplasts in a short period of time. The yield of the protoplasts from thalli was much lower than that from suspension cultured cells as already reported (Bopp and Vicktor, 1988). Although longer incubation with the cell wall digesting enzymes or centrifugation at higher speed resulted in higher yield of the protoplasts, they caused to decrease live protoplasts with intact nuclei. The material thalli should be fresh, at least 3-4 weeks after germination from gemma. (2) Embedding protoplasts into gel plug. Two materials, protoplasts and nuclei were also compared. Although the final DNA concentration from nuclei embedded in the gel plug was 10 times as high as from protoplasts, the 95% of resulting library contained small insert clones (below 20 kb). This might be caused by trapping of smaller DNA fragment with in the HMW DNA when a large amount of partially digested DNA was loaded to the gel (Osoegawa, et al., 1998). And isolation nuclei instead of protoplast may cause increase of such smaller DNA fragments because of physical and chemical damages to nuclei. Twice in size-fractionation is recommended by Osoegawa, but when it was applied to the liverwort nucleus gel plug, twice size-fractionation caused serious loss of HMW DNA. The liverwort nuclei gel plug might have not contained large enough amount of DNA for twice size-fractionation. The final solution adopted in this paper was; (3) Electrophoresis of protoplast gel plug at the condition shown in the methods so as to allow the HMW DNAs with size ranging from 100 kb to 200 kb to be stacked. With this method, the trapping of smaller DNA fragment was mostly avoided and relatively high concentration of HMW DNA was obtained. The resulting library contained more than 60% large insert clones (above 20 kb).

Summary of the libraries constructed is given in Table 3-1. In the table, the small insert clones (below 20 kb) are counted as non-insert clones so as to keep the average insert size

high. The total numbers of clones with sufficiently long (average insert size: 90 kb) nuclear genomic inserts were 22,000 and 2,3000 for the female and male libraries, respectively. These correspond to genome coverage of 7-fold, and the libraries are expected to cover all unique nuclear genomic sequence at probability of 99.9% (P=0.999) according to the following equation; $N = \ln (1-P) / \ln (1-I/G)$ (*N*: number of necessary clones, *P*: probability to contain all unique sequence, *I*: average insert size in kb, *G*: genome size in kb).

Table 3-1. Summary of PAC libraries of *Marchantia polymorpha* female and male.

	Stored microplate	Stored clones	Rate of large insert clones	Clones with large insert	Contamination of organellar clones	Clones with nuclear DNA insert	Average insert size
Female	652	62592	50%	approx. 32,000	30%	approx. 22,000	100 kb
Male	1,104	105,984	30%	approx. 33,000	30%	approx. 23,000	90 kb

Isolation of male specific genomic clone

The male library was searched for male specific clones by differential screening using ^{32}P-labeled male total DNA and female total DNA as probes. Twenty-two clones were screened out of approx. 4,600 clones.

Three of the 22 clones specifically hybridized to male total DNA but not to female total DNA. One of such clones, pMM4G7, was selected for further analyses. The result of genomic Southern blot analysis is shown in Fig. 1A. Two *Bam*HI fragments, 2.5-kb and 0.7-kb were observed specifically in the male genomic DNA. From intensity observed in the autoradiogram, it was presumed that these DNA fragments were accumulated in the male genome.
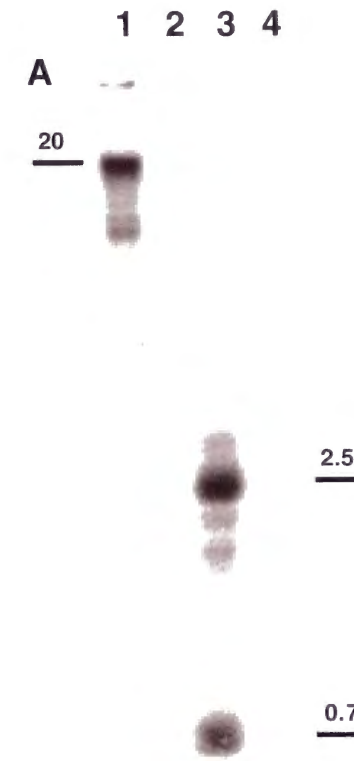
Fig. 3-1. Genomic Southern blot analysis of a male specific clone, pMM4G7. Lane 1. Male genomic DNA digested with *Eco*RI. Lane 2. Female genomic DNA digested with *Eco*RI. Lane 3. Male genomic DNA digested with *Bam*HI. Lane 4. Female genomic DNA digested with *Bam*HI. The bars with numbers indicate the intense male specific bands with their molecular size in

pMM4G7 was localized on the Y chromosome

A new cell line (MEc) in suspension culture from the male thallus line (MEt) was used to prepare chromosomes for FISH. Although this cell line was reported to be unstable in karyotype (chapter 1), Y chromosome can be constantly observed both from haploid cell and diploid cell. Thus, FISH was performed using this line and biotinylated pMM4G7. Signals of FITC representing hybridization of pMM4G7 were detected on the Y chromosomes both in a haploid cell (Fig. 3-2A, B) and in a diploid cell (Fig. 3-2C). This indicates that pMM4G7 was derived from the Y chromosome. As the signal of pMM4G7 was mostly covered over the Y chromosome, it appeared to contain considerable amount of sequence homologous to pMM4G7.
