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Physical Mapping of the 5S Ribosomal RNA Gene in Citreae of Aurantioideae Species using Fluorescence *in situ* Hybridization

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The location of the 5S ribosomal RNA gene (rDNA) in species from six genera of the Citreae of Aurantioideae was determined using fluorescence *in situ* hybridization (FISH). A 5S rDNA probe was labeled with biotin-16-dUTP. The probe was detected using a fluorescein isothiocyanate (FITC)-avidin conjugate with chromosomes counterstained with propidium iodide (PI). When the chromosomes were observed under a G filter, PI-stained chromosomes were classified into the following five types based on the number and position of PI-positive (+) bands; B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands and Dst: type D with a satellite chromosome. Two 5S rDNA sites were located in type D chromosomes in *Citropsis gabunensis*, whereas in *Citrus reshni*, *Fortunella japonica*, *Clymenia polyandra* and *Swinglea glutinosa* they were found in type E chromosomes. In *Poncirus trifoliata*, three rDNA sites were located together at the proximal PI (+) band of a type B chromosome, one site was located in the proximal region of a type D chromosome, and two sites were located together at the PI (+) band of a type D chromosome.

Key Words: Citrinae, citrus, FISH, karyotype, rDNA, Rutaceae.

Introduction

In citrus, it has been shown that some chromosomes can be distinguished despite similar morphology by means of fluorescent staining, such as chromomycin A_3 (CMA) (Guerra, 1993). Citrus chromosomes have been found to exhibit a high degree of diversity and heterozygosity, an understanding of which has shed light on the phylogenic relationships of *Citrus* and its related genera (Befu et al., 2001; Cornelio et al., 2003; Guerra et al., 2000; Miranda et al., 1997b; Yamamoto et al., 2007, 2008a, 2008b; Yamamoto and Tominaga, 2003).

Fluorescence *in situ* hybridization (FISH), for use in the physical mapping of genes, is an important technique for chromosome analysis. Detection of ribosomal RNA gene (rDNA) sites using FISH offers essential information on phylogenic relationships and on the evolution of given species (Fukui et al., 1994). In citrus, the number and location of rDNA sites in various accessions have been detected (Brasileiro-Vidal et al., 2007; Carvalho et al., 2005; Matsuyama et al., 1996; Miranda et al., 1997a; Morares et al., 2007a, 2007b; Ollitrault et al., 2000; Pedrosa et al., 2000; Roose et al., 1998). However, these results were limited to the genus *Citrus* to *Poncirus* and *Fortunella*, two very closely related genera. Prior to the present study, FISH of rDNA sites of other *Citrus* relatives had not been undertaken.

Several studies have demonstrated the relationship between chromosomal regions containing rDNA sites and heterochromatic regions stained by CMA (Brasileiro-Vidal et al., 2007; Carvalho et al., 2005; Matsuyama et al., 1996; Miranda et al., 1997a; Morares et al., 2007a, 2007b; Pedrosa et al., 2000). These combined analyses have been more informative than any single analysis of just rDNA sites or CMA staining alone. Although CMA staining is a very powerful tool for chromosome identification, the speed with which such staining fades reduces its efficacy. Preedasuttijit et al. (2007) reported that the chromosome banding pattern produced in *Poncirus* by propidium iodide (PI) and 4'-6-diamidinio-2-phenylindole (DAPI) double staining was identical to that produced by CMA. In addition,

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heterochromatin was detected in *Fortunella* by FISH following PI counterstaining (Miranda et al., 1997b).

In our previous report on CMA staining (Yamamoto et al., 2008b), we described that Aurantioideae genera and species, including *Citrus* and its relatives, show a unique chromosome configuration, and that in general more advanced species have more heterochromatic regions. In the present study, we first examined the number and location of rDNA sites in six genera, including *Citrus*, using FISH, and then characterized chromosomes containing rDNA sites to heterochromatic regions by PI counterstaining.

