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Application of Fluorescent Staining of Chromosomes to Genetic Studies in Citrus

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ABSTRACT

The application of staining with the guanine-cytosine specific fluorochrome chromomycin A_3 (CMA) of citrus chromosomes to genetic studies is reviewed. When CMA staining is performed, the existence of characteristic CMA banding patterns with high levels of diversity and heterozygosity in citrus chromosomes is demonstrated. Similar CMA banding patterns have been observed in related species. Thus, CMA banding patterns of chromosomes have been considered to provide very important information for phylogenic studies in citrus. Chromosomal identification of citrus has also progressed rapidly by means of the CMA banding method. Chromosomes exhibiting the same CMA banding pattern were separated and classified using the relative sizes of fluorescent bands as indices. All nine chromosomes of haploid citrus were identified by this method. Analysis of the chromosomal configuration of parental materials and their progeny was efficient for genetic improvement in citrus. Characteristic chromosomes could be used as a marker for the parent-progeny relationship since these chromosomes are transmitted from the parents. In particular, CMA chromosome staining is a very powerful tool for ploidy manipulation. The advantage of using CMA staining is that the entire chromosomal configuration can be clearly observed at once.

Keywords: breeding, CMA, genetic resources, haploid, karyotype, phylogenic relationship
Abbreviations: CMA, chromomycin A_3; **DAPI**, 4'-6-diamidino-2-phenylindole; **FISH**, Fluorescence *in situ* hybridization; **GISH**, Genomic *in situ* hybridization

CONTENTS

INTRODUCTION

Chromosomal analysis is important for genetic and biotechnological studies including breeding, genomic analysis, somatic hybridization, and ploidy manipulation (Lamb *et al*. 2006). In citrus (2n=18), one of the most important fruit trees cultivated in temperate and subtropical regions, these studies are essential for genetic improvement. The behavior of chromosomes in meiosis has been studied for some time. In particular, it was elucidated that several meiotic aberrations of chromosomes caused seedless or few seeds in citrus (Nakamura 1943; Naithani and Raghuvanshi 1958; Raghuvanshi 1962; Iwamasa 1966). On the other hand, chromosomal analyses of the mitosis of citrus have not progressed though chromosomal counts of many *Citrus* species and have mainly been conducted for polyploidy breeding (Nakamura 1934; Krug and Bacchi 1943; Tachikawa *et al*. 1961). For chromosome counts, chromosome samples were usually made by paraffin section or the squash method combined with aceto-carmine or aceto-orcein staining (Krug 1943; Oiyama 1981). Guerra *et al*. (1997) analyzed chromosome number, position, and number of secondary constrictions and satellites by means of the squash method with Giemsa staining, and found high structural variability and heterozygosity of citrus chromosomes. However, in genneral, conventional methods have been suitable for the investigation of chromosome numbers, but not for chromosome identification because of the small chromosome size and the similarity in chromosome morphology.

Kurata and Omura (1978) developed the enzymatic maceration method for making clearly shaped chromosomes. This method is quite useful for preparing good chromosomes in plants with small chromosomes (Fukui 1996). Ito *et al*. (1992) and Kitajima (2001) applied this method to citrus, and obtained good preparations.

The identification of morphologically similar chromosomes could be possible because several banding techniques which are more effective than conventional staining techniques have been developed (Friebe *et al*. 1996). Of these methods, C-banding and fluorescent banding using the base-specific binding fluorochrome were applied to citrus chromosome analysis. Heterochromatin patterns were detected and some chromosomes could be identified by C-banding (Guerra 1985; Liang 1988).

The guanine-cytosine (GC)-specific fluorochrome chromomycin A_3 (CMA) and adenine-thymine (AT)-specific fluorochrome 4'-6-diamidino-2-phenylindole (DAPI) banding methods are quite reliable and useful for identifying chromosomes of various plants (Schweizer 1976; Hizume *et al*. 1989; Hizume 1991). *Citrus* CMA/DAPI chromosome banding patterns were first reported by Guerra (1993). Citrus chromosomes showed CMA positive-bands but not

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Invited Review

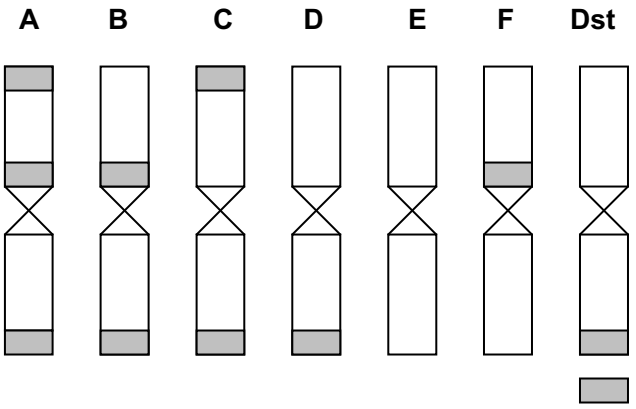

Fig. 1 Schematic representation of chromosome types in citrus according to the position of CMA-positive bands. A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, F: one proximal band, Dst: type D with a satellite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

DAPI positive-bands. The CMA banding pattern of each chromosome was classified into several types. All materials showed a large amount of heterochromatin and heterozygosity. The following studies (Miranda *et al*. 1997a; Befu *et al*. 2000; Cornelio *et al*. 2003; Carvalho *et al*. 2005) reported almost the same chromosomal characteristics of *Citrus* and related genera. In addition, Befu *et al*. (2000) reported that not all DAPI negative-bands correspond to CMA positive-bands. It seems that CMA staining is more useful than DAPI staining for chromosomal identification in citrus from these results.

Fluorescence *in situ* hybridization (FISH) using the ribosomal RNA gene (rDNA) as a probe has been conducted in citrus and rDNA sites were located in some CMA positive-bands (Matsuyama *et al*. 1996; Miranda *et al*. 1997b; Roose *et al*. 1998; Pedrosa *et al*. 2000; Carvalho *et al*. 2005). TGG repeated-sequences in chromosomes were also detected (Matsuyama *et al*. 1999).

Of the above mentioned methods, CMA banding combined with the enzyme maceration method is considered to be easy, reliable, informative, and useful for chromosomal analyses in citrus. The utilization of CMA banding for studies of genetics, breeding, and genetic resources in citrus is discussed in this review.

APPLICATION OF CMA BANDING TO PHYLOGENIC STUDIES IN CITRUS

CMA banding patterns of many citrus accessions were elucidated after Guerra's first report (1993) (Miranda *et al*. 1997a; Befu *et al*. 2000, 2001, 2002; Cornelio *et al*. 2003; Yamamoto and Tominaga 2003; Carvalho *et al*. 2005; Yamamoto *et al*. 2005, 2007). Those studies indicated that CMA-positive bands are reproducible and can be detected in metaphase chromosomes. A high degree of diversity and heterozygosity were demonstrated in citrus chromosomes. Chromosomes were classified into the following seven types based on the number and position of CMA-positive bands (Miranda *et al*. 1997a; Befu *et al*. 2000; Yamamoto and Tominaga 2003; Yamamoto *et al*. 2007): A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, F: one proximal band, and Dst: type D with a satellite chromosome (**Figs. 1, 2**).