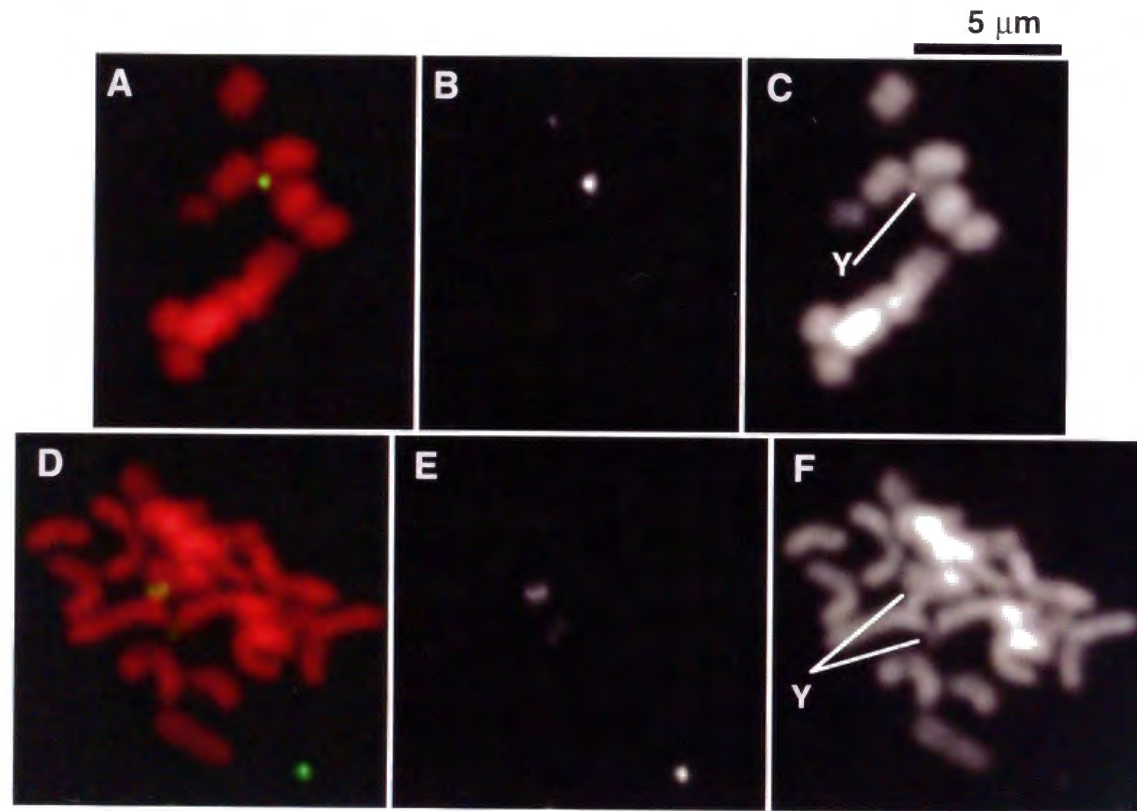
Fig. 3-2. FISH analyses of the male specific clone pMM4G7. A-C. A haploid cell line suspension culture derived from the male thallus line. D-F. A diploid male cell from the same line. A. Overlaid image of the signal of probe DNA detected by FITC and chromosome image stained with PI. B. The signal of probe DNA detected by FITC. C. Chromosome image stained with PI. D. Overlaid image of the signal of probe DNA detected by FITC and chromosome image stained with PI. E. The signal of probe DNA detected by FITC. F. Chromosome image stained with PI.

Repeated sequences specific to Y chromosome

The Y-derived PAC clone, pMM4G7, contained a 40-kb insert. Digested with *Bam*HI and separated in 1% agarose gel containing ethidium bromide, a 2.5-kb fragment appeared too intense for its size (Fig. 3-3). This indicates that this clone contained multi-copied sequences of 2.5-kb fragments, which correspond to the 2.5-kb band shown in the genomic Southern blot analysis (Fig. 3-1).