Materials and Methods

Plant materials and chromosome preparation

In this study, six species belonging to six genera [*Citropsis gabunensis* (Engl.) Swingle et. M. Kell, *Citrus reshni* hort. ex Tanaka, *Fortunella japonica* (Lour.) Swingle, *Poncirus trifoliata* (L.) Raf., *Clymenia polyandra* (Tan.) Swingle, and *Swinglea glutinosa* (Blanco) Merr.] were used (Table 1). The materials used in this study were preserved at Faculty of Agriculture, Kagoshima University and Saga University, Japan. Roots of young seedlings were the source of the material from polyembryonic *Citrus reshni*, *Fortunella japonica*, and *Poncirus trifoliata*, while in the case of *Citropsis gabunensis*, *Clymenia polyandra*, and *Swinglea glutinosa*, young leaves of about 3–5 mm in length from adult trees were used. Fourteen, nine and eight seedlings were used in *Citrus reshni*, *Fortunella japonica*, and *Poncirus trifoliata*, respectively. Although nucellar and zygotic seedlings were not distinguished prior to chromosomal analysis, the reproducibility of PI banding patterns of each species was confirmed. Seeds were germinated in Petri dishes at 25°C in the dark. Root tips of about 1 cm in length and young leaves were excised, immersed in 2 mM 8-hydroxyquinoline at 10°C for 4 h in the dark, fixed in methanol-acetic acid (3 : 1), and stored at −20°C.

Enzymatic maceration and air drying were performed as described by Fukui (1996) with minor modifications. The root tips or young leaves were washed in distilled water to remove the fixative and were then macerated in an enzyme mixture containing 1 or 2% Cellulase Onozuka RS, 0.75 or 1.5% Macerozyme R200 (Yakult, Tokyo, Japan), 0.15 or 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd, Tokyo, Japan), and 1 mM EDTA, pH 4.2, at 37°C for 45–60 min.

Chromosomes were stained with 2% Giemsa solution (Merck Co., Darmstadt, Germany) in 1/30 M phosphate buffer (pH 6.8) for 15 min, rinsed with distilled water, air dried, and then mounted with xylene. After confirmation of each chromosome position on the slide glass, the chromosomes were de-stained with 70% methanol.

Fluorescence in situ hybridization

In accordance with the procedure described in Carvalho et al. (2005), the 5S rDNA probe was amplified from genomic DNA of *Citrus reshni* by PCR using a pair of primers (5'-GTG CGA TCA TAC CAG CAC TAA TGC ACC GG-3' and 5'-GAG GTG CAA CAC CAG GAC TTC CCA GGA GG-3') that were based on 5S rDNA sequences of *Glycine max* (L.) Merrill (Gottlob-McHugh et al., 1990). The 5S rDNA probe obtained was labeled with biotin-16-dUTP (Roche, Manheim, Germany).

FISH was performed according to the method of Ohmido and Fukui (1996). The biotinylated probe was hybridized to chromosomal rDNA *in situ* and detected with a fluorescein isothiocyanate (FITC)-avidin conjugate (Vector, California, USA) by fluorescence microscopy. FITC signals were visualized using a B filter. Chromosomes were counterstained with 0.5 μg·mL^{-1} PI and visualized using a G filter.

Table 1. Species belonging to Citreae of Aurantioideae used in this study, their PI banding pattern and number of 5S rDNA sites on somatic chromosomes.

Subtribe	Group	Genera and species	Common name	PI banding pattern[z]	No. of 5S rDNA sites
Citrinae					
	Near citrus fruit trees				
		Citropsis gabunensis (Engl.) Swingle et. M. Kell	Gabon cherry-orange	16D + 2E	2
	True citrus fruit trees				
		Citrus reshni hort. ex Tanaka	Cleopatra	10D + 8E	2
		Fortunella japonica (Lour.) Swingle	Round kumquat	1B + 9D + 2Dst + 6E	2
		Poncirus trifoliata (L.) Raf.	Trifoliate orange 'Rubidoux'	3B + 9D + 6E	6
		Clymenia polyandra (Tan.) Swingle	Clymenia	2C + 8D + 8E	2
Balsamocitrinae					
	Tabog group				
		Swinglea glutinosa (Blanco) Merr.	Tabog	18E	2

[z] B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without band, Dst: type D with a satellite chromosome.

