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Distribution patterns of rDNAs and telomeres and chromosomal rearrangement between two cytotypes of *Lysimachia mauritiana* L. (Primulaceae)

Yoshiko Kono[1,2], Yoshikazu Hoshi[3]*, Hiroaki Setoguchi[4], Masatsugu Yokota[5] and Kazuo Oginuma[6]

[1]Graduate School of Human Health Science, Kochi Women's University, 5-15 Eikokuji-cho, Kochi 780-8515, Japan
[2]Herbarium (HAST), Biodiversity Research Center, Academia Sinica, Taipei 115, Taiwan
[3]Department of Plant Science, School of Agriculture, Tokai University, Kawayou, Minamiaso-mura, Aso-gun, Kumamoto 869-1404, Japan
[4]Graduate School of Human and Environmental Studies, Kyoto University, Yoshidanihonmatsu-cho, Sakyo-ku, Kyoto 606-8501, Japan
[5]Laboratory of Ecology and Systematics, Faculty of Science, University of Ryukyus, 1 Senbaru, Nishihara-cho, Nakagami-gun, Okinawa 903-0213, Japan
[6]Department of Biology, Faculty of Human Life and Environmental Science, Kochi Women's University, 5-15 Eikokuji-cho, Kochi 780-8515, Japan

Abstract — Two cytotypes of *Lysimachia mauritiana* collected from Japanese mainland (JM-type) and the Sakishima group (SR-type), located in the southernmost of the Ryukyu Archipelago of Japan, were cytogenetically analyzed. JM- and SR-types showed karyotype formulae of 2n=20(4**m**)=4**m**+2sm+4st+10t and 2n=18(6**m**)=6**m**+2sm+10t, respectively. In FISH analysis, a signal of 5S rDNA was observed at the interstitial region of long arm on a pair of t-chromosome in both cytotypes. On the other hand, different sizes of 45S rDNA signals were observed on the distal and proximal regions of **m**-, sm-, st- and t-chromosomes in JM-type, and of **m**- and t-chromosomes in SR-type. Also in SR-type, a tiny 45S rDNA signal was shown at the interstitial region of long arm on sm-chromosome. Telomere signals in two cytotypes appeared at both ends of all chromosomes, and at internal sites of certain chromosomes. The big telomere sequence signal at the proximal region coincided with the small 45S rDNA signal in JM-type. These results gave us a fusion/fission hypothesis with two st- or one **m**-chromosome(s) for new intraspecific karyotype formation of JM-type or SR-type.

Key words: Chromosomal polymorphism, FISH, Karyotype variation, *Lysimachia mauritiana*

INTRODUCTION

Lysimachia mauritiana L. (Primulaceae), which is biennial and herb species, has wide distribution in the temperate and subtropical zones of both hemispheres (Iwatsuki *et al*. 1993). In Japan, the populations of the species can be frequently seen on rocky place in seacoasts from the north to the south in the most islands or archipelagos. Cytogeographical investigations of this species have been mainly conducted in the population of East Asia, including the Japanese Archipelago. These studies for more than half a century led us to common understanding that mitosis of *L. mauritiana* had species-specific karyotype with four longer and landmark metacentric chromosomes, six submeta- or subtelocentric and ten telocentric chromosomes, displaying the formula '2n=20=4m+2sm+4st+10t' (Tanaka and Hizume 1978) or '2n=20(4**m**)' (Kono *et al*. 2008) as 'Japan mainland-type (JM-type)' (Fig. 1A).

*Corresponding author: e-mail: yhoshi@agri.u-tokai.ac.jp

However, recent study of *L. mauritiana* in the Sakishima group, located in the southernmost of the Ryukyu Archipelago of Japan, revealed that whole individuals in the almost islands belonging to this group showed only single karyotype differ substantially from previous studies, displaying the formula '2n=18=6**m**+2sm+10t' or '2n=18(6**m**)' (OGINUMA *et al.* 2004; KONO *et al.* 2010) as 'Southern Ryukyu-type (SR-type)' (Fig. 1B). Thus, up to the present date we recognize two major cytotypes as cytogenetically important materials to infer a chromosome differentiation of this species.

Fluorescence *in situ* hybridization (FISH) of tandemly-repeated DNA sequences, such as the ribosomal RNA genes (rDNAs) and telomeric sequence repeats are useful tools for chromosome or genome characterizations, and for studying inter- and intra species-level relationships in a large number of plant taxa (HESLOP-HARRISON 2000). There is, however, no report employing FISH for *L. mauritiana*. Therefore, extended application of the technique would provide further insight into cytogenetic differentiation or chromosome changes between two cytotypes in the species.

In this study, we investigated the 5S and 45S rDNAs, and telomere arrays in *L. mauritiana* by chromosomal physical FISH mapping to gain further insight into the chromosome rearrangement between two cytotypes in the Japanese Archipelago. We will discuss the chromosomal changing with tandem repeat sequences in this species.

MATERIALS AND METHODS

Plant materials - Two cytotypes with 2n=18 (6**m**) and 20(4**m**) of *Lysimachia mauritiana* were collected in Japan. These plants used were cultivated in the greenhouse of Kochi Women's University. Voucher specimens investigated were deposited in RYU, the herbarium of University of the Ryukyus.

Slide preparation - The methods of pretreatment, fixation and staining for chromosome preparation of OGINUMA and NAKATA (1988) were used with slight modification. After root tips were pretreated with 2 mM 8-hydroxyquinoline for 6 h at about 18°C, they were fixed in a mixture of ethanol and acetic acid (3:1), washed with distilled water, and then macerated in an enzymatic mixture containing 0.02% Cellulase Onozuka R-10 (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan) and 0.015% Pectolyase Y-23 (Seishin Pharmaceutical Co., Tokyo, Japan) for 60 min at 37°C. After washing with distilled water, root tips were put onto glass slide, and squashed in 1 N HCl. The preparations were air-dried for 24 h at room temperature after removal of coverslips with dry ice.

DNA extraction, amplification and purification - Total genomic DNA was isolated from

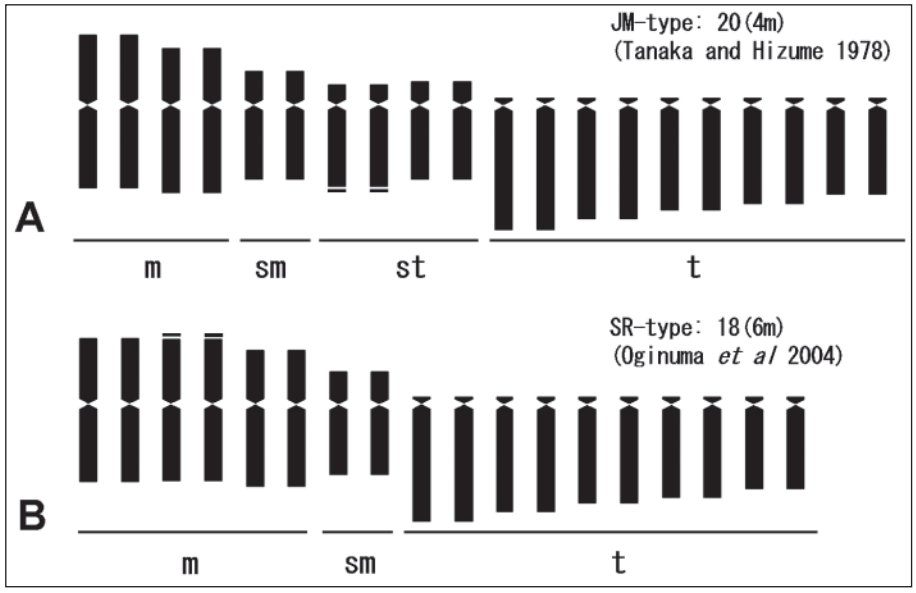

Fig. 1 — Previously reported karyotypes of two cytotypes in *Lysimachia mauritiana*. (A) JM-type (Tanaka and Hizume 1978). (B) SR-type (Oginuma *et al.* 2004).

young leaves following modified cetyltrimethylammonium bromide (CTAB) method (DOYLE and DOYLE 1990). The samples were ground into powder with liquid nitrogen and homogenized in the buffer containing 0.1 M Tris-HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB and 0.5% mercaptoethanol. The homogenate was extracted three times with an equal volume of chloroform-isoamyl alcohol (24:1) for 15 min each and the DNA was precipitated with an equal volume of isopropyl alcohol at room temperature. The DNA was treated with DNase-free RNase A (0.2 mg/ml) at 37°C for one hour followed by extractions with phenol-chloroform and chloroform. With extracted DNA, 5S rDNA and the 18S rDNA sequences were amplified by polymerase chain reaction (PCR) using primer sets of 5S F (5'-CGGTGCATTAATGCTGGTAT-3') and 5S R (5'-CCATCAGAACTCCGCAGTTA-3'), 18S F (5'-AACCTGGTTGATCCTGCCAGT-3') and 18S R (5'-TGATCCTTCTGCAGGTTCACCTAC-3'), respectively. *Arabidopsis*-type telomere concatemer was synthesized using primers $(TTTAGGG)^5$ and $(CCCTAAA)^5$ in the absence of template DNA (IJDO *et al.* 1991). The cycle profile was an initial denaturation of 94°C (4 min), 35 cycles with 94°C (30 sec), 57°C (30 sec) and 72°C (60 sec), and a final extension step of 72°C for 5 min. PCR products were column-purified using QIAquick PCR Purification Kit (QIAGEN, Tokyo, Japan) or gel-purified using GeneClean III (BIO 101, CA, USA).

DNA probes and labelling - To track the chromosomal locations of the 5S rDNA, 45S rDNA and telomere arrays, the 5S rDNA unit, the 45S rDNA sequences, and synthesized telomere concatemer were used as FISH probes, respectively. Sequence identity of the 5S and 45S rDNA fragments were confirmed by DNA sequencing. 5S and 45S rDNA fragments were biotin-labelled by random primed labelling technique (FEINBERG and VOGELSTEIN 1983) using Biotin-High Prime (Roche Applied Science, IN, USA), or labelled in PCR with tetramethyl-rhodamine-5-dUTP (Roche Applied Science). Telomere concatemer were labelled with digoxigenin (DIG)-11-dUTP by DIG-Nick Translation mix (Roche) by nick translation.

Fluorescence in situ hybridization (FISH) - Chromosome preparation was treated with 100 µg ml^{-1} RNase A (NIPPON GENE Co., Ltd., Tokyo, Japan) for 120 min at 37°C in a humid chamber. After dehydration in a graded series of ethanol, a hybridization mixture containing 50% formamide, 10% dextran sulfate and DNA probes was dropped onto the slides. The preparation was sealed, denatured for 3 min at 78°C, and then incubated for 16 h at 37°C. Subsequently, the slides were rinsed in 2 × SSC at 42°C for 10 min, 0.2 × SSC at 42°C for 10 min, and 2 × SSC / 0.2% Tween-20 at room temperature for 10 min twice. The slides were blocked with 5% bovine serum albumin in 2 × SSC / 0.2% Tween-20 for 60 min at 37°C. Biotin-labelled and DIG-labelled probes were detected with streptavidin-Alexa Fluor 488 (Invitrogen, CA, USA) and anti-digoxigenin-rhodamine (Roche) in 2 × SSC, respectively, for 2 h at 37°C in a humid chamber. The slides were washed in 2

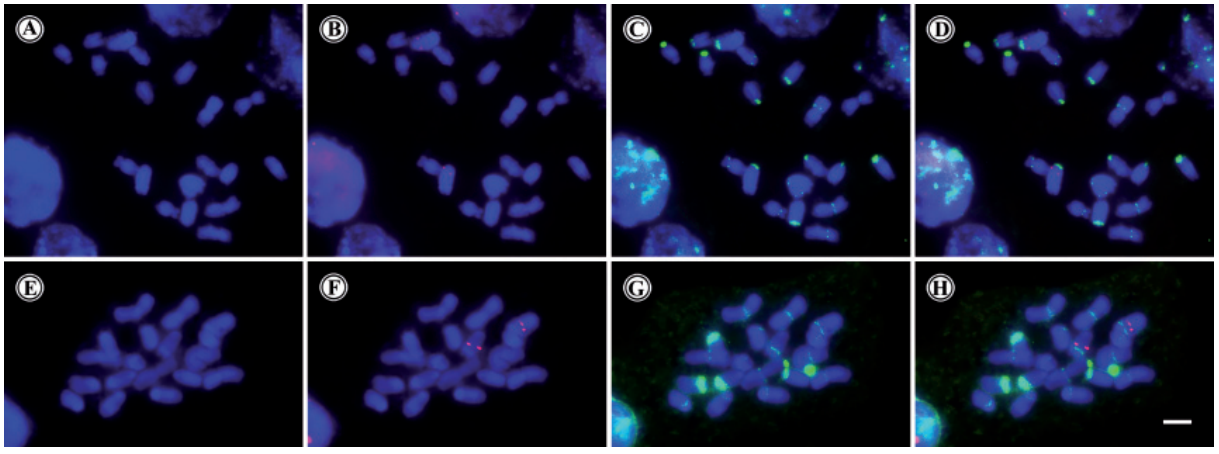

Fig. 2 — Dual FISH to detect 5S rDNA (*red*) and 45S rDNA (*green*) sites on mitotic metaphase chromosome spreads of two cytotypes of *Lysimachia mauritiana*. (A-D) JM-type. E-H: SR-type. (A and E) DAPI-counterstained chromosomes. (B and F) 5S rDNA hybridization sites detected by red rhodamine. (C and G) 45S rDNA hybridization sites detected by Alexa Fluor 488. (D and H) Merged image with DAPI, 5S and 45S rDNAs signals. Bar = 5 µm.

× SSC / 0.2% Tween-20 for 10 min twice, and 2 × SSC for 10 min twice at room temperature. The preparations were then mounted in Vectashield mounting medium containing 1.5 µg/ml 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Inc., CA, USA). Chromosome images were taken by a digital camera (CoolSNAP: Roper Scientific, Inc., Chiba, Japan) on a microscope (Olympus BX51; Olympus, Tokyo, Japan).

Karyotype analysis - To determine the FISH signals on chromosomes, at least 30 cells were observed for each accession. Chromosome length was obtained from digital images of 10 metaphase chromosome complements. The relative length of chromosomes was calculated by (length of each chromosome) / (total length of all chromosomes of the complement) × 100. Categories of chromosome morphology on the basis of the position of centromeres followed LEVAN *et al*. (1964).

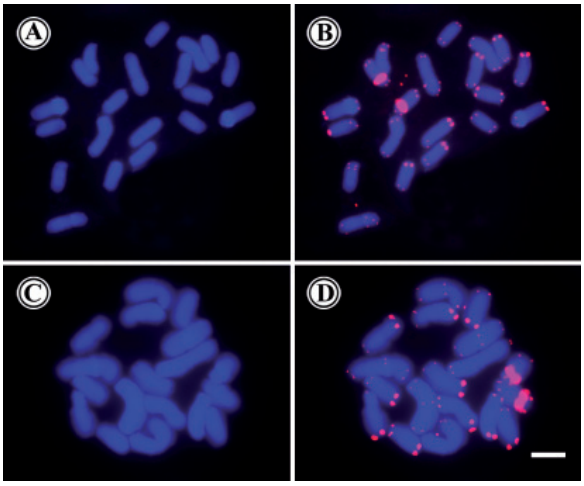

Fig. 3 — Dual FISH to detect telomere repeat sequences signals on mitotic metaphase chromosome spreads of two cytotypes of *Lysimachia mauritiana*. (A and B) JM-type. (C and D) SR-type. (A and C) DAPI-counterstained chromosomes. B and D: Telomere repeat sequences signals detected by red rhodamine. Bar = 5 µm.

Since some chromosomes were quite characteristic and could be easily recognized as landmarks in the metaphase complements of *L. mauritiana*, the karyotype formula for this species was defined on the basis of the centromere position and relative length (KONO *et al*. 2008). Metacentric chromosomes with relative length more than 5.4 were additionally classified as longer-medians indicated by bold letter "**m**" in karyotype formula. The chromosome numbers and numbers of "**m**" were used as markers to be simplified the karyotypic polymorphism because more than a hundred various karyotypes were observed in this species.

RESULTS

Karyomorphological characters - Mitotic chromosome number of JM-type was 2n=20, while the chromosome number of SR-type was 2n=18. The karyotype of JM-type consisted of four **m**-, two sm-, four st- and ten t-chromosomes. In contrast, the karyotype of SR-type consisted of six **m**-, two sm- and ten t-chromosomes. Four and six **m**-chromosomes with relative length more than 5.4 were found in JM-type and SR-type, respectively. Thus, the karyotype formulae were determined as 2n=20(4**m**)=4**m**+2sm+4st+10t in JM-type, and as 2n=18(6**m**)=6**m**+2sm+10t in SR-type. Satellites in the chromosome complement were located on the long arms of the two st-chromosomes in JM-type, and on the short arms of the two longer **m**-chromosomes in SR-type, respectively.

rDNAs localization on the chromosome set - The results of rDNA fluorescent *in situ* hybridization (FISH) are shown in Figs. 2 and 4, and Table 1. Certain chromosomes could be paired or identified in both cytotypes. A strong signal of 5S rDNA probe was observed at the interstitial region of long arm on a pair of t-chromosome in both cytotypes (red signals in Figs. 2 and 4).

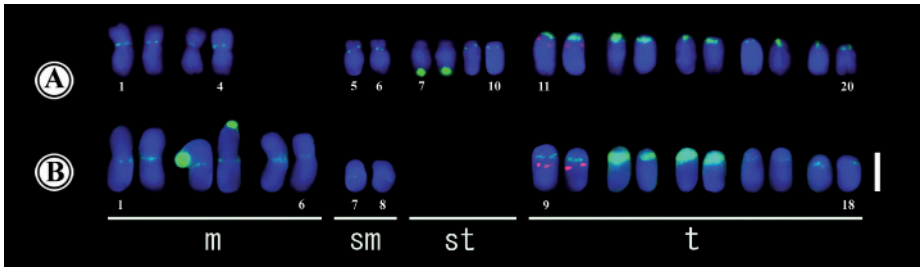

Fig. 4 — Alignments of mitotic metaphase chromosomes with dual FISH signals of 5S (*red*) and 45S (*green*) rDNAs. (A) JM-type. (B) SR-type. Bar = 5 µm.

On the other hand, different sizes of 45S rDNA signals were observed on the distal and proximal regions of **m**-, sm-, st- and t-chromosomes in JM-type, and of **m**- and t-chromosomes in SR-type. A pair of sm-chromosome in SR-type had tiny signal on the interstitial region of long arm. Quite stable and major signals were observed in a pair of st-chromosome in JM-type, and a pair of **m**-chromosome in SR-type.

JM-type had a major signal at the distal region of long arm on a pair of st-chromosome, a tiny signal at the proximal region of two pairs of **m**-chromosome, small signals at the proximal region of a pair of sm- and another st-chromosomes, and tiny or unstable signals at the proximal regions of long arms on five pairs of t-chromosomes (green signals in Figs. 2 and 4).

In contrast, SR-type had a major signal at the distal region of short arm on a pair of **m**-chromosome, tiny signals at the proximal regions of three pairs of **m**-chromosomes, a small signal at the interstitial region of long arm on a pair of sm-chromosome, and tiny signals at the proximal regions of long arms on five pairs of t-chromosomes (green signals in Figs. 2 and 4).

T*elomere sequence distribution pattern* - Telomere FISH signals appeared at both ends of all chromosomes (Table 1), satellites and proximal regions of certain chromosomes in two cytotypes examined (red signals in Figs. 3 and 5). Both cytotypes had two different sizes of internal telomere signals: A big signal at the proximal region of a pair of sm-chromosome, and tiny signals at the proximal regions of one or two pairs of **m**- and st-chromosomes (red signals in Figs. 3 and 5). In JM-type, the big telomere sequence signal at the proximal region coincided with the small 45S rDNA signal (Table 1).

DISCUSSION

Karyotype differentiation - Two cytotypes in this study are karyotypically stable in their own native islands. The new karyotype called SR-type was found in the Sakishima group by OGINUMA *et al.* (2004) and KONO *et al.* (2010), and was quite stable in most of islands of this group. The exception to this was Yonagunijima Island, which had chromosome number of 2n=16 with chromosomal polymorphism, possessing 6, 7 or 8 longer **m**-chromosomes (OGINUMA *et al.* 2004). Considering the geographical factor with minority population and the high variability of chromosome morphology, the all karyotypes in Yonagunijima Island might derive from the karyotype with 2n=18(6**m**) as seen in other islands of the Sakishima group. There were some common features between JM- and SR-types: The somatic chromosomes, which could be classified to three groups by centromere position, were referable to either meta-, submeta-/subtelo- or

TABLE 1 — Summary of the FISH signals on haploid chromosomes of *Lysimachia mauritiana*.

Cytotype	Haploidal Karyotype formula	Karyotype formula(e) bearing FISH signals					
		45S rDNA		5S rDNA		Telomere	
		Major	Minor	Major	Minor	Major	Minor
JM-type	2**m**+1sm+2st+5t	1st	2**m**+1sm+1st+5t	1t	-	1sm	2**m**+1sm+2st+5t
SR-type	3**m**+1sm+5t	1**m**	3**m**+1sm+5t	1t	-	1sm	3**m**+1sm+5t

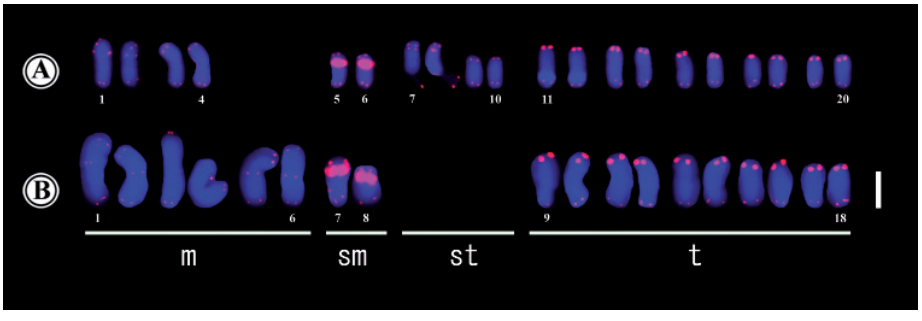

Fig. 5 — Alignments of mitotic metaphase chromosomes with FISH signals of telomere repeat sequences (*red*). A: JM-type. B: SR-type. Bar = 5 μm.

telocentric groups. However, the basic chromosome number and chromosomal location of satellite are quite different each other. JM-type had one st-chromosome with satellite, while SR-type had one **m**-chromosome with satellite in haploid genome set. Moreover, SR-type showed no st-chromosomes, but showed six **m**-chromosomes, instead of four in JM-type. These results allow us to infer an involvement in satellite chromosome changing for karyotype modification.

Chromosome natures with rDNAs - Our FISH analysis revealed a single locus of 5S rDNA at the interstitial region of long arm on t-chromosome and multiple loci of 45S rDNA on distal, interstitial and proximal regions. Ribosomal RNA (rRNA) genes in angiosperms are present in hundreds to thousands of copies (ROGERS and BENDICH 1987), and are clustered as tandem repeats at one or more loci (ÁLVAREZ and WENDEL 2003). These genes comprise two well-defined multigene families: 45S rDNA consists of 18S, 5.8S and 25S rRNAs, external transcribed spacers, internal transcribed spacers, and intergenic spacer, while 5S rDNA consists of 5S rRNA and non-transcribed spacers (LONG and DAWID 1980; ROGERS and BENDICH 1987). Generally, the two gene clusters are not linked (HEMLEBEN and GRIERSON 1978), and are localized independently on different chromosomes per haploid genome (FUKUI *et al*. 1994; LEE and SEO 1997). In *L. mauritiana*, because each chromosome possessed one or two signal(s) of 45S rDNA probe, the 5S rDNA locus was linked with a minor site of 45S rDNA on a pair of t-chromosome. However, the major and stable 45S rDNA signal, which was considered only the active site of nucleolar organizer region in *L. mauritiana* genome, was localized independently on different set of the chromosome from that of 5S rDNA gene. We suggest that minor and unstable 45S rDNA loci on pericentromeric regions in all chromosomes might be inactive. Thus, the two gene clusters of rDNAs in *L. mauritiana* also fall into the general category as seen in many other plant species studied previously.

The 5S and 45S rDNA arrays of *L. mauritiana* make chromosomes useful as markers for easy identification in telocentric chromosome. Moreover, the 45S rDNA locus with the largest size in the loci was located on secondary constriction with satellite chromosome. Although intraspecific differentiation of two cytotypes must be

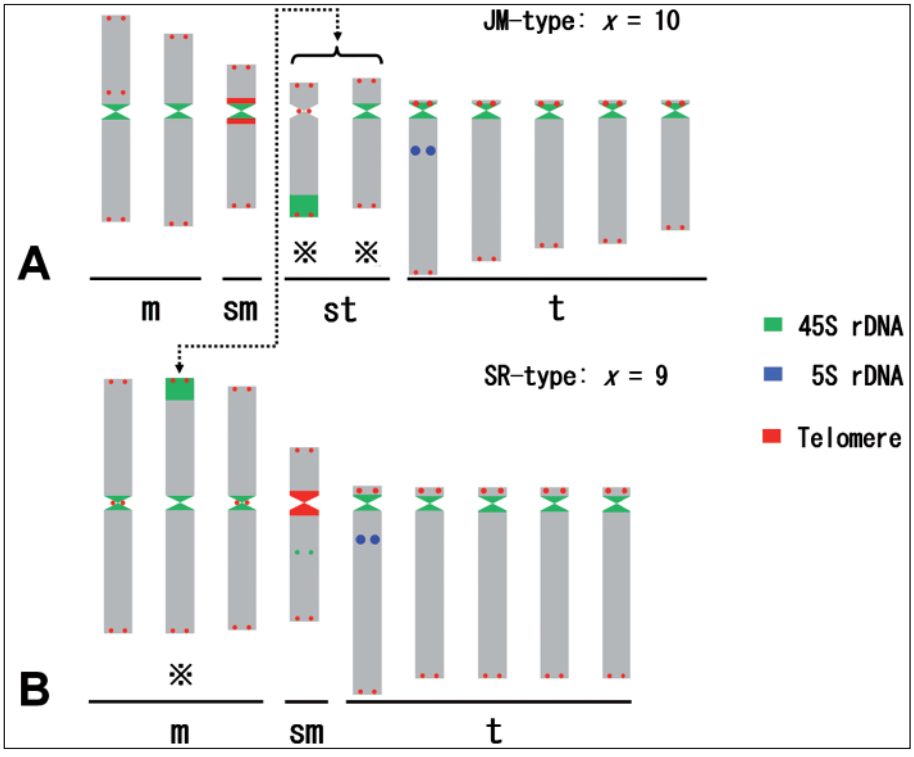

Fig. 6 — Haploidal FISH-karyotypes of two cytotypes in *Lysimachia mauritiana*. A: JM-type. B: SR-type. Fusion/fission hypothesis with two st- or one **m**-chromosome(s) for new intraspecific karyotype formation is summarized.

considered carefully, these novel data gave us a simply fusion/fission hypothesis, as summarized in Fig. 6, with two st- or one **m**-chromosome(s) for new intraspecific karyotype formation of JM-type or SR-type.

On the other hand, because of a high possibility of mobility of rDNA with transposable elements (SCHUBERT and WOBUS 1985; DUBCOVSKY and DVORAK 1995; RASKINA *et al*. 2004), multi 45S rDNA loci detected as minor signals at centromeric regions on several chromosomes suggest that complicated genome rearrangement occur before or during speciation event of *L. mauritiana*. The copy number and chromosomal location of rDNA could be rapidly changed in plant genomes (SCHUBERT and WOBUS 1985; RAINA and MUKAI 1999), and chromosomal rDNA distribution often varies among closely-related taxa, even if within species (GARRIDO *et al*. 1994). Variation in the amount of rDNA genes has demonstrated to be on the basis of most of these polymorphisms, as revealed by *in situ* hybridization (WARBURTON and HENDERSON 1979; KING *et al*. 1990; CASTRO *et al*. 1998). The dynamics of rDNA clusters might be regarded as a strong indicator for significant intra-genomic processes with chromosomal repatterning (JIANG and GILL 1994; RASKINA *et al*. 2004; RASKINA *et al*. 2008).

Chromosome repatterning with internal telomere sequence repeat - Telomere repeat sequences in our FISH results located not only on the ends of all chromosomes, but also near to centromere or proximal regions on some chromosomes. Big telomere site was same to 45S rDNA colocalized position at proximal region on sm-chromosome in JM-type, but not in SR-type. This organization is rather rare case in plant genome. In many organisms, telomere repeat sequences have been located at the ends of chromosomes (MEYNE *et al*. 1990; PETRACEK *et al*. 1990; RICHARDS *et al*. 1991). In contrast, non-terminal telomere repeat sequences at the internal sites are known in some cases, and are suggested to mediate chromosome fusions and fissions (FRY and SALSER 1977; MEYNE *et al*. 1990; RICHARDS *et al*. 1991). Especially, internal telomeres at proximal region near centromere position are considered to support large karyotype change with Robertsonian fusion from telocentric chromosomes (HOLMQUIST and DANCIS 1980; COX *et al*. 1993). Thus, as in the case of multi rDNA loci, the internal residues of telomere repeat sequences on *L. mauritiana* might be an evidence of chromosome rearrangement or karyotype repatterning with their large structural changes during genome differentiation of the species.

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