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Fluorescent Staining and Fluorescence *in situ* Hybridization of rDNA of Chromosomes in Pear (*Pyrus* spp.)

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Fluorescent banding patterns of pear chromosomes were determined from samples taken from root tips of open-pollinated seedlings of *Pyrus pyrifolia* (Burm. F) Nakai (Japanese pear), *P. calleryana* Decne. (Callery pear), *P. pyrifolia*×*P. ussuriensis* var. *aromatica* (a hybrid of Japanese pear and Iwateyamanashi), and *P. mikawana* Koidz. (Toyotomi Nashi). All accessions used in this study had 2n=34 chromosomes. Chromomycin A_3 (CMA)-positive (+) bands were observed in telomeric positions of four chromosomes in all accessions. 4'-6-diamidino-2-phenylindole (DAPI)-negative bands (–) corresponded with CMA+ bands. Fluorescence *in situ* hybridization (FISH) was conducted using open-pollinated seedlings of 'Osa Gold' (*Pyrus pyrifolia*) and Toyotomi Nashi (*P. mikawana*) as materials. Four out of six 18S-5.8S-25S ribosomal RNA gene (rDNA) sites corresponded with CMA+/DAPI– bands. The 5S rDNA sites were detected in centromeric positions of two chromosomes. Two centromeric 5S rDNA and six telomeric 18S-5.8S-25S rDNA sites were located on different chromosomes as determined from the results of multi-color FISH.

Key Words: CMA, DAPI, FISH, McFISH, ribosomal RNA gene.

Introduction

Chromosome information is an important parameter to elucidate the stability of a plant at the cellular level and also for genetic and biotechnological studies. Chromosomes are the first dataset that leads to an understanding of the genetics of any species. Although pears (*Pyrus* spp.) are one of the most important fruit tree species cultivated in temperate regions, their study has not progressed compared with other temperate zone fruit trees such as persimmon and peach (Choi et al., 2003a, b; Yamamoto et al., 1999a, b; Zhuang et al., 1990); therefore, we analyzed the chromosome of pears. We performed fluorescent staining using base-specific binding fluorochrome, guanine-cytosine (GC)-specific chromomycin A_3 (CMA) and adenine-thymine (AT)-specific 4'-6-diamidino-2-phenylindole (DAPI), and fluorescence *in situ* hybridization (FISH) of 18S-5.8S-25S ribosomal RNA gene (rDNA) of pears (Yamamoto et al., 2010a, b).

In fluorescent staining, all open-pollinated seedlings of six cultivars from three species [*Pyrus pyrifolia* (Burm. F) Nakai (Japanese pear) 'Osa Gold' and 'Niitaka', *P. communis* L. (European pear) 'La France' and 'Max Red Bartlett', and *P. bretschneideri* Rehder (Chinese pear) 'Enli' and 'Yali'] showed the same chromosome configuration: CMA-positive (+) and DAPI-negative (–) bands were observed in telomeric positions of four chromosomes (Yamamoto et al., 2010a). FISH analysis revealed the 18S-5.8S-25S rDNA sites of open-pollinated seedlings of Japanese pear 'Osa Gold' and European pear 'La France' in telomeric positions of six chromosomes (Yamamoto et al., 2010b).

However, there are still unresolved issues in fluorescent staining and FISH of rDNA in pears: 1) the divergence of the chromosome configuration by CMA/DAPI staining, 2) the relationship between CMA+/DAPI– bands and 18S-5.8S-25S rDNA sites, 3) the number and position of 5S rDNA sites, and 4) the relationship between 5S rDNA and 18S-5.8S-25S rDNA sites. Thus, we conducted CMA/DAPI staining, FISH and multi-color FISH (McFISH) of rDNA to clarify the above-mentioned issues in the present study.

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Materials and Methods

Fluorescent staining

Japanese pears 'Osa Gold' (accession number: JP110825 of NIAS Genebank, Japan, http://www.gene.affrc.go.jp/index_en.php) and 'Saitama 8' (JP113809) [*Pyrus pyrifolia* (Burm. F.)] (Iketani, H., personal communication), Callery pear 'Aichi Mamenashi' (JP113537) (*P. calleryana* Decne.), a hybrid of Japanese and Iwateyamanashi 'Iwate 7 go' (JP113801) (*P. pyrifolia* × *P. ussuriensis* var. *aromatica*) (Iketani, H., personal communication), and Toyotomi Nashi (JP113771) (*P. mikawana* Koidz.) were used. The materials used in this study were obtained from the National Institute of Fruit Tree Science, Tsukuba, Ibaraki, Japan. In the present study of fluorescent staining, native species of Japan were mainly used as materials. The detailed results of fluorescent staining of 'Osa Gold' have already been reported (Yamamoto et al., 2010a).

Roots of young seedlings from open-pollinated fruits were the source of the material used in this study. Seeds were germinated in Petri dishes at 12°C in the dark. Root tips of approximately 1 cm in length 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 (Yamamoto et al., 2010b). The root tips were washed in distilled water to remove the fixative and macerated in an enzyme mixture containing 4% Cellulase Onozuka RS, and 1% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd., Japan) and 1 mM EDTA, pH 4.2, at 37°C for 3 h.

Chromosomes were stained with 2% Giemsa solution (Merck Co., 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's position on the slide glass, the chromosomes were destained with 70% methanol.

Fluorescent staining was performed according to the method of Hizume (1991) with minor modifications (Yamamoto et al., 2010a). The slides were preincubated for 30 min in McIlvaine buffer (6.6 mM citric acid and 88.2 mM Na_2HPO_4, pH 7.0) and treated with 0.1 g·L^{-1} distamycin A in the buffer for 10 min. The slides were incubated for 10 min in buffer containing 5 mM $MgSO_4$, and then stained for 60 min with 0.5 g·L^{-1} CMA in buffer containing 5 mM $MgSO_4$. The slides were also incubated for 10 min in buffer containing 5 mM $MgSO_4$, and then stained for 30 min with 1.0 mg·L^{-1} DAPI in the buffer. The slides were incubated for 10 min in the buffer and then mounted using SlowFade (Eugene, USA). Chromosomes stained with CMA and DAPI were observed under a fluorescence microscope (ECLIPSE 80i, Nikon, Japan) with a microscope digital camera (DP71, Olympus, Japan) using BV and UV filter cassettes, respectively.

Fluorescence in situ hybridization (FISH)

For detection of the rDNA sites, two kinds of rDNA probe were used in this study: 1) a 9.0 kb fragment including a full-length 18S-5.8S-25S rDNA repeat unit of wheat (Barker et al., 1988; Gerlach and Bedbrook, 1979) (accession number: X07841, DDBJ/GenBank/EMBL) and 2) in accordance with the procedure described in Choi et al. (2003b), a 5S rDNA probe of 0.6 kbp that was amplified from genomic DNA of 'Hosui' (*Pyrus pyrifolia*) by PCR using a pair of primers (5S-F: 5'-GGATGCGATCATACCAGCAC-3' and 5S-R: 5'-GGGAATGCAACACGAGGACT-3'), which were designed by Fukui et al. (1994). PCR amplification was performed in a 50 μL reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM each dNTP, 25 pmol each forward and reverse primer, 25 ng genomic DNA, and 2.5 units Taq polymerase (Invitrogen, USA). Amplification was conducted under the following conditions: initial denaturation at 94°C for 1 min, followed by 35 cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, for denaturation, annealing and primer extension, respectively. Sequence data of partial 5S ribosomal RNA gene of pear have been submitted to DDBJ/GenBank/EMBL under the following accession number: AB621370.

The 5S rDNA probes were labeled with biotin-16-dUTP (Roche, Germany) by a random priming method. The 18S-5.8S-25S rDNA probes were labeled with biotin-16-dUTP or digoxigenin-11-dUTP (Roche) by a random priming method.

FISH was performed according to the method of Ohmido and Fukui (1996) with minor modifications (Yamamoto et al., 2010b). The biotinylated probe was hybridized to chromosomal rDNA *in situ* and detected with a fluorescein isothiocyanate (FITC)-avidin conjugate (Vector Laboratories, USA) by fluorescence microscopy. FITC signals were visualized using a B filter. Chromosomes were counterstained with 2.0 mg·L^{-1} DAPI or 0.1 g·L^{-1} propidium iodide (PI) and visualized using a UV or G filter, respectively.

McFISH of 5S and 18S-5.8S-25S rDNA was performed according to the method of Shishido et al. (2001). The biotinylated 5S rDNA probe and digoxigenin-labeled 18S-5.8S-25S rDNA were hybridized to chromosomal rDNA *in situ*. The hybridization mixture (each 100 ng labeled probe per slide dissolved in a mixture of equal parts of 50% formamide and 2 × SSC) was denatured for 10 min at 90°C and then immediately cooled down to 0°C. The chromosome samples were denatured in 50% formamide/2 × SSC for 6 min at 70°C with the hybridization mixture on a thermal cycler with a flat aluminum bed (PC816, Astec, Japan) and then hybridized for 18 h at 37°C. After hybridization, they were washed twice in 2 × SSC, once in 50% formamide/2 × SSC and once in 4 × SSC, each for 10 min at 42°C. Chromosome samples were blocked with 5%

bovine serum in BT buffer (0.1 M sodium hydrogen carbonate, 0.05% Tween-20, pH 8.3) at 37°C for 5 min. Fluorescein (FITC)-avidin (1%, Vector Laboratories) in 1% bovine serum albumin (BSA) in BT buffer was dropped onto the chromosome samples, which were then incubated at 37°C for 60 min. After the FITC-avidin solution had been rinsed with BT buffer three times at 40°C for 5 min each, samples were blocked with 5% goat serum albumin in BT buffer at 37°C for 5 min. Biotinylated anti-avidin (1%; Vector Laboratories) and 10% sheep anti-digoxigenin rhodamine (Roche) in 5% goat serum in BT buffer was dropped onto the chromosome samples, which were then incubated at 37°C for 60 min. After washing with BT buffer three times at 37°C for 5 min each, they were blocked with 5% bovine serum in BT buffer at 37°C for 5 min. Then, 1% FITC-avidin and 1% anti-sheep-Texas Red (Vector Laboratories) in BSA in BT buffer was dropped onto them, and they were incubated at 37°C for 60 min. After rinsing twice with BT buffer and once with 2 × SSC at 40°C for 5 min each, the chromosome samples were finally counterstained with 2.0 mg·L^{-1} DAPI in an antifadant solution (Vector Shield; Vector Laboratories). FITC signals (5S rDNA) were visualized using a B filter. Rhodamine/Texas Red signals (18S-5.8S-25S rDNA) were visualized using a G filter. Chromosomes counterstained with DAPI were visualized using a UV filter.

In both FISH and McFISH, the chromosome samples were observed with a fluorescence microscope (ECLIPSE 80i, Nikon) with a microscope digital camera (DP71, Olympus). Signal images were analyzed by imaging software (DP-BSW Ver. 03.01, Olympus). From the preparation derived from seedlings of 'Osa Gold' stained with DAPI, seven cells were selected for use in determining chromosome length.

Results and Discussion

All seedlings from five accessions used in this study had 2n = 34 chromosomes. When CMA staining was performed, four out of 34 chromosomes exhibited CMA-positive (+) bands, and 30 chromosomes had no CMA+ or negative (–) bands in the eight to ten seedlings of each accession. The four CMA+ bands were located in telomeric regions of the four chromosomes (Fig. 1). These four CMA+ bands corresponded with DAPI– bands (Fig. 1). Since CMA and DAPI are GC- and AT-specific, respectively, the CMA+/DAPI– regions are considered to be GC-rich regions of chromosomes.