Results

PI-stained chromosomes were classified into the following five types based on the number and position of PI-positive (+) bands. Each type of chromosome was classified according to the types of CMA banding patterns (Befu et al., 2000; Miranda et al., 1997b; Yamamoto and Tominaga, 2003; Yamamoto et al., 2007); B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, and Dst: type D with a satellite chromosome (Fig. 1). Chromosomes with very light or barely visible bands were designated as type E in accordance with Guerra (1993). Types A (two telomeric and one proximal band) and F (one proximal band) were not observed. Each species used in this study exhibited high chromosomal variability as indicated by chromosomal patterns (Fig. 2).

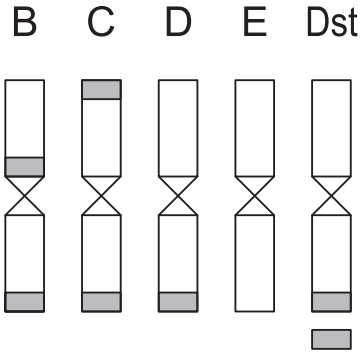

Fig. 1. Schematic representation of chromosome types according to the position of PI-positive bands. B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, Dst: type D with a satellite chromosome. The gray regions indicate PI-positive bands.

In *Citropsis gabunensis*, a species of Citrinae closely related to true citrus fruit trees, the PI banding pattern was 16D+2E. The PI banding patterns of species actually belonging to the true citrus fruit trees group were 10D +8E in *Citrus reshni*, 1B+9D+2Dst+6E in *Fortunella japonica*, 3B+9D+6E in *Poncirus trifoliata*, and 2C+ 8D+8E in *Clymenia polyandra*. For *Swinglea glutinosa*, a member of Balsamocitrinae, the PI banding pattern was 18E (Fig. 2 and Table 1).

In all the species examined in this study, two 5S rDNA sites were located in the telomeric regions of chromosomes. In *Citropsis gabunensis*, these two sites were located in type D chromosomes, whereas they were found in type E chromosomes in *Citrus reshni*, *Fortunella japonica*, *Clymenia polyandra*, and *Swinglea glutinosa*. Among six 5S rDNA sites in *Poncirus trifoliata*, three sites were located together at the proximal PI (+) band of a type B chromosome, one site was located in the proximal region of a type D chromosome, and two sites were located together at the PI (+) band of a type D chromosome (Figs. 3 and 4, Table 1).

Discussion

The PI banding pattern of *Fortunella* chromosomes was similar to that produced by CMA staining (Miranda et al., 1997a), in spite of differences between the staining agents, i.e, CMA is a guanine-cytosine (GC)-specific