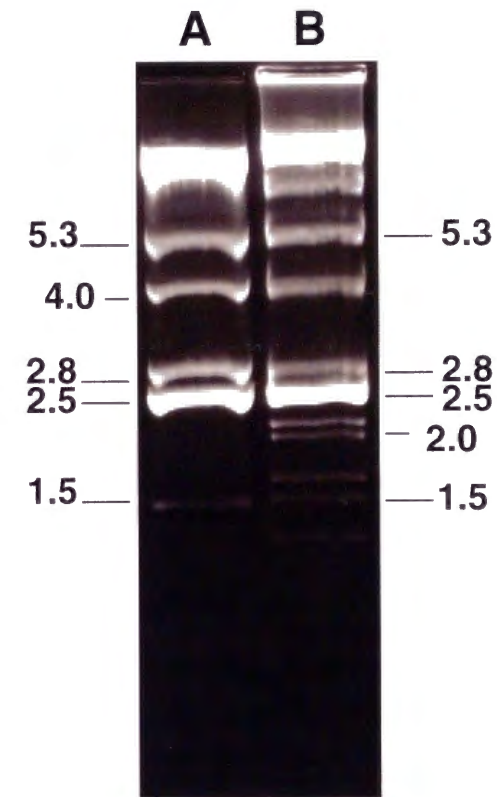
Fig. 3-3. BamHI digestion of pMM4G7 with pMM29D7. Lane A. pMM4G7 digested with BamHI and NotI. Lane B. pMM29D7. Digested with BamHI. The 2.5-kb band suggests that they are multi-copied. The band pattern similarity implies that pMM29D7 clone overlaps with pMM4G7 clone.

Other *Bam*HI fragments (1.5 kb, 2.8 kb, and 5.3 kb) and a 4.0-kb *Bam*HI-*Not*I fragment as well as the 2.5-kb fragments were subcloned and both end of each insert was sequenced, including pMM4G7 itself. Surprisingly not only the 2.5-kb fragments but also the other *Bam*HI fragments and one end of pMM4G7 contained highly conserved sequences at each side (Fig. 3-4). The complete sequence of one of the *Bam*HI 2.5-kb fragments revealed that the presence of subrepeats in the *Bam*HI fragment. *Mbo*I 400-bp repeats, which contains a *Mbo*I site in each unit and extends to approximately 400 bp each, are directly repeated 4 times in the *Bam*HI 2.5-kb fragment. *Hae*III 69-bp repeats, which contains a *Hae*III site in each unit and extends to 68-9 bp each, are repeated only once in the 2.5-kb fragments but they are repeated 4 times in the 2.8-kb fragments. These sequences showed no significant similarity to the sequences in the database (GenBank). These results indicated that there are novel repeat sequences, which contain *Bam*HI site. Some of them are tandemly repeated in a 2.5-kb or 2.8-kb fragment and the rests are separated in different lengths.

It has been reported that sex chromosomes of some organisms also contain sex chromosome specific repeat sequences. The sequence obtained from micro-dissected Y_1 and Y_2 chromosomes of *Rumex acetosa* contained sequences which specifically accumulated on X, Y_1 and Y_2 chromosomes by FISH (Ruiz Rejon, et al., 1994). A randomly amplified polymorphic DNA (RAPD) fragment specific to male *Cannabis sativa* contained a retrotransposon-like repeat sequence that was specific to the Y chromosome (Sakamoto and Satoh, 1998). A BAC clone derived from the W sex chromosome of a silk worm, *Bombyx mori* was shown to have many kinds of retrotransposon-like repeat sequences (Ohbayashi, et al., 1998).