In situ hybridization with the 18S-5.8S-25S rDNA probe revealed signals on six chromosomes of 'Osa Gold' and Toyotomi Nashi seedlings. The six signal sites were located in telomeric regions of the six chromosomes (Fig. 2). As reported previously (Yamamoto et al., 2010b), the 18S-5.8S-25S rDNA sites were stable, and there was no difference among the seedlings from open pollination.

In a previous (Yamamoto et al., 2010a) and the present study, the chromosome configuration of open-pollinated seedlings of ten accessions from five species and one interspecific hybrid were clarified by CMA/DAPI staining. Almost all seedlings showed the same CMA+/DAPI– bands and no species or varietal difference in the CMA/DAPI banding pattern was observed. This result agrees with the results of some *Prunus* (peach, Japanese plum, and apricot) CMA/DAPI banding patterns, which showed no variation among seedlings, cultivars, and species (Yamamoto, unpublished; Yamamoto et al., 1999a). Moreover, the number and locations of 18S-5.8S-25S rDNA sites were stable among the seedlings of Japanese and European pear, and Toyotomi Nashi (Yamamoto et al., 2010b). It could be concluded that the divergence of chromosome configuration seems to be very low or non-existent in view of the 18S-5.8S-25S rDNA sites and fluorescent banding patterns, at least among the accessions and species used in this study.

Four CMA+/DAPI– bands corresponded with 18S-5.8S-25S rDNA sites (Fig. 3). This result agrees with the results of peach and *Ciser*, which showed CMA+ bands that corresponded with 18S-5.8S-25S rDNA sites (Galasso et al., 1996; Yamamoto et al., 1999b) although not all CMA+ bands were 18S-5.8S-25S rDNA sites in *Citrus* (Carvalho et al., 2005; Matsuyama et al., 1996). These results indicate that the rDNA sites of pear are a region with a high proportion of GC content (Schweizer, 1976).

The sequence identity between partial 5S rDNA of pear amplified in the present study and *Arabidopsis thaliana* 5S rDNA (accession number: AJ307356, DDBJ/GenBank/EMBL) was 89%. From this result, it can be concluded that the amplified sequence in the present study was partial 5S rDNA. The 5S rDNA sites were detected in centromeric positions of two chromosomes of 'Osa Gold' and Toyotomi Nashi seedlings (Fig. 4). The 5S rDNA sites were stable, and there was no difference among the seedlings from open pollination. Two centromeric 5S rDNA and six telomeric 18S-5.8S-25S rDNA sites were located at different chromosomes, as determined from the results of McFISH (Fig. 4).

Some studies reported the variability of 18S-5.8S-25S rDNA sites and stability of 5S rDNA sites in congeneric species (Adams et al., 2000; Liu et al., 2003; Vaio et al., 2005). Variability of 18S-5.8S-25S rDNA sites was not detected in Japanese and Chinese pear (Yamamoto et al., 2010b), and Toyotomi Nashi. Thus, it could be considered that the variability of 5S rDNA sites is absent or low in pears, although European pears were not used as materials in the present study.

This is the first report on the number and locations of 5S rDNA sites in pear. Two rDNA (18S-5.8S-25S and 5S rDNA) sites could be revealed in the present and previous (Yamamoto et al., 2010b) studies. The

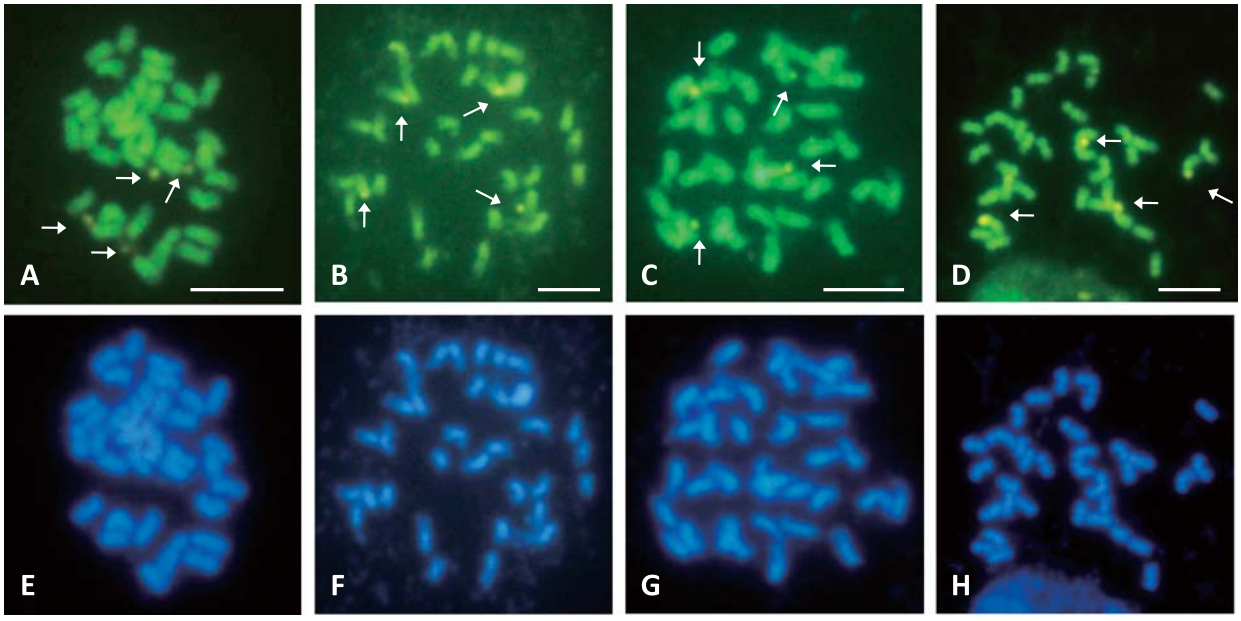

Fig. 1

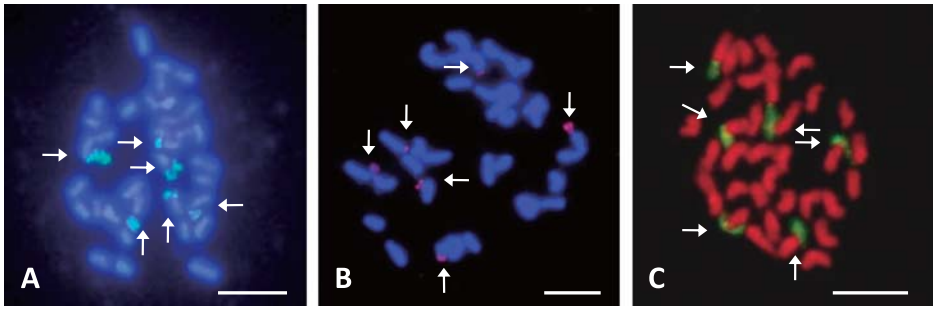

Fig. 2

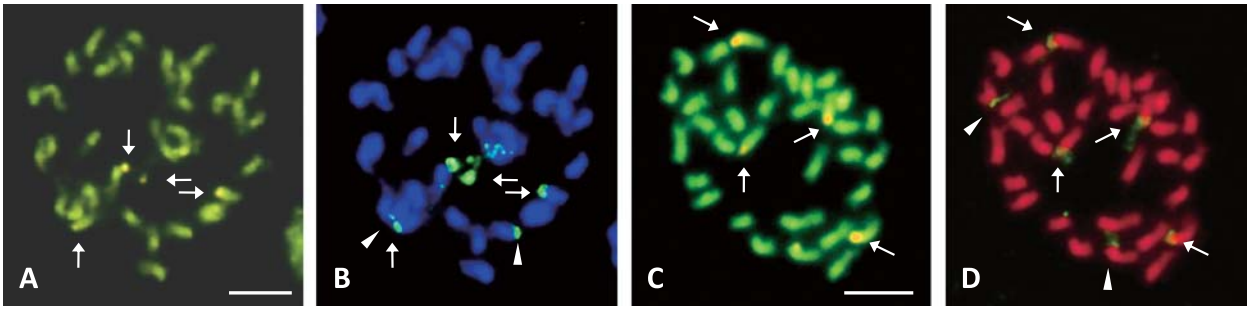

Fig. 3

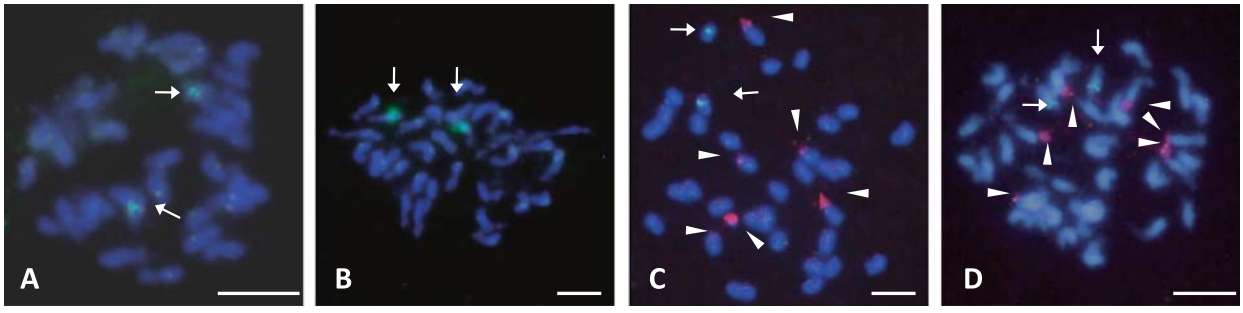

Fig. 4

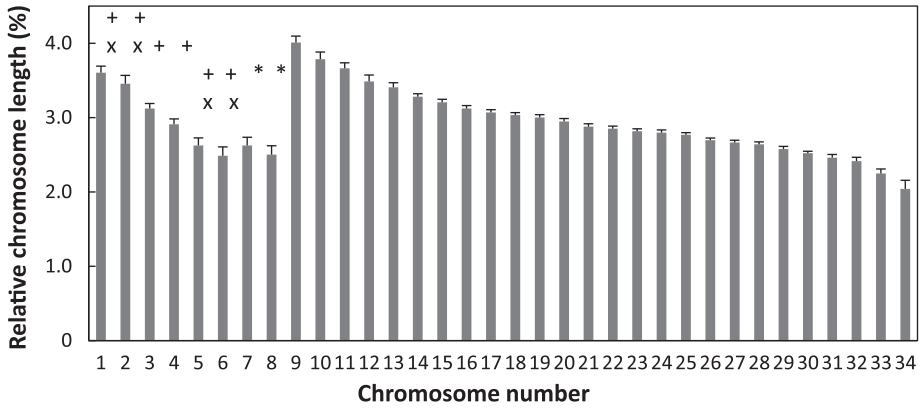

Fig. 5. The relative length (% of the total length) of each 34 chromosome derived from seedlings of Japanese pear 'Osa Gold' [*Pyrus pyrifolia* (Burm. F.)]. +: Chromosome with 18S-5.8S-25S rDNA site, *: Chromosome with 5S rDNA site, ×: Chromosome with CMA positive band.

18S-5.8S-25S and 5S rDNA sites have been detected in several fruit tree species (Choi et al., 2003b; Corredor et al., 2004; de Melo and Guerra, 2003; Maghuly et al., 2010; Pedrosa et al., 2000; Schuster et al., 1997) since their detection is useful for chromosome identification, phylogenetic studies, and so on; thus, the results of the present study are considered to be the first step for applying chromosome information to these studies.

Figure 5 shows the relative length of each of the 34 chromosomes derived from seedlings of 'Osa Gold'. The length of chromosomes ranged from 4.0% to 2.0%. The six 18S-5.8S-25S rDNA sites were located on two relatively long (3.6 and 3.5%), two medium (3.1 and 2.9%), and two relatively short (2.6 and 2.5%) chromosomes. The two 5S rDNA sites were located on relatively short (2.6 and 2.5%) chromosomes which do not possess 18S-5.8S-25S rDNA sites.

18S-25S (the same locus as 18S-5.8S-25S) rDNA and 5S rDNA sites in apple (*Malus* × *domestica* Borkh.) have been reported (Schuster et al., 1997) with 2n = 34 chromosomes belonging to the same subfamily Maloideae of the family Rosaceae. The 18S-25S rDNA and 5S rDNA sites in apples were detected in telomeric regions of eight chromosomes and centromeric regions of two chromosomes, respectively. The rDNA sites of pears and apples are quite similar. The similarity of linkage maps between pears and apples was also reported (Yamamoto et al., 2007). It could be concluded that there is a syntenic relationship between pears and apples on the basis of these results, despite some divergence of chromosome configuration between them.

In conclusion, we demonstrated the stability of CMA+/DAPI– bands, the relationship between CMA+/DAPI– bands and 18S-5.8S-25S rDNA sites, the number and location of 5S rDNA sites, and the relationship between 5S rDNA and 18S-5.8S-25S rDNA sites on pear chromosomes. These results and previous studies (Yamamoto et al., 2010a, b) could provide fundamental information on pear chromosomes, and are considered to contribute to the progress of breeding and genome studies in pears. In addition, detection of the physical locations of useful gene loci has progressed in important crops and vegetables (Iwano et al., 1998; Ohmido et al., 1998). Therefore, physical mapping of useful genes, such as for scab resistance and black spot disease susceptibility (Terakami et al., 2006, 2007), should be conducted in pears.

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Fig. 1. CMA- and DAPI-stained chromosomes derived from seedlings of pears. A and E: 'Saitama 8' [*Pyrus pyrifolia* (Burm. F.)]; B and F: 'Aichi Mamenashi' (*P. calleryana* Decne.); C and G: 'Iwate 7 go' (*P. pyrifolia* × *P. ussuriensis* var. *aromatica*); D and H: Toyotomi Nashi (*P. mikawana* Koidz.). A, B, C, and D: stained with CMA; E, F, G, and H: stained with DAPI. Arrows indicate CMA-positive bands. Bars represent 5 μm.

Fig. 2. FISH of 18S-5.8S-25S probes on somatic chromosomes of pear seedlings. A: 'Osa Gold' [*Pyrus pyrifolia* (Burm. F.)]; B and C: Toyotomi Nashi (*P. mikawana* Koidz.). Arrows indicate rDNA sites. Counterstaining of A and B, and C are DAPI and PI, respectively. Bars represent 5 μm.

Fig. 3. Sequential application of CMA staining and FISH with 18S-5.8S-25S rDNA probe on somatic chromosomes of pear seedlings. A and B: 'Osa Gold' [*Pyrus pyrifolia* (Burm. F.)]; C and D: Toyotomi Nashi (*P. mikawana* Koidz.). A and C: stained with CMA; B and D: FISH using 18S-5.8S-25S rDNA as the probe. Arrows in A and C indicate the CMA-positive (+) bands. Arrows and arrowheads in B and D indicate rDNA sites corresponding with CMA+ bands and not corresponding with those bands, respectively. Counterstaining of B and D are DAPI and PI, respectively. Bars represent 5 μm.

Fig. 4. FISH with 5S rDNA and multi-color FISH with 5S and 18S-5.8S-25S rDNA probes on somatic chromosomes of pear seedlings. A, B, and D: 'Osa Gold' [*Pyrus pyrifolia* (Burm. F.)]; C: Toyotomi Nashi (*P. mikawana* Koidz.). A and B: 5S rDNA; C and D: 5S rDNA and 18S-5.8S-25S rDNA. Arrows and arrowheads indicate 5S rDNA and 18S-5.8S-25S rDNA sites, respectively. Counterstaining: DAPI. Bars represent 5 μm.

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