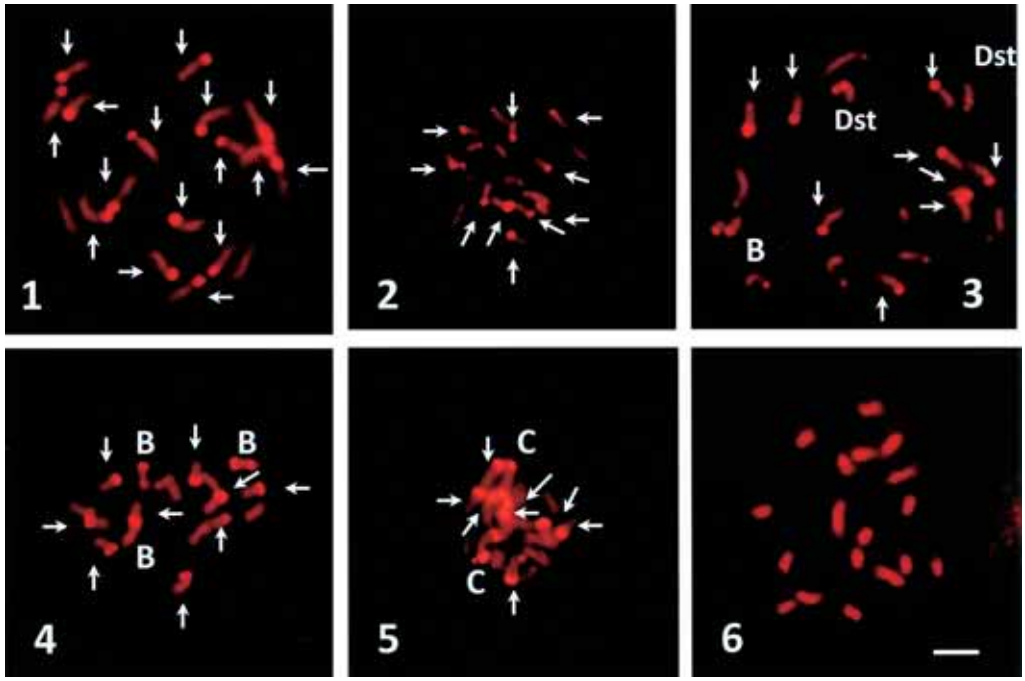

Fig. 2. PI staining of somatic chromosomes in species belonging to Citreae of Aurantioideae. 1: *Citropsis gabunensis*, 2: *Citrus reshni*, 3: *Fortunella japonica*, 4: *Poncirus trifoliata*, 5: *Clymenia polyandra*, 6: *Swinglea glutinosa*. Arrows indicate type D chromosome. B, C, D, and Dst: See Figure 1. Bar in 6 represents 5 μm for all figures.

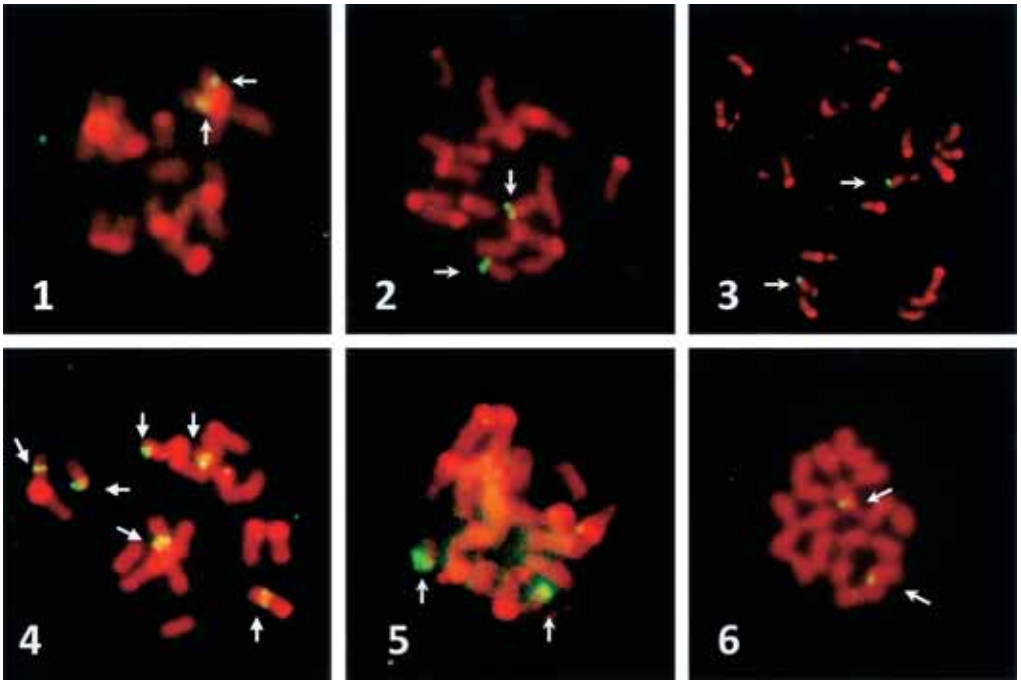

Fig. 3. FISH of 5S rDNA probe to somatic chromosomes from species belonging to Citreae of Aurantioideae. 1: *Citropsis gabunensis*, 2: *Citrus reshni*, 3: *Fortunella japonica*, 4: *Poncirus trifoliata*, 5: *Clymenia polyandra*, 6: *Swinglea glutinosa*. Arrows and green signals indicate rDNA sites. Counterstaining: PI.

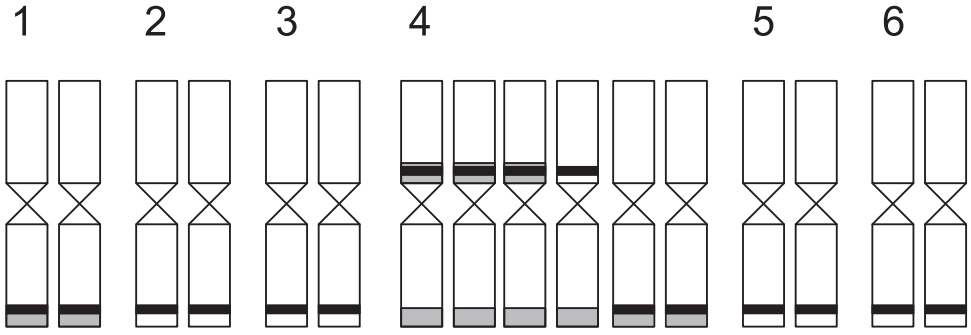

Fig. 4. Schematic representation of chromosomes possessing 5S rDNA sites, and regions of PI-positive bands in species belonging to Aurantioideae. 1: *Citropsis gabunensis*, 2: *Citrus reshni*, 3: *Fortunella japonica*, 4: *Poncirus trifoliata*, 5: *Clymenia polyandra*, 6: *Swinglea glutinosa*. The black and gray regions indicate 5S rDNA sites and PI-positive bands, respectively.

fluorochrome (Schweizer, 1976) while PI is a non-base specific fluorochrome. The PI banding patterns of *Citropsis gabunensis*, *Clymenia polyandra*, and *Swinglea glutinosa* in the present study were identical to those of CMA described in previous reports (Yamamoto et al., 2008a, 2008b). A resemblance was found between the present PI results and the previous CMA banding patterns of *Citrus reshni*, *Fortunella japonica*, and *Poncirus trifoliata* (Befu et al., 2000; Brasileiro-Vidal et al., 2007; Cornelio et al., 2003; Kunitake et al., 2005; Yamamoto and Tomoinaga, 2003). In these species, a few chromosomes designated as types B and D following CMA staining were designated as types D and E following PI staining. The regions causing different designations, and which were stained by CMA but not PI, appear to be GC-rich but, in view of the effects of CMA and PI staining, were not condensed chromosomal regions. PI staining is a very easy and useful chromosome identification method in FISH analysis in Citreae, although the detection of positive bands was slightly better with CMA staining. It could be contended that PI is therefore superior to DAPI counterstaining, which does not result in a clear chromosome banding pattern (Roose et al., 1998) when FISH is not combined with CMA staining. In addition, the chromosome banding pattern obtained from double staining using PI and DAPI was identical to that of CMA staining in *Poncirus trifoliata* (Preedasuttijit et al., 2007). PI/DAPI double counterstaining seems to be effective in FISH of *Citrus* and its relatives.

In all six species used in this study, two 5S rDNA sites were located in telomeric regions of two chromosomes. An additional four sites were also detected in a proximal region in *Poncirus trifoliata*. In

various *Citrus* species and accessions, the 5S rDNA sites were also located in telomeric regions of two chromosomes (Carvalho et al., 2005; Moraes et al., 2007a, 2007b).