```
4G7B2.5kb-I      1 GGATCCGGAT GCCGAATATA TTGAAAAAAC ATATAAGTAA AAAAAGTAAG   50
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7NB4.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I     51 AAATGAAAAC CATGTGAAAA ACATAAAGCA ATAAATAAAA ATATTAAGTA  100
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7NB4.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb         :::::::::: :::::::::: :::::::::: :::::::::: :::::::N:

4G7B2.5kb-I    101 ATATACTAAA GGACATATCA AAAAACAAAG AAGAAAATAC AAAACCATAT  150
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7NB4.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb          :::::::::: ::N::::N: :::::::::: :::N:::N: ::::::::::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7-T7             ---------- ---------- ---------- -::::::::: ::::::::::

4G7B2.5kb-I    151 AAAAACATAA AGCAATAAAT AAAAACTCAA GAGACGACTG ACGACTGACT  200
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::A:::: :::::::::: :::::::::: ::::::::::
4G7NB4.0kb         :::::::::: :::::A:::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb          :::::::::: :::::::::: N::::::::: :G:::::::: N:::::::::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::N:::: ::::::::::
29D7B2.0kb         :N::::::: :::::::::: :::::::::: :::N:::: ::::::::::
4G7-T7             :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    201 CGACTGGTCT GACTGCGGAC TGACGGGGTC AGTCCGCAGT CCGACCGTCG  250
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::::::: ::::------ ---------- ---------
4G7NB4.0kb         :::::::::: :::::::::: ::::------ ---------- ---------
4G7B2.8kb          :::N::::: :::::::G: NN:::::::: :::::::TN :G::::N::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb         :::::::::: :::::::::: :::::::::: :::::::::: ::::::::T
4G7-T7             :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    251 GTCGTCAGTC GAGTTCTTGT CATTCCTCAG TGACCCTATA TATAAACCCT  300
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          ---------- ---------- ---------- ---------- ----------
4G7NB4.0kb         ---------- ---------- ---------- ---------- ----------
4G7B2.8kb          :::::::N:: :::::::::N :::::::::: ::::::N::: ::::::::::
4G7B1.5kb          :::::::::: :::::::::: :T::::::: :::::::::: ::::::::::
29D7B2.0kb         :::::::N: :::N::::: ::N:::N:: :::::::::: N:N:N::::
4G7-T7             :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
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```
4G7B2.5kb-I    301 AAACCCGCTC TCCCTCTCGC CACTACGCAC TCTCGCTCTC GAATTTGCCC   350
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          ---------- ---------- ---------- ---------- ----------
4G7NB4.0kb         ---------- ---------- ---------- ---------- ----------
4G7B2.8kb          :::::::G:: :::::::::G N:A::A:G:: :::::G:::: ::::::TG::
4G7B1.5kb          :::::::::: :::::::::: :::::::::: :::::::::: :---------
29D7B2.0kb         :::::::::: ::::::::N :::::::::: :::N:T:N:: ::::::::::
4G7-T7             :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    351 GCTCTCGGCT CTCACTCTCC GCTCTCGCTC CTCCTCTCGT CTCGTTCCTG   400
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          ---------- ---------- ---------- ---------- ----------
4G7NB4.0kb         ---------- ---------- ---------- ---------- ----------
4G7B2.8kb          :G:::N:G: ::::-:::: :G::::N:: T:G::N:GTC T:::::::::
4G7B1.5kb          ---------- ---------- ---------- ---------- ----:::::
29D7B2.0kb         :::::N::N :N:::::N: :G::N:::NN :N::N:NN:: :::N::::
4G7-T7             :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    401 CGTTCATCGA TCTCTTCAAT GCGTGGATGG AGAAATCACA CTATTCAATC   450
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          ---------- ---------- ---------- -ACT:G:::: ::::::::::
4G7NB4.0kb         ---------- ---------- ---------- -ACT:G:::: ::::::::::
4G7B2.8kb          ::::N::N:N ::::::::=>
4G7B1.5kb          :::::::::: :::::::::T :::::::::: :::::::::: ::::::::::
29D7B2.0kb         N::N::N:: ::N::N:::T :::::::::: N:N:N:::NN ::::N:::N:
4G7-T7             :::::::::: :::::::::T :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    451 TCTTCCCTGG AGGAAAACCT CTTGATGATT TGATGATTGA AATCACGCTC   500
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: :::::T:::: :::::::::: :::::::::: ::::::A::T
4G7NB4.0kb         :::::::::: :::::T:::: :::::::::: :::::::::: ::::::A::T
4G7B1.5kb          :::::::::: :::G:T:::: :::::::=>
29D7B2.0kb         :N::::::::: :::=>
4G7-T7             :::::::::: :::AA::::: ::::::AT: :::::::::: ::::::::::

4G7B2.5kb-I    501 TTAACTCGTC AAATAATCAA GAGGTGTTCC TCCACGGAAG AGATTAAATA   550
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::::::::: ::::TG:::: :::::::::: :::::::::: ::::::::::
4G7NB4.0kb         :::::::::: ::::TG:::: :::::::::: :::::::::: ::::::::::
4G7-T7             ::::::::-: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    551 AAACCATAAA GGAATATACT AAAGCACATA TTACAAAACA AAGAAGAAAA   600
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb          :::A:::::G :T:::::T:A :::::::::: A:::::::T: :::T::T:::
4G7NB4.0kb         :::A:::::G :T:::::T:A :::::::::: A:::::::T: :::T::T:::
4G7-T7             :::::::::: :::::::::: :::::::CAT A:TAC:::AC ::AG:AG:::

4G7B2.5kb-I    601 TACAAAACCA TATAAAAACA TAAGGGCAAT AAATAAAAAC TCAAGCGACG   650
4G7B2.5kb-II       :::::::::C C:ANTTTTN: ANTTTTT=>
4G7B5.3kb          :::::::::: AC:C::=>
4G7NB4.0kb         :::::::::: AC:C::AA:: :::A-::AA: :::::::::: :::::::NG:
4G7-T7             ATAC:::A:C ATAT::AAAC AT:A::GCAA T://
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4G7B2.5kb-I    651 -ACTGACGAC TGACTCGACT GGTCCGACTG CGGACTGACC CCGTCAGTCC   700
4G7NB4.0kb         N::::::::: :::::::::: ::::T::::: :::::::::: ::::::::::

4G7B2.5kb-I    701 GCAGTCCGAC CGTCAGTCGT CAGTCGTGTT CTTGTCTTTC CTCAGTGACC   750
4G7NB4.0kb         :::::::::: :::::::::: ::::::A::: ::::::A::: ::::::::::

4G7B2.5kb-I    751 CTATATATAA ACCCTAAACC CGCTCTCCCT CTCGCCACTA CGCACTCTCG   800
4G7NB4.0kb         :::::::::: :::::::::: ::G:::::::: :::TN::::: ::G:::::::

4G7B2.5kb-I    801 CTCTCGAATT TGCCCGCTCT CGTCTCGTTC CTGCGTTCAT CGATCTCTTC   850
4G7NB4.0kb         G::://

4G7B2.5kb-I    851 AATGCGTGGA TGGAGAAATC ACACTATTCA ATCTCTTCCC TGGAGGAATA   900

4G7B2.5kb-I    901 CCTCTTGATG ATTTGATGAT TGAAATCACA CTCTTAACTC GTCAAATAAT   950

4G7B2.5kb-I    951 CAAGAGGTGT TCCTCCACGG AAGAGATTAA ATAAAAACAT TAAGCAATAT   1000

4G7B2.5kb-I   1001 ACTAAAGCAC ATATTACAAA ACAAAGTAGT AAATACAAAA CCAACTAAAA   1050

4G7B2.5kb-I   1051 ACATAAGGGC AATAAATAAA AACTCAAGCG ACGACTGACG ACTGACTCGA   1100

4G7B2.5kb-I   1101 CTGGTCCGAC TGCGGACTGA CCCCGTCAGT CCGCAGTCCG ACCGTCAGTC   1150

4G7B2.5kb-I   1151 GTCAGTCGAG TTCTTGTCTT TCCTCAGTGA CCCTATATAT AAACCCTAAA   1200

4G7B2.5kb-I   1201 CCCGCTCTCC CTCTCGCCAC TACGCACTCT CGCTCTCGTT CCTGCGTTCA   1250

4G7B2.5kb-I   1251 TCGATCTCTT CAATGCGTGG ATGGAGAAAT CACACTATTC AATCTCTTCC   1300

4G7B2.5kb-I   1301 CTGGAGGAAT ACCTCTTGAT GATTTGATGA TTGAAATCAC ACTTTTAACT   1350

4G7B2.5kb-I   1351 CGTCAGATAG TCAAGAGGTG TTCCTCCACG GAAGAGATTA AATAAAAACA   1400

4G7B2.5kb-I   1401 TAAGGTAATA TTCAAAAGCA CATATTACAA AATAAAGTAG TAAATACAAA   1450

4G7B2.5kb-I   1451 ACCAACTCAA AACATAAAGC AATAAATAAA AACTCAAGCG ACGACTGACG   1500

4G7B2.5kb-I   1501 ACTGACTCGA CTGGTCTGAC TGCGGACTGA CCCCGTCAGT CCGCAGTCCG   1550

4G7B2.5kb-I   1551 ACCGTCAGTC GTCAGTCGAG TTCGATGCGA CCGTGGCCCT CGTCCATACG   1600

4G7B2.5kb-I   1601 AGGAGCGATG TCAGCAGTAC GAGGTGAACC CTAAAACCTA AACCCTCATT   1650
4G7B2.5kb-II       <=:C:::T :::::::::: :::T::::: :::::::T:: ::::::::::

4G7B2.5kb-I   1651 TCTTTACATG TTAGTTATCG AATATTTTCT GAAATTCTCT CTTTAGATGT   1700
4G7B2.5kb-II       :::::::::: ::::::C:: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I   1701 GACTTTTTAT ATCTTTCATA GAATGATTAT TTTCATGTGC TTGTTTCTTT   1750
4G7B2.5kb-II       :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb                                                          <=T:::
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4G7B2.5kb-I    1751 CTGAATATAT ATTTGTTATT TTTTTTATTT TCTTAATTAT CTCATGATAA  1800
4G7B2.5kb-II        :::::::::: :::::::::: ::::N::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: ::::::A:::: ::::NA:::: :::::::::: ::::::T::T

4G7B2.5kb-I    1801 TCAAAAAAAT AAGATAACCA AACCCCCCGG GACCCTTTCG TCGACGTGAT  1850
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::N: :G:T::::: :::::::::: N::::::::: ::::::::::

4G7B2.5kb-I    1851 GTCCATCACG TCCGGACCGG TGTAACCCAG ATCCGAGAAG ACCGCTGCGG  1900
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    1901 TTCAGCGCCC TTGGCACGGA AGAGGTCTGG GATGGGTGAC CTCCTCTCCG  1950
4G7B2.5kb-II        ::A::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          ::A::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    1951 TGGAACTTCC GCAGCTCATA AACATGAAAA AGTTCCTCAA GTCCTGTTTG  2000
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb                                                     <= ::::::N::
4G7B2.8kb                                                        <=AANN:::T
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::AA GT:::::::N

4G7B2.5kb-I    2001 TTTTATAAA- -------TAT ATATATATAT ATACATGTAG ATTTCTATTT  2050
4G7B2.5kb-II        :::::::::- -------::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           NN:::::::-- -------::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :NAA:NTTT- -------::A T:::::::::: :::::::GT :::::::::
4G7B1.5kb                        <= ATATATA::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          CCC::N::AT ATATATA::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2051 GTATTTGTAT TTCTATTTTT AGAGGGTCAA TTTTATGAAG GCGACCGTGG  2100
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::::::: :::::::::: ::G::A:::: ::C:::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: :::::::::: :::::::::- ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2101 CCCCTCGGAC CCTGGTCCAT ACGAGGAACG ATGTCAGCAG TACGAGGTAC  2150
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::::::: :::C::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2151 GGAGGATATG CGACCTT--- ---------- ---------- ----------  2200
4G7B2.5kb-II        :::::::::: :::::::--- ---------- ---------- ----------
4G7B5.3kb           :::::::::: :::::::--- ---------- ---------- ----------
4G7B2.8kb           ::C::::::: :::::G:GGC CCCTCGGACC CCCGTCCATA CCAGGAACGA
4G7B1.5kb           :::::::::: :::::::--- ---------- ---------- ----------
29D7B2.0kb          :::::::::: :::::::--- ---------- ---------- ----------
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4G7B2.5kb-I    2201 ---------- ---------- ---------- ---------- ----------  2250
4G7B2.5kb-II        ---------- ---------- ---------- ---------- ----------
4G7B5.3kb           ---------- ---------- ---------- ---------- ----------
4G7B2.8kb           TGTCAGCAGT ACGAGGTACG GCGGATATGC GACTGTGGCC CCTCGGACCC
4G7B1.5kb           ---------- ---------- ---------- ---------- ----------
29D7B2.0kb          ---------- ---------- ---------- ---------- ----------

4G7B2.5kb-I    2251 ---------- ---------- ---------- ---------- ----------  2300
4G7B2.5kb-II        ---------- ---------- ---------- ---------- ----------
4G7B5.3kb           ---------- ---------- ---------- ---------- ----------
4G7B2.8kb           CGTCCATACC AGGAACGATG TCAGCAGTAC GAGGTACGGA GGATATGCGA
4G7B1.5kb           ---------- ---------- ---------- ---------- ----------
29D7B2.0kb          ---------- ---------- ---------- ---------- ----------

4G7B2.5kb-I    2301 ---------- ---------- ---------- ---------- ----------  2350
4G7B2.5kb-II        ---------- ---------- ---------- ---------- ----------
4G7B5.3kb           ---------- ---------- ---------- ---------- ----------
4G7B2.8kb           CCGTGGCCCC TCGGACCCTC GTCCATACGA GAAACGATGT CAGCAGTACG
4G7B1.5kb           ---------- ---------- ---------- ---------- ----------
29D7B2.0kb          ---------- ---------- ---------- ---------- ----------

4G7B2.5kb-I    2351 ---------- ---------- ---GGCCCGT CGTACTATAC GAGGTAAAAA  2400
4G7B2.5kb-II        ---------- ---------- ---:::::::: :::::::::: ::::::::::
4G7B5.3kb           ---------- ---------- ---:::::::: :::::::A:: ::::::::::
4G7B2.8kb           AGGTACGGAG GATATGCGAC CTT::::::: :::::::::: ::::::::::
4G7B1.5kb           ---------- ---------- ---:::::::: :::::::::: ::::::::::
29D7B2.0kb          ---------- ---------- ---:::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2401 TCTGGCGCTG CCCCTGGCGC ATTCCTAAAC CCTAGTTGGG CAGTCCCTTT  2450
4G7B2.5kb-II        :::::::::: :::::::::: ::::::GGG- ---:::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: ::::::GGG- ---:::::::: ::::::::::
4G7B2.8kb           :::::::::: :::::::::: ::::::GGG- ---:::::::: :::::T::::
4G7B1.5kb           :::::::::: :::::::::: ::::::GGG- ---:::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: ::::::GGG- ---:::::::: ::::::::::

4G7B2.5kb-I    2451 GTTCGAGTGA GAGGGACGAA AGGAGGAAAG AAATGTACCC GAGTGTGCGC  2500
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2501 CTCGCCAAGT AGGAGCGTTG GGGGCGCGCG TCTCGCGGTG CGTTGAACCC  2550
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
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4G7B2.5kb-I    2551 GGCCGGGCGA AATGGGGGAG ATTTGGGTAG TGAGACTGAG CGCAATTTGG  2600
4G7B2.5kb-II        :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: :::::::::: :::::::::: ::::::::::

4G7B2.5kb-I    2601 ATTTTTACCA CCCAGAAGTT TCGCGGATCC  2650
4G7B2.5kb-II        :::::::::: :::::::::: ::::::::::
4G7B5.3kb           :::::::::: :::::::::: ::::::::::
4G7B2.8kb           :::::A:::: :::::::::: ::::::::::
4G7B1.5kb           :::::::::: :::::::::: ::::::::::
29D7B2.0kb          :::::::::: :::::::::: ::::::::::
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Fig. 3-4. Nucleotide sequence comparison of respective end of _Bam_HI fragments from pMM4G7 and pMM29D7. A major _Bam_HI-2.5-kb fragment, 4G7B2.5kb-I, was sequenced completely by a shotgun method. The other fragments were aligned using 4G7B2.5kb-I as core sequence. Another _Bam_HI-2.5-kb fragment from pMMM4G7, 4G7B2.5kb-II. 4G7B5.3kb: A 5.3-kb _Bam_HI fragment from pMM4G7. 4G7NB4.0kb: A 4.0-kb fragment from SP6 promoter side of pMM4G7. When 4G7NB4.0kb was sequenced from _Bam_HI side it had highly conserved sequence with 4G7B5.3kb. 4G7B2.8kb: One of the 2.8-kb _Bam_HI fragments from pMM4G7. 4G7B1.5kb: A 1.5-kb _Bam_HI fragments from pMM4G7. 29D7B2kb. A 2.0-kb _Bam_HI fragment from pMM29D7. G7-T7: The sequence appeared when pMM4G7 were directly sequenced from T7 prompter end.

Walking on Y chromosome from pMM4G7

Chromosome walking was performed using two strategies, colony hybridization using the high-density membranes already prepared for differential screening, and PCR screening as described in methods. Using the _Bam_HI 2.5-kb fragment as an Y chromosome specific probe, 20 clones were screened from 4600 clones. Five of them showed similar band pattern with pMM4G7 when digested with _Bam_HI. One clone, pMM29D7 (ca. 100-kb insert) contained almost all bands shown in pMM4G7 (Fig. 3-3). The clone appeared to contain all region of pMM4G7. Then, the sequencing of pMM29D7 from both ends were performed. The SP6 end appeared to have similar sequence with the _Bam_HI 2.5-kb repeat. Thus, a PCR primer set was designed for the other T7 end. The PCR primer set was used as sequence tagged site (STS) marker and contig clones were screened using the clone pools described above. Several candidates of contig clones were obtained and appeared to contain another kind of repeat sequence. Some of them were confirmed to be real contig by cutting map of rare cutting restriction enzymes (Fig. 3-5).
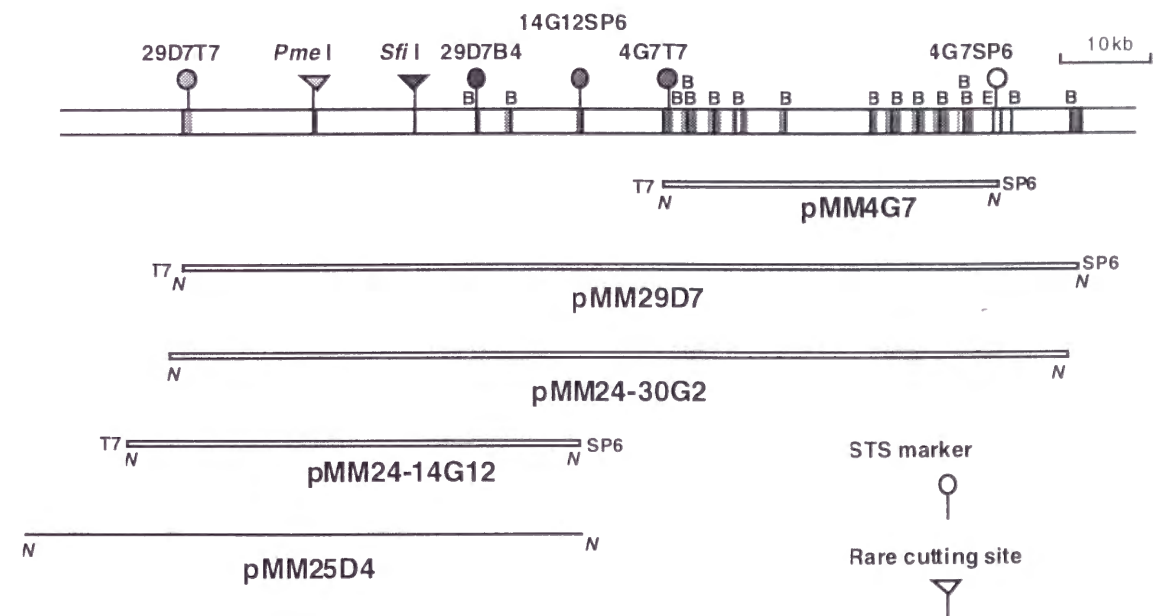
Fig. 3-5. Partial contig map of Y chromosome. Sequence tagged site (STS) markers and rare cutting restriction enzyme sites are indicated by circles and triangles, respectively.

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Summary

Chapter I

Marchantia polymorpha L. is a dioecious liverwort and has small sex chromosomes (female: n=8+X, male: n=8+Y). The total nuclear genome size is also small, thus it is a potential model plant for the research of sex chromosome in the bryophytes. In this study, we examined accurate genome size and sex chromosome sizes of *M. polymorpha*.

Two male (Mc, MEc) and two female (Fc, FDc) cell lines of suspension culture and a pair of thallus lines (MEt, FEt) were analyzed for their genome sizes and chromosome numbers. The genome sizes of the female and the male thallus lines were both 280±8 Mb by a flow cytometry. In the cultured cell lines, however, variations were observed between 270±20 to 630±20. The size of the X and Y sex chromosomes were estimated to be 10±2 Mb and 5±2 Mb, respectively by a simple photon counting method. One cause of the genome size variation was appeared to be polyploidy and aneuploidy of the cell lines. Another cause was estimated to be the changes of the copy number of the repeated sequences. The copy numbers of the rDNA repeat showed a correlation with the genome sizes per haploid. Fluorescence *in situ* hybridization (FISH) revealed that not only the copy number but also the loci of the rDNA repeat were varied from cell line to cell line.

Chapter II

5S ribosomal RNA genes (rDNA) usually locates independently from rDNA repeat (45S rDNA) containing 18S, 5.8S and 26S ribosomal RNA genes in higher eukaryotes, whereas they are co-localized in the 45S rDNA repeat units in some lower eukaryotes such as fungi and protozoa. The 45S and the 5S rDNAs of Southern blot and fluorescence *in situ* hybridizations revealed that almost all the 5S rDNAs coexist with the 45S rDNAs on the genome of a liverwort, *Marchantia polymorpha*. Actually a single repeat unit of the 45S

rDNA (16,103 bp in length) also revealed that the repeat unit contained the 5S rDNA. To our knowledge, this is the first report on the co-localization of 5S and 45S rDNA in land plants. Furthermore, we detected 5S rDNA in the 45S rDNA sequence of a moss, *Funaria hygrometrica*. These findings suggest that there has been structural re-organization of the rDNAs after divergence of the bryophytes from the other plant species in the course of evolution.

Chapter III

A dioecious liverwort, *Marchantia polymorpha* has unusually small sex chromosomes (X: 10±2 Mb, Y: 5±2 Mb). In order to isolate sex-chromosome-specific DNA sequences, large-insert genomic libraries of the male and the female were constructed using a P1-derived artificial chromosome, pCYPAC2 as a vector. The final scale of the library was 7-fold genome coverage, which is expected to covers all unique sequences at 99.9%. Then, male-specific clones were isolated from the male library by a differential screening using male genomic DNA and female genomic DNA as probes. One clone, named pMM4G7 with 40-kb insert was specifically hybridized to male genomic DNA when it was used as a probe in a genomic Southern blot analysis. Finally, it was confirmed that the clone was derived from Y chromosome by a fluorescence *in situ* hybridization. Partial sequencing of the clone revealed that this region of Y chromosome was covered with male-specific repeated sequences.

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I wish I could have gathered as many liverworts as possible during my three years on the stone.

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Takefumi Sone