Table 1 summarizes the CMA banding patterns of somatic chromosomes of various *Citrus* species (Matsuyama *et al*. 1996; Miranda *et al*. 1997; Befu *et al*. 2000; Pedrosa *et al*. 2000; Befu *et al*. 2001, 2002; Yamamoto and Tominaga 2003, 2004b; Yamamoto *et al*. 2005, 2007). CMA banding patterns of *Poncirus* and *Fortunella* were also revealed (**Table 2**) (Miranda *et al*. 1997; Befu *et al*. 2000; Kunitake *et al*. 2005). Guerra (1993), Cornelio *et al*. (2003) and Carvalho *et al*. (2005) elucidated the CMA banding patterns of approximately 30 *Citrus* accessions. There is a slight difference in the classification of chromosomes between the studies included in **Table 1** and Guerra (1993), Cornelio *et al*. (2003) and Carvalho *et al*. (2005); e.g. Guerra (1993) distinguished type D with large band from same type with small one, and they (Guerra 1993; Cornelio *et al*. 2003; Carvalho *et al*. 2005) described type FL which is the largest chromosome with absent or very small band. Thus, **Table 1** excludes their results.

The *Citrus* species exhibited high chromosomal variability with characteristic banding patterns, and a similar pattern has been observed in related accessions. Type D and E chromosomes were predominant in almost all *Citrus* species. Befu *et al*. (2001) suggested that types D and E are the basic chromosome types in citrus, and types A, B and C arose from type D by inversion and/or translocation. *C. medica*, *C. limon*, *C. aurantifolia* and mandarins possessed large numbers of type D and E chromosomes in general. In particular, all 18 chromosomes of *C. reshni* and *C. sunki* belonged to types D and E. On the other hand, large total numbers of type A, B, and C chromosomes were observed in *C. maxima*. Few mandarin and papeda possessed type F. The type Dst chromosome was detected in only two species in the subgenus *Papeda* and not at all in subgenus *Citrus*.

Numerical taxonomic studies (Barrett and Rhodes 1976; Handa and Oogaki 1985), biochemical studies (Handa *et al*. 1986) and DNA analyses (Yamamoto *et al*. 1993; Nicolosi *et al*. 2000) have revealed that *C. medica*, *C. maxima*, and *C. reticulata* of Swingle's system (1943) are the basic species of the subgenus *Citrus*. Other species, such as *C. sinensis*, *C. paradis*, and *C. limon*, are of hybrid origin. In addition, papedas, non-edible citrus, have played an important role in the development of edible *Citrus* species (Hirai and Kajiura 1987; Federici *et al*. 1998; Nicolosi *et al*. 2000).

C. medica, the chromosomal configuration of both accessions was 2B+8D+8E. This might indicate that each homologous chromosome had the same CMA-positive band as *C. medica* in contrast to many *Citrus* species in which some homologous chromosomes did not exhibit the same CMA-positive band. It is considered that this result reveals the non-hybrid origins of *C. medica*.

Large total numbers of type A, B, and C chromosomes have been considered a characteristic CMA configuration in *C. maxima* though some accessions do not possess the type B chromosome. Five to seven types of A, B and C chromosomes were observed in five accessions (**Table 1**).

C. reticulata of Swingle's system (mandarin) has many accessions with marked differences in morphological and biochemical traits (Torres *et al*. 1978; Handa and Oogaki 1985), much higher than that found in *C. maxima* and *C. medica*. There was also wide variation in the CMA configuration of *C. reticulata*, but numbers of type A, B, and C chromosomes were generally lower. Cornelio *et al*. (2003) stated that the simplest karyotype (only D and E chromosomes) was a candidate to represent *C. reticulata* as a true species. In addition they considered that type C may be the original because many species of mandarin have it. Mandarin accessions having types A and B were speculated to be hybrids and from the lime-lemon-citron-pummelo group. This hypothesis agreed with previous studies (Handa *et al*. 1986; Herrero *et al*. 1996; Nicoloci *et al*. 2000); *C. nobilis* having types A and B was considered to be a hybrid of sweet orange and mandarin. *C. tachibana*, a small fruit mandarin originating in Japan, possessed characteristic type F chromosomes. This was observed only in *C. tachibana* and its relatives in the subgenus *Citrus*. Chloroplast DNA and mitochondrial DNA of *C. tachibana* slightly differ from those of *C. reticulata* of Swingle's system (Yamamoto *et al*. 1993; Nicolosi *et al*. 2000). These results on DNA and our chromosomal analysis suggest that *C. tachibana* separated from other mandarins at an early date.

The ancestors of species considered to be of hybrid origin according to isozyme and DNA analyses (Hirai and Kajiura 1987; Nicolosi *et al*. 2000; Gulsen and Roose 2001)