The numbers of CMA-positive regions in *Fortunella* chromosomes are very high (Miranda et al., 1997b); thus, this genus is considered to be more advanced because in general, the quantity of heterochromatin (CMA-positive) tends to increase through evolution (Ikeda, 1988). *Citropsis* seems to be an ancestor of *Citrus* (Swingle and Reece, 1967). Its chromosomes possessed many more heterochromatin regions than those of other near and primitive citrus fruit trees (Yamamoto et al., 2008b). Chromosome evolution which increased heterochromatin regions seems to have occurred. It is considered that *Clymenia* is the most primitive of all the genera of true citrus fruit trees due to its morphological traits (Swingle and Reece, 1967). Its chromosome configuration seemed to be the primitive type of true citrus fruit trees (Yamamoto et al., 2008a). *Swinglea glutinosa*, which lacks a heterochromatin rich chromosome, is considered to have the most primitive chromosome configuration in Aurantioideae (Yamamoto et al., 2008b).

The results of the present study obtained from the analysis of primitive and advanced species, and from previous studies of various *Citrus* (Carvalho et al., 2005; Moraes et al., 2007a, 2007b) indicate that two 5S rDNA sites located in telomeric regions are fundamental feature of chromosomes in Citreae species.

There were found to be six 5S rDNA sites in chromosomes of *Poncirus trifoliata*, which has already been reported by Brasileiro-Vidal et al. (2007). This species was found to possess two telomeric 5S rDNA sites, which seems to be a fundamental feature of Citreae species. The number of proximal sites probably increased to four after an ancestor of *Poncirus* became separated from other species belonging to true citrus fruit trees, since other closely related genera such as *Citrus* and *Fortunella* do not possess these sites up to the present data (Carvalho et al., 2005; Moraes et al., 2007a, 2007b; Pedrosa et al., 2000; Roose et al., 1998). Variation in the numbers of 5S rDNA sites was reported in *Diospyros* and *Oryza* species (Choi et al., 2003; Fukui et al., 1994). Geographical variability in the number of rDNA sites was observed in both genera. The distribution of *Poncirus* is the most northerly among true citrus fruit trees. Furthermore, from a morphological aspect, *Poncirus* is distinct from *Citrus* and *Fortunella* because of its deciduous and trifoliate leaves. DNA analysis also revealed genetic divergence between *Poncirus* and both *Citrus* and *Fortunella* (Nicolosi et al., 2000; Yamamoto et al., 1993).

Chromosomes with telomeric 5S rDNA sites were homozygous in all species analyzed in the present study. These chromosomes were type D in *Citropsis gabunensis* and *Poncirus trifoliata*, but type E in *Citrus reshni*, *Fortunella japonica*, *Clymenia polyandra*, and *Swinglea glutinosa*. Of the four proximal 5S rDNA sites of *Poncirus trifoliata*, three were on a type B chromosome, and one was on a type D chromosome; however, all four 5S rDNA sites were located on CMA-stained B chromosomes (Brasileoro-Vidal et al., 2007). As mentioned above, a few CMA-positive bands could not be detected as PI-positive bands in this study. In view of the CMA banding pattern, the four chromosomes possessing 5S rDNA sites appear to be two pairs of homologous chromosomes (Brasileoro-Vidal et al., 2007). In addition, two 5S rDNA sites have been found in CMA-stained type D chromosomes in *Citrus reshni* (Moraes et al., 2007).

This study demonstrates the similarity of 5S rDNA sites in Citreae, despite every species exhibiting a unique PI and CMA banding pattern. There seems to be two telomeric 5S rDNA sites which constitute a fundamental feature of the chromosome configuration in Citreae; however, *Poncirus trifoliata* exhibits unique 5S rDNA sites. Although only one probe was used in this study, it is clear that the use of multicolor FISH using more than one probe would be very informative; therefore, further FISH analysis using more species and probes should be conducted to clarify the phylogenic relationships among Aurantioideae.

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