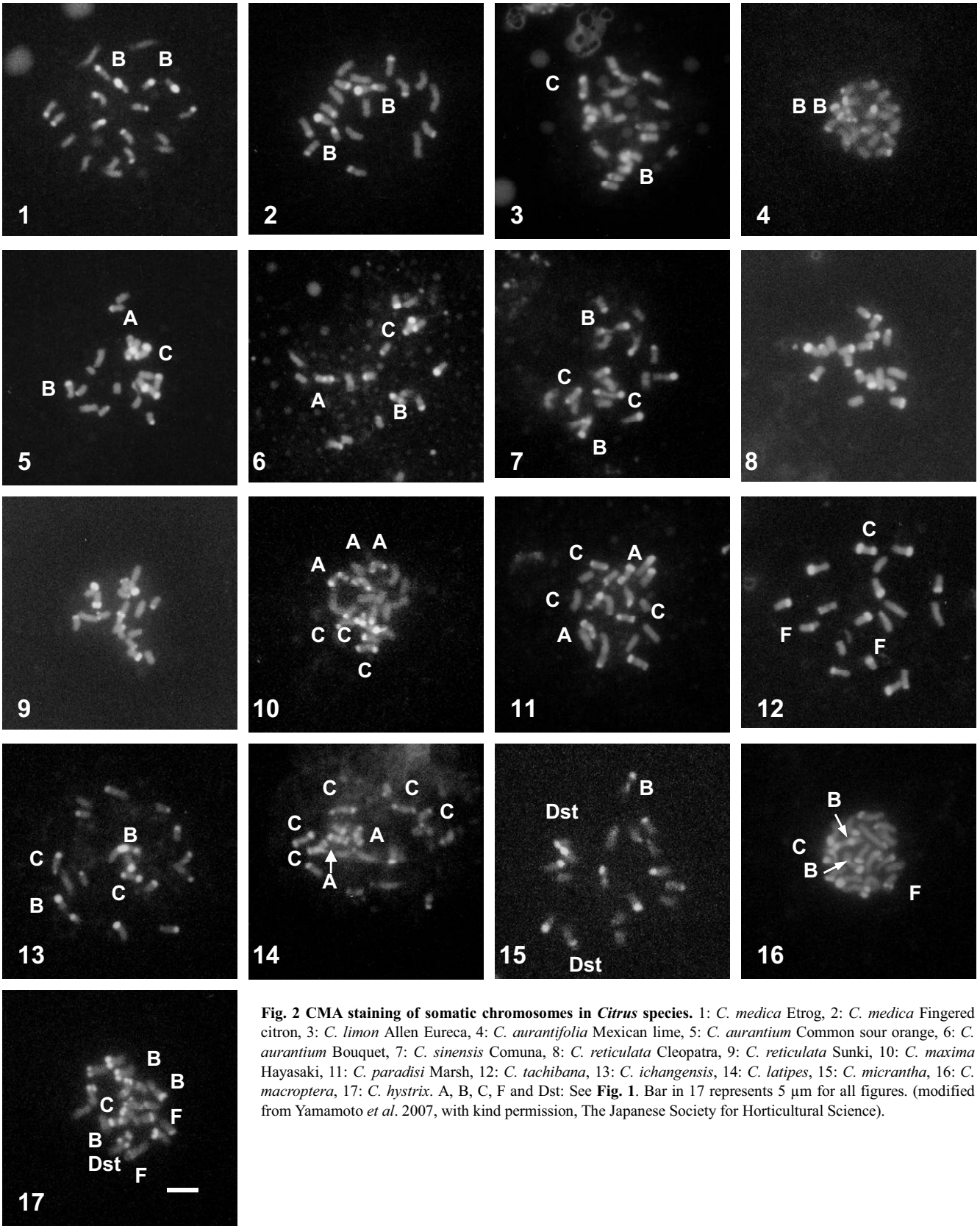

Fig. 2 CMA staining of somatic chromosomes in *Citrus* species. 1: *C. medica* Etrog, 2: *C. medica* Fingered citron, 3: *C. limon* Allen Eureca, 4: *C. aurantifolia* Mexican lime, 5: *C. aurantium* Common sour orange, 6: *C. aurantium* Bouquet, 7: *C. sinensis* Comuna, 8: *C. reticulata* Cleopatra, 9: *C. reticulata* Sunki, 10: *C. maxima* Hayasaki, 11: *C. paradisi* Marsh, 12: *C. tachibana*, 13: *C. ichangensis*, 14: *C. latipes*, 15: *C. micrantha*, 16: *C. macroptera*, 17: *C. hystrix*. A, B, C, F and Dst: See **Fig. 1**. Bar in 17 represents 5 μm for all figures. (modified from Yamamoto *et al*. 2007, with kind permission, The Japanese Society for Horticultural Science).

seem to be as follows: *C. limon*: *C. aurantium* and *C. medica*, *C. aurantifolia*: *C. micrantha* and *C. medica*, *C. aurantium*, *C. sinensis*, *C. hassaku*, *C. natsudaidai* and *C. iyo*: *C. maxima* and *C. reticulata*, *C. paradisi*: *C. maxima* and *C. sinensis*, *C. tankan*: *C. reticulata* and *C. sinensis*. The number of each type of chromosome (chromosome configuration) of each species was intermediate between those of each ancestoral species. The type A chromosomes of *C. aurantium* and *C. paradisi* were considered to be derived from *C. maxima*. The resemblance is found in the CMA banding patterns of *C. medica* and those of its putative progeny *C. limon* and *C. aurantifolia*; a number of types B, D, and E are similar.

C. micrantha and *C. hystrix* belonging to the subgenus *papeda*, possessed a characteristic type of chromosome Dst that was not observed in any species of the subgenus *Citrus*. The type F chromosome found only in *C. tachibana* in the subgenus *Citrus* was observed in *C. macroptera* and *C. hystrix*. Since these two species of papeda were not close to *C. tachibana* (Federici *et al*. 1998; Nicolosi *et al*. 2000), it probably arose independently in papeda and *C. tachibana*. The chromosomal configurations suggested that *C. micran-*

Table 1 CMA banding patterns of somatic chromosomes of various *Citrus* species.

Latin name		Common name	CMA banding pattern[3]	Reference[4]
Swingle's system[1]	Tanaka's system[2]			
Subgenus *Citrus*				
Citron				
Citrus medica L. var. ethrog Engl.	*C. medica* L. var. ethrog Engl.	Etrog Citron	2B+8D+8E	7
C. medica L. var. *sarcodactylis* (Hoola van Nooten) Swingle	*C. medica* L. var. *sarcodactylis* (Hoola van Nooten) Swingle	Fingered Citron	2B+8D+8E	2, 7
Lemon, lime and their relatives				
C. limon (L.) Burm. f. .	*C. limon* (L.) Burm. f.	Allen Eureka	1B+1C+8D+8E	7
C. spp.	*C. jambhiri* Lush.	Rough Lemon	1B+11D+6E	5
C. spp.	*C. jambhiri* Lush.	Rough Lemon	1B+8D+9E	11
C. aurantifolia (Christm.) Swingle	*C. aurantifolia* (Christm.) Swingle	Mexican Lime	2B+9D+7E	7
Sour orange				
C. aurantium L.	*C. aurantium* L.	Daidai	1B+2C+8D+7E	3
C. aurantium L.	*C. aurantium* L.	Common sour orange	1A+1B+1C+7D+8E	7
C. aurantium L.	*C. aurantium* L.	Bouquet	1A+1B+1C+7D+8E	7
Sweet orange and its relatives				
C. sinensis (L.) Osbeck	*C. sinensis* (L.) Osbeck	Trovita	2B+2C+7D+7E	4, 5
C. sinensis (L.) Osbeck	*C. sinensis* (L.) Osbeck	Washington Navel	2B+2C+7D+7E	1, 5
C. sinensis (L.) Osbeck	*C. sinensis* (L.) Osbeck	10 cultivars	2B+2C+7D+7E	6
C. sinensis (L.) Osbeck	*C. sinensis* (L.) Osbeck	Comuna	2B+2C+7D+7E	7
C. spp.	*C. tankan* hort.ex Tanaka	Tarumizu 1 go	1A+1B+1C+8D+7E	8
Mandarin				
C. reticulata Blanco	*C. reticulata Blanco*	Ponkan cv. Yoshida	1B+1C+9D+7E	3
C. reticulata Blanco	*C. reticulata Blanco*	Ponkan cv. Yoshida	1B+1C+10D+6E	9
C. reticulata Blanco	*C. reshni* hort. ex Tanaka	Cleopatra	15D+3E	9
C. reticulata Blanco	*C. tangerina* hort. ex Tanaka	Dancy	1C+10D+7E	9
C. reticulata Blanco	*C. sunki* (Hayata)hort. ex Tanaka	Sunki	12D+6E	9
C. reticulata Blanco	*C. succosa* hort. ex Tanaka	Honchiso (Ben di zao)	1A+1B+10D+6E	5
C. reticulata Blanco	*C. succosa* hort. ex Tanaka	Honchiso (Ben di zao)	1A+1C+10D+6E	9
C. reticulata Blanco	*C. kinokuni* hort. ex Tanaka	Mukaku-Kishu	1C+8D+9E	3
C. reticulata Blanco	*C. kinokuni* hort. ex Tanaka	Kinokuni	1C+8D+9E	9
C. reticulata Blanco	*C. deliciosa* Ten.	Mediterranean cv. Tardivo di Ciaculli	1C+10D+7E	9
C. reticulata Blanco	*C. suhuiensis* hort. ex Tanaka	Shikaikan (Szu ui kom)	1B+10D+7E	9
C. reticulata Blanco	*C. nobilis* Lour.	King	1A+1B+1C+8D+7E	9
C. reticulata Blanco	*C. nobilis* Lour.	Kunenbo	1A+1B+2C+5D+9E	9
C. reticulata Blanco	*C. clementina* hort. ex Tanaka	Clementine	1B+1C+10D+6E	10
C. reticulata Blanco	*C. keraji* hort. ex Tanaka	Kabuchi	2B+2C+5D+7E+2F	9
C. reticulata Blanco	*C. keraji* hort. ex Tanaka	Keraji	1A+1B+7D+8E+1F	9
C. reticulata Blanco	*C. leiocarpa* hort. ex Tanaka	Koji	1C+8D+9E	2
C. reticulata Blanco	*C. leiocarpa* hort. ex Tanaka	Koji	2B+1C+6D+9E	9
C. reticulata Blanco	*C. unshiu* Marcow.	Satsuma mandarin cv. Juman	2C+8D+8E	9
C. reticulata Blanco	*C. unshiu* Marcow.	Satsuma mandarin cv. Okitsu Wase	1A+1C+8D+8E	2, 9
C. reticulata Blanco	*C. depressa* Hayata	Shiikuwasha	1C+10D+6E+1F	9
C. tachibana (Makino) Tanaka	*C. tachibana* (Makino)Tanaka	Tachibana	1C+10D+5E+2F	9
Pummelo				
C. maxima (Burm.) Merr.	*C. maxima* (Burm.) Merr.	Shadenyu	3A+2C+7D+6E	5
C. maxima (Burm.) Merr.	*C. maxima* (Burm.) Merr.	Tosa-Buntan	1A +1B +5C+2D+9E	1
C. maxima (Burm.) Merr.	*C. maxima* (Burm.) Merr.	Ban-okan	2A +1B +3C+3D+9E	2
C. maxima (Burm.) Merr.	*C. maxima* (Burm.) Merr.	Tanikawa-Buntan	3A+2C+4D+9E	3
C. maxima (Burm.) Merr.	*C. maxima* (Burm.) Merr.	Hayasaki	3A+3C+4D+8E	7
Pummelo relatives				
C. paradisi Macfad.	*C. paradisi* Macfad.	Duncan	2A+1B+1C+6D+8E	2
C. paradisi Macfad.	*C. paradisi* Macfad.	Marsh	2A+3C+6D+7E	7
C. spp.	*C. hassaku* hort. ex Tanaka	Hassaku	1A+1C+8D+8E	8
C. spp.	*C. tamurana* hort. ex Tanaka	Hyuganatsu	2A+2C+5D+9E	8
C. spp.	*C. natsudaidai* Hayata	Kawano Natsudaidai	1A+2C+7D+8E	8
C. spp.	*C. iyo* hort.ex Tanaka	Miyauchi Iyokan	1A+1B+1C+8D+7E	8
Yuzu and its relatives				
C. ichang-austera hybrid	*C. junos* Sieb. ex Tanaka	Yamame	2B+1C+11D+4E	8
C. spp.	*C. spaerocarpa* hort. ex Tanaka	Kabosu	3B+2C+5D+8E	8
C. spp.	*C. sudachi* hort. ex Shirai	Sudachi	1B+2C+9D+6E	8
Subgenus *Papeda*				
C. ichangensis Swingle	*C. ichangensis* Swingle	Ichang papeda	2B+2C+12D+2E	7
C. latipes (Swingle) Tanaka	*C. latipes* (Swingle) Tanaka	Khasi papeda	2A+5C+8D+3E	7
C. micrantha Wester	*C. micrantha Wester*	Biasong	1B+11D+4E +2Dst	7
C. macroptera Montr.	*C. macroptera* Montr.	Melanesian papeda	2B+1C+11D+3E+1F	7
C. hystrix DC.	*C. hystrix* DC.	Purutt	3B+1C+8D+3E+2F+1Dst	7

[1] Swingle (1943), [2] Tanaka (1977), [3] A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without band, F: one proximal band, Dst: type D with a satellite chromosome.

[4] 1: Befu *et al.* (2000), 2: Befu *et al.* (2001), 3: Befu *et al*. (2002), 4: Matsuyama *et al.* (1996), 5: Miranda *et al*. (1997), 6: Pedrosa *et al*. (2000), 7: Yamamoto *et al*. (2007), 8: Yamamoto *et al*. (2005), 9: Yamamoto and Tominaga (2003), 10: Yamamoto and Tominaga (2004b), 11: Yamamoto (unpublished).

Table 2 CMA banding patterns of somatic chromosomes of *Poncirus* and *Fortunella*.

Latin name	Common name	CMA banding pattern[1]	Reference[2]
Poncirus trifoliata (L.) Raf.	Trifoliate orange	2B+10D+8E	3
		4B+8D +6E	1
Fortunella hindsii (Champ. ex Benth.) Swingle	Kinzu	2B+16D	2, 3
Fortunella japonica (Lour.) Swingle	Round kumquat	2B+2C+16D	3
Fortunella japonica (Lour.) Swingle	Round kumquat	1A+1B+1C+16D+1F	2
Fortunella margarita (Lour.) Swingle	Oval kumquat	1A+1B+2C+13D+1F	2
Fortunella crassifolia Swingle	Meiwa kumquat	2A+1B+2C+12D+1F	2

[1] A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without band.
[2] 1: Befu *et al.* (2000), 2: Kunitake *et al*. (2005), 3: Miranda *et al.* (1997).

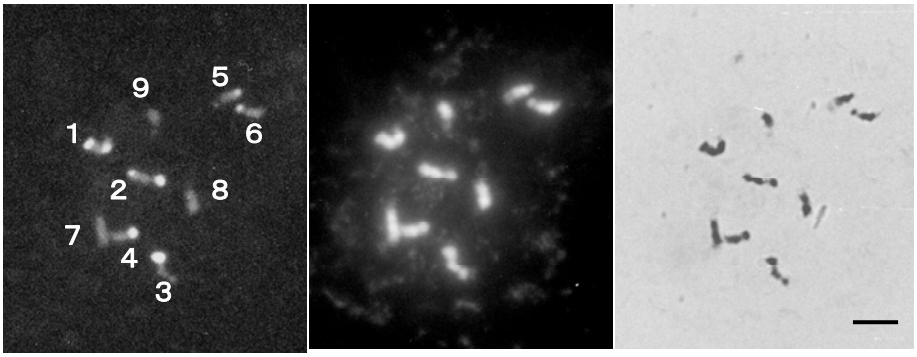

Fig. 3 CMA, DAPI and Giemsa stained chromosomes of haploid Clementine. Left: stained with CMA (each number corresponds to the number in **Table 3**), center: stained with DAPI, right: stained with Giemsa. Bar represents 5 µm. (from **Yamamoto M, Tominaga S** (2004a) *Scientia Horticulturae* **101**, 201-206, with kind permission, Elsevier, ©2004).

Table 3 Choromosome identification of haploid Clementine accordimg to CMA, DAPI and Giemsa staining (according to **Yamamoto M, Tominaga S** (2004a) *Scientia Horticul-turae* **101**, 201-206, with kind permission, Elsevier, ©2004).

No. [1]	CMA band[2]	Intensity of CMA band	Region of very light CMA (+) spot	Region of DAPI (-) band	Length of chromosome[3] (µm)
1	B[4]				3.1 ± 0.15
2	C[4]				3.1 ± 0.19
3	D	Heavy[4]			3.1 ± 0.17
4	D	Intermediate[4]			2.7 ± 0.21
5	D	Light	Opposite terminal region to CMA (+) band[4]	Two terminal regions[4]	2.4 ± 0.24
6	D	Light	Nothing[4]	One terminal region[4] (corresponding to CMA (+) band)	2.3 ± 0.15
7	E				3.3 ± 0.25[4]
8	E				2.4 ± 0.17[4]
9	E				1.8 ± 0.16[4]

[1] Each number correponds to the chromosome in **Fig. 3**.
[2] B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without band.
[3] Average in 10 cells
[4] Each chromosome could be identified using this item.

tha, *C. macroptera*, and *C. hystrix* were differentiated from the subgenus *Citrus*. This result agreed with that of Nicolosi *et al*. (2000) who revealed the distance between those three species of papeda and subgenus *Citrus* by using DNA markers. Analyses of DNA (Federici *et al*. 1998; Nicolosi *et al*. 2000), Fraction I protein (Handa *et al*. 1986), and isozymes (Hirai and Kajiura 1987), showed the genetic similarity between *C. latipes* and *C. maxima*. Federici *et al*. (1998) supposed that *C. latipes* was of non-hybrid origin according to RFLP data. *C. latipes* was the only species that possessed the type A chromosome in the subgenus *Papeda*. It seems that *C. latipes* was the ancestor of *C. maxima*, and that the type A chromosome of *C. maxima* was derived from *C. latipes*. The resemblance in total numbers of type A and C chromosomes of *C. latipes* and *C. maxima* seems to support this concept. Although *C. ichangensis* was a distinct species and very different from most other subgenus *Citrus* and papeda species according to DNA analysis (Federici *et al*. 1998), its cpDNA was close to *C. reticulata* (Nicolosi *et al*. 2000). Hirai and Kajiura (1987) suggested a similarity of isozyme banding patterns between *C. ichangensis* and Yuzu. Resemblance was found in the CMA banding patterns of *C. ichangensis* and Yuzu. *C. ichangensis* may therefore be very closely related to Yuzu.

Fortunella and *Poncirus*, very closely related *Citrus*, showed contrastive CMA banding patterns. Two to nine type E chromosomes were observed in all *Citrus* and *Poncirus*, but not in *Fortunella*. These results suggested less divergence between *Citrus* and *Poncirus* compared with *Fortunella*. In some cases, heterochromatin (CMA(+)) volume probably tends to increase with evolution (Deumling and Greihuber 1982; Ikeda 1988). *Fortunella* was considered to have the most advanced morphological characterics (Tanaka 1954). These results are consistent with the hypothesis (Miranda *et al*. 1997a).

The CMA banding patterns in the chromosomes of 17 species belonging to 15 genera of Rutaceae subfamily Aurantioideae (=Citroideae) including *Citrus* were analyzed by Guerra *et al*. (2000). Generally more basal genera exhibited very small amounts of heterochromatin (CMA (+)), whereas relatively advanced genera displayed numerous large CMA (+) bands.

KARYOTYPING

Although the use of fluorescent banding, such as CMA and/or DAPI used for chromosome staining, has allowed the identification of chromosomes with morphological similarities according to their banding patterns, not all chromosomes could be distinguished. Chromosomes exhibiting the same fluorescence banding pattern were separated and classified using the relative intensity/size of fluorescent bands as indices. These differences were stable and reproducible thus, were used to distinguish between same types of citrus chromosomes (Befu *et al*. 2002; Yamamoto and Tominaga 2004a).

Befu *et al*. (2002) analyzed CMA banding patterns of chromosomes in four cultivars; 3A+2C+4D+9E in 'Tani-

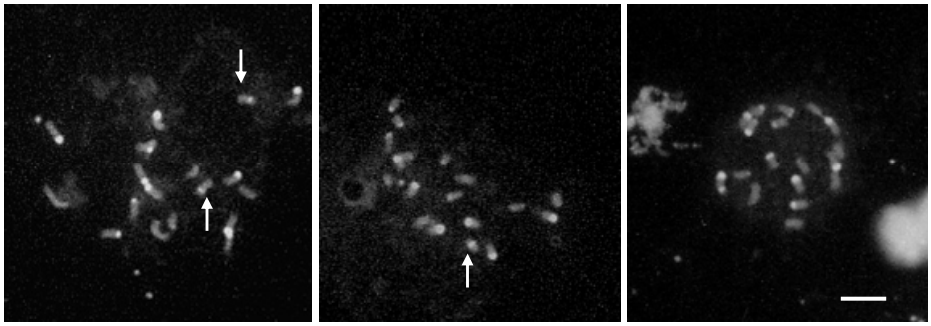

Fig. 4 CMA staining of somatic chromosomes in seedlings from *C. kinokuni* X *C. tachibana*. Left: two type F chromosomes, center: one type F chromosome, right: no type F chromosome. Arrows indicate type F chromosomes. Bar represents 5 μm.

kawa-Buntan' (*C. maxima*), 1B+1C+9D+7E in 'Yoshida ponkan' (*C. reticulata*), 1C+8D+9E in 'Mukaku-Kishu' (*C. kinokuni*) and 1B+2C+8D+7E in sour orange (*C. aurantium*). Types C and B chromosomes could be categorized into more than one group according to relative intensity/sizes of CMA (+) regions as indices. In addition, a few chromosomes with the same CMA banding pattern were classifiable on the basis of chromosome length. The CMA banding patterns were 1A-I+2A-II+1C-I+1C-II+4D+9E in 'Tanikawa-Buntan' (*C. maxima*), 1B+1C+7D-I+1D-II+1D-III+7E in 'Yoshida ponkan' (*C. reticulata*), 1C+5D-I+3D-II+8E-I+1E-II in 'Mukaku-Kishu' (*C. kinokuni*), and 1B+1C-I+1C-II+4D-I+2D-II+2D-III+7E in sour orange (*C. aurantium*).

Not all 18 chromosomes of diploid citrus were identified though the relative intensity/sizes of CMA (+) regions are useful as indices for classification. Chromosomal identification in haploid cells should be easier than that in diploid cells since they have a lower chromosome number (2n=9). The identification is greatly facilitated in other species using haploid plants (Fukui and Iijima 1991).

Yamamoto and Tominaga (2004a) identified the karyotype of haploid Clementine (*C. clementina* hort. ex Tanaka) produced by Oiyama and Kobayashi (1993) by means of sequential Giemsa/CMA/DAPI staining. Using CMA staining, haploid Clementine chromosomes were classified into four types based on the number and position of the CMA-positive bands: type B, C, D and E. One type B chromosome, one type C chromosome, four type D chromosomes, and three type E chromosomes were observed (**Fig. 3**). Type D chromosomes were categorized into three groups according to the relative intensity of the CMA-positive (+) bands. One chromosome was heavily stained, another was intermediate in staining intensity, and two chromosomes were lightly stained. Although the length of the two chromosomes with light CMA (+) bands was almost identical (**Table 3**), the chromosomes could be separately identified since one had a very light CMA (+) spot in the terminal region opposite the CMA (+) band, a characteristic that the other lacked. The two chromosomes could also be distinguished by DAPI staining. One chromosome had DAPI negative (-) bands in both terminal regions, while the other had one terminal DAPI (-) band which corresponded to a CMA (+) region (**Fig. 3**). Based on chromosome length, type E chromosomes could be divided into three types: long (3.3 μm), medium (2.4 μm), and short (1.8 μm) (**Table 3**).

The data shows that using haploid Clementine as plant material, citrus chromosome identification can be efficiently made by an estimation of the relative intensity of CMA-positive regions on the chromosomes.

Since high heterozygosity is a characteristic of citrus chromosomes, making it very difficult to identify all 18 chromosomes by karyotype analysis, haploid citrus plants are useful for identifying chromosomes. Several haploid plants have been produced in citrus (Hidaka *et al*. 1979; Oiyama and Kobayashi 1993; Germanà *et al*. 1994; Toolapong *et al*. 1996; Germanà and Chiancone 2001). The identification of their chromosomes is considered to be very important because almost all species possesses characteristic CMA banding patterns.

In Giemsa, CMA and DAPI staining, it is difficult to identify the position of the centromeres. By contrast, the discrimination of centromeres was easier when quinacrine mustard (QM) staining was used compared to Giemsa staining (Befu *et al*. 2000). QM therefore is useful for chromosomal identification. Kitajima *et al*. (2001) conducted the Multi-color GISH (Genomic *in situ* hybridization) of satsuma mandarin (*C. unshiu*) chromosomes hybridized with labeled total DNA of satsuma mandarin and 'Tosa-Buntan' (*C. maxima*). Fourteen out of 18 chromosomes could be identified by this method. Therefore the sequential Giemsa/QM/CMA/DAPI staining technique and/or multicolor GISH seems to be very effective for future chromosomal identification in citrus.

APPLICATION OF CMA BANDING TO GENETICS AND BREEDING

Since citrus showed high variability of CMA banding patterns in their chromosomes, characteristic and small numbers of chromosomes such as types A, B, C and F would be marker chromosomes in genetic studies. Inheritance and/or segregation of marker chromosomes were confirmed using Tachibana (*C. tachibana*) possessing characteristic type F chromosomes was used as parental materials (Yamamoto unpublished). CMA banding patterns in hybrids derived from Kinokuni (*C. kinokuni*) (1C+8D+9E) in CMA banding pattern crossed with Tachibana (1C+10D+5E+2F) were observed. Numbers of type F chromosomes of hybrids were classified into three types; none, one and two (**Fig. 4**). This result demonstrated the segregation of type F chromosomes and the usefulness of characteristic chromosomes as markers for genetic and breeding studies in citrus.

Yang *et al*. (2002) estimated the chromosome pairing set of 'Tosa-Buntan' (*C. maxima*) according to the CMA banding patterns of hybrids derived from a 'Tosa-Buntan' (1A+1B+5C+2D+9E) X 'Suisho-Buntan' (3A+3C+3D+9E) cross. From the results of 13 kinds of chromosomal configurations of 38 hybrids, the pairing set of chromosome type in 'Tosa-Buntan' was estimated as AB+CC+CC+CD+DE+EE+EE+EE+EE or AB+CC+CC+DD+CE+EE+EE+EE+EE. A few triploid or tetraploid hybrids appeared from both diploid parents in citrus (Esen and Soost 1971; Geraci *et al*. 1975; Yang *et al*. 2002). Yang *et al*. (2002) also estimated the origin of the unreduced gamete from the chromosome configuration of polyploid hybrids.

CMA banding patterns of chromosomes are useful for analyzing configurations in citrus somatic hybrids as well as hybrid seedlings (Miranda *et al*. 1997c). They detected putative structural alterations of one chromosome in one somatic hybrid produced from *C. jambhiri* + *C. reticulata*.

The production of pure lines (homozygous plant) is essential for the advancement of citrus breeding. However, it is very difficult to obtain pure lines by conventional repeated self-pollination because of a long juvenile period. Therefore, haploid and doubled haploid plants are valuable. It is possible that the configuration of CMA-stained chromosomes could be used for the detection of chromosome doubling in haploid cells. Yamamoto and Tominaga (2004b) used a mixoploid plant (variant) with both haploid and diploid cells which arose from haploid Clementine (*C. clementina*) produced by Oiyama and Kobayashi (1993). The chromosomal configuration was 2B+2C+8D+6E for the diploid cells of the mixoploid plant, 1B+1C+4D+3E for the haploid

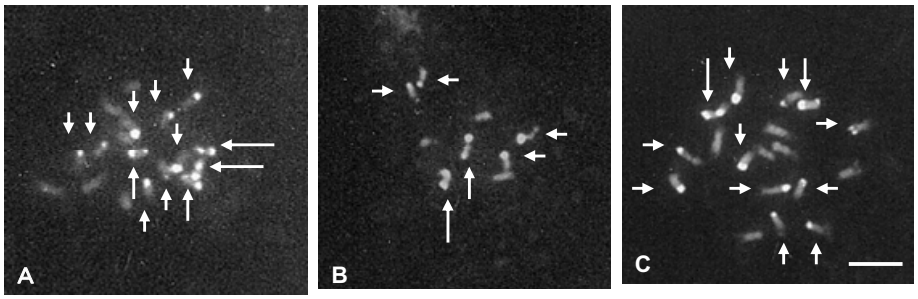

Fig. 5 CMA staining of somatic chromosomes in (A) diploid cells of the mixoploid plant, (B) haploid Clementine and (C) diploid Clementine. Long, medium and short arrows indicate B, C and D type chromosomes, respectively. Bar in (C) represents 5 µm for all figures. (from Yamamoto and Tominaga 2004b, with kind permission, Japanese Society for Breeding).

plant, and 1B+1C+10D+6E for Clementine (**Fig. 5**). By CMA staining of the chromosomes it could be determined that the diploid cells of the variant were doubled haploid cells. The chromosomal configuration of the diploid cells of the variant differed from that of the original diploid Clementine. The number of chromosomes of each type in the diploid cells of the variant (2B+2C+8D+6E) was twice that in the haploid plant (1B+1C+4D+3E). As mentioned above, the configuration of the CMA-stained chromosomes of almost all the accessions of *Citrus* showed a high degree of heterozygosity, i.e., some homologous chromosomes did not show the same CMA-positive band. In these accessions, each haploid plant derived from a gamete is likely to differ in chromosomal configuration, due to the occurrence of segregation of the CMA-positive band in homologous chromosomes. The homologous chromosomes of the doubled haploid cells obtained from these haploid plants showed the same CMA-positive band, in contrast to the configuration in the original diploid plants. Therefore, a CMA analysis of chromosomes appears to be a very suitable method for the identification of doubled haploids in citrus. In previous studies (Germanà *et al*. 1994, 2000), isozyme and RAPD analyses were used for the identification of doubled haploids in citrus. Compared with these methods, the advantage of a chromosomal analysis with CMA staining is that whole chromosomes can be clearly observed in one image. A doubled haploid plant produced by a haploid 'Banpeiyu' (*C. maxima*) was also confirmed based on chromosome configuration analysis by CMA staining (Yahata *et al*. 2005). The haploid was composed of 1A+1B+1C+2D+4E, and that of the doubled haploid was 2A+2B+2C+4D+8E.

CONCLUSION

A high degree of diversity and heterozygosity were detected in CMA-stained chromosomes in *Citrus* and related genera. Other fruit trees such as peach (*Prunus persica* (L.) Batch) showed no variation of CMA banding patterns in the chromosomes and the same CMA banding pattern of homologous chromosomes (Yamamoto *et al*. 1999). The diversity and heterozygosity of CMA banding patterns are unique characteristics of citrus chromosomes, and thus a very powerful tool for genetic studies in citrus.

Since compared with other staining methods, CMA staining is reliable, informative and relatively easy, large amounts of material can be analyzed. Therefore, CMA banding analysis of citrus chromosomes was useful for elucidation of phylogenic relationships and evolution of *Citrus* and related genera and karyotyping and utilization of markers for breeding.

However, identification of all 18 chromosomes of *Citrus* and related genera is difficult by CMA staining alone. CMA staining combined with multicolor GISH (Kitajima *et al*. 2001) or multicolor FISH (Murata *et al*. 1997) could become a suitable tool for identifying all the chromosomes in citrus, and provide useful information on genetics in citrus.

REFERENCES

Barrett HC, Rhodes AM (1976) A numerical taxonomic study of affinity relationships in cultured *Citrus* and its close relatives. *Systematic Botany* **1**, 105-136

Befu M, Kitajima A, Ling YX, Hasegawa K (2000) Classification of 'Tosa-Buntan' pummelo (*Citrus grandis* [L.] Osb.), 'Washington' navel orange (*C. sinensis* [L.] Osb.) and trifoliate orange (*Poncirus trifoliata* [L.] Raf.) chromosomes using young leaves. *Journal of the Japanese Society for Horticultural Science* **69**, 22-28

Befu M, Kitajima A, Hasegawa K (2001) Chromosome composition of some citrus species and cultivars based on the chromomycin A_3 (CMA) banding patterns. *Journal of the Japanese Society for Horticultural Science* **70**, 83-88

Befu M, Kitajima A, Hasegawa K (2002) Classification of the citrus chromosomes with same types of chromomycin A banding patterns. *Journal of the Japanese Society for Horticultural Science* **71**, 394-400

Carvalho R, Soares Filho WS, Brasileiro-Vidal AC, Guerra M (2005) The relationships among lemons, limes and citron: a chromosomal comparison. *Cytogenetic Genome Research* **109**, 276-282

Cornelio WTMN, Figueiroa ARS, Santos KGB, Carvalho R, Soares Filho WS, Guerra M (2003) Chromosomal relationships among *Citrus reticulata* Blanco, its hybrid and related species. *Plant Systematics and Evolution* **240**, 149-161

Deumling B, Greilhuber J (1982) Characterization of heterochromatin in different species of the *Scilla siberica* group (Liliaceae) by *in situ* hybridization of satellite DNAs and fluorochrome banding. *Chromosoma* **84**, 535-555

Esen A, Soost RK (1971) Unexpeced triploids in *Citrus*: their origin, identification and possible use. *Journal of Heredity* **62**, 410-414

Federici CT, Fang DQ, Scora RW, Roose ML (1998) Phylogenic relationships within the genus *Citrus* (*Rutaceae*) and related genera as revealed by RFLP and RAPD analysis. *Theoretical and Applied Genetics* **96**, 812-822

Friebe B, Endo TR, Gill BS (1996) Chromosome-banding methods. In: Fukui K, Nakayama S (Eds) *Plant Chromosome*. *Laboratory Methods*, CRC Press, Florida, pp 123-153

Fukui K (1996) Plant chromosome at mitosis. In: Fukui K, Nakayama S (Eds) *Plant Chromosome*. *Laboratory Methods*, CRC Press, Florida, pp 1-17

Fukui K, Iijima K (1991) Somatic chromosome map of rice by imaging methods. *Theoretical and Applied Genetics* **81**, 589-596

Geraci G, Esen A, Soost RK (1975) Triploid progenies from 2x X 2x crosses of citrus cultivars. *Journal of Heredity* **66**, 177-178

Germanà MA, Chiancone B (2001) Gynogenetic haploids of *Citrus* after *in vitro* pollination with triploid pollen grains. *Plant Cell, Tissue and Organ Culture* **66**, 59-66

Germanà, MA, Reforgiato Recupero G, Russo MP (2000) Preliminary characterization of several doubled haploids of *Citrus clementina* cv. Nules. *Acta Horticulture* **535**, 183-190

Germanà MA, Wang YY, Barbagallo MG, Iannolino G, Crescimanno FG (1994) Recovery of haploid and diploid plantlets from anther culture of *Citrus clementina* Hort. ex Tan. and *Citrus reticulata* Blanco. *The Journal of Horticultural Science* **69**, 473-480

Guerra M (1985) Cytogenetics of Rutaceae. III. Heterochromatin patterns. *Caryologia* **38**, 335-346

Guerra M (1993) Cytogenetics of Rutaceae. V. High chromosomal variability in *Citrus* species revealed by CMA/DAPI staining. *Heredity* **71**, 234-241

Guerra M, Galvao K, Santos B, Silva AEM, Ehrendorfer F (2000) Heterochromatin banding patterns in Ruraceae-Aurantioideae – A case of parallel chromosomal evolution. *American Journal of Botany* **87**, 735-747

Guerra M, Pedrosa A, Silva AEB, Cornelio MTM, Santos K, Soares Fiho WS (1997) Chromosome number and secondary construction variation in 51 accessions of a citrus germplasm bank. *Brazillian Journal of Genetics* **20**, 489-496

Gulsen O, Roose ML (2001) Chloroplast and nuclear genome analysis of the parentage of lemons. *Journal of the American Society for Horticultural Science* **126**, 210-215

Handa T, Ishizawa Y, Oogaki C (1986) Phylogenic study of Fraction I protein in the genus *Citrus* and its close related genera. *Japanese Journal of Genetics* **61**, 15-24

Handa T, Oogaki C (1985) Numerical taxonomic study of *Citrus* L. and *Fortunella* Swingle using morphological characters. Application of multivariate analysis. *Journal of the Japanese Society for Horticultural Science* **54**, 145-154

Herrero R, Asims MJ, Pina JA, Carbonell EA, Navarro L (1996) Genetic diversity in the orange subfamily Aurantioideae. II. Genetic relationships among genera and species. *Theoretical and Applied Genetics* **93**, 1327-1334

Hidaka T, Yamada Y, Shichijo T (1979) *In vitro* differentiation of haploid plants by anther culture in *Poncirus trifoliate* (L.) Raf. *Japanese Journal of Breeding* **29**, 248-254
Hirai M, Kajiura I (1987) Genetic analysis of leaf isozymes in citrus. *Japanese Journal of Breeding* **37**, 377-388
Hizume M (1991) Analysis of plant chromosomes using a fluorescent banding method. *Plant Cell Technology* **3**, 78-83
Hizume M, Ohgiku A, Tanaka A (1989) Chromosome banding in the genus *Pinus*. II. Interspecific varirtion of fluorescent banding patterns in *P. densiflora* and *P. thunbergii*. *The Botanical Magazine, Tokyo* **102**, 25-36
Ikeda H (1988) Karyomorphological studies on the genus *Crepis* with special reference to C-banding pattern. *Journal of Science for Hiroshima University, Series B, Division 2* **22**, 65-117
Ito Y, Omura M, Nesumi H, Yoshida T (1992) Improvement of preparation and observation methods for *Citrus* chromosomes. *Bulletin of Fruit Tree Research Station* **23**, 57-66
Iwamasa M (1966) Studies on the sterility in genus *Citrus* with special reference to the seedlessness. *Bulletin of the Horticultural Station, Japan, Series B* **6**, 1-81
Kitajima A, Befu M, Hidaka Y, Hotta T, Hasegawa K (2001) A chromosome preparation method using young leaves of citrus. *Journal of the Japanese Society for Horticultural Science* **70**, 191-194
Kitajima A, Nishihara M, Hasegawa K (2001) Chromosome identification in satsuma mandarin (*Citrus unshiu* Marc.) by multi-color GISH. *Journal of the Japanese Society for Horticultural Science* **70** (Suppl. 1), 208
Krug CA (1943) Chromosome numbers in the subfamily Aurantioideae with special reference to the genus *Citrus*. *Botanical Gazette* **103**, 602-611
Krug CA, Bacchi O (1943) Triploid varieties of citrus. *Journal of Heredity* **43**, 277-283
Kunitake H, Haraguchi K, Yahata M (2005) Studies on phylogenic relationship in the genus *Fortunella*. *Journal of the Japanese Society for Horticultural Science* **74** (Suppl. 1), 476-478
Kurata N, Omura T (1978) Karyotype analysis in rice. 1. A new method for identifying all chromosome pairs. *Japanese Journal of Genetics* **53**, 251-255
Lamb JC, Kato A. Yu W-C, Han F-P, Albert PS, Birchler JA (2006) Cytogenetics and chromosome analytical techniques. In: Teixeira da Silva JA (Ed) *Floriculture, Ornamental and Plant Biotechnology: Advances and Topical Issues* (1st Edn, Vol IV), Global Science Books, London, UK, pp 245-248
Liang G (1988) Studies on the Giemsa C-banding patterns of some *Citrus* and its related genera. *Acta Genetica Sinica* **15**, 409-415
Matsuyama T, Akihama T, Ito Y, Omura M, Fukui K (1996) Characterization of heterochromatic regions in 'Trovita' orange (*Citrus sinensis* Osbeck) chromosomes by the fluorescent staining and FISH method. *Genome* **39**, 941-945
Matsuyama T, Akihama T, Ito Y, Omura M, Fukui K (1999) Distribution of TGG repeat-related sequences in 'Trovita' orange (*Citrus sinensis* Osbeck) chromosomes. *Genome* **42**, 1251-1254
Miranda M, Ikeda F, Endo T, Moriguchi T, Omura M (1997a) Comparative analysis on the distribution of heterochromatin in *Citrus*, *Poncirus* and *Fortunella* chromosomes. *Chromosome Research* **5**, 86-92
Miranda M, Ikeda F, Endo T, Moriguchi T, Omura M (1997b) rDNA sites and heterochromatin in Meiwa kumquat (*Fortunella crassifolia* Swing.) chromosomes revealed by FISH and CMA/DAPI staining. *Caryologia* **50**, 333-340
Miranda M, Ikeda F, Endo T, Moriguchi T, Omura M (1997c) Chromosome markers and alterations in mitotic cells from interspecific *Citrus* somatic hybrids analysed by fluorochrome staining. *Plant Cell Reports* **16**, 807-812
Murata M, Heslop-Harrison JS, Motoyoshi F (1997) Physical mapping of the 5S ribosomal RNA genes in *Arabidopsis thaliana* by multi-color fluorescence *in situ* hybridization with cosmid clones. *The Plant Journal* **12**, 31-37
Naithani SP, Raghuvanshi SS (1958) Cytogenetical studies in the genus *Citrus*. *Nature* **181**, 1406-1047
Nakamura M (1934) Discovery of tetraploid citrus: Its significance in karyology and breeding of citrus fruits. *Journal of the Japanese Society for Horticultural Science* **10**, 217-220
Nakamura M (1943) Cytological and ecological studies on the genus *Citrus* with special reference to the occurrence of sterile pollen grains. *Memoirs of the Faculty of Science and Agriculture, Taihoku University* **27**, 53-159
Nicolosi E, Deng ZN, Gentile A, La Malfa S, Continella G, Tribulato E (2000) Citrus phylogeney and genetic origin of important species as investigated by molecular markers. *Theoretical and Applied Genetics* **100**, 1155-1166
Oiyama I (1981) A technique for chromosome observation in root tip cells of citrus. *The Bulletin of the Fruit Tree Research Station* **D3**, 1-7
Oiyama I, Kobayashi S (1993) Haploids obtained from diploid X triploid crosses of citrus. *Journal of the Japanese Society for Horticultural Science* **62**, 89-93
Pedrosa A, Schweizer D, Guerra M (2000) Cytological heterozygosity and hybrid origin of sweet orange [*Citrus sinensis* (L.) Osbeck]. *Theoretical and Applied Genetics* **100**, 361-367
Raghuvanshi SS (1962) Cytogenetical studies in genus *Citrus*, *Citrus assamensis*. *Caryologia* **15**, 143-149
Roose ML, Schwarzacher T, Heslop-Harrison JS (1998) The chromosomes of *Citrus* and *Poncirus* species and hybrids: identification of characteristic chromosomes and physical mapping of rDNA loci using *in situ* hybridization and fluorochrome banding. *The Journal of Heredity* **89**, 83-86
Schweizer D (1976) Reverse fluorescent chromosome banding with chromomycin and DAPI. *Chromosoma* **58**, 307-324
Swingle WT (1943) The botany of *Citrus* and its wild relatives in the orange subfamily. In: Webber HJ, Batchelor LD (Eds) *The Citrus Industry* (Vol 1), University of California, Berkeley, pp 128-474
Tachikawa T, Tanaka Y, Hara S (1961) Investigations on the breeding of citrus trees. Part I. Study on the breeding of triploid citrus varieties. *The Bulletin of Citrus Experimental Station of Shizuoka Prefecture* **4**, 33-46
Tanaka T (1954) *Species Problems in Citrus*, Society for Promotion of Science, Tokyo, 152 pp
Tanaka T (1977) Fundamental discussion of citrus classification. *Studia Citrologica* **14**, 1-6
Toolapong P, Komatsu H, Iwamasa M (1996) Triploids and haploid progenies derived from small seeds of 'Banpeiyu', a pummelo, crossed with 'Ruby red' grapefruit. *Journal of the Japanese Society for Horticultural Science* **65**, 255-260
Torres AM, Soost RK, Deidenhofen U (1978) Leaf isozyme as genetic markers in citrus. *American Journal of Botany* **65**, 869-881
Yahata M, Kunitake H, Yabuya T, Yamashita K, Kashihara Y, Komatsu H (2005) Production of a doubled haploid pummelo using colchicine treatment of axillary shoot buds. *Journal of the American Society for Horticultural Science* **130**, 899-903
Yamamoto M, Asadi Abkenar A, Matsumoto R, Nesumi H, Yoshida T, Kuniga T, Kubo T, Tominaga S (2007) CMA banding patterns of chromosomes in major *Citrus* species. *Journal of the Japanese Society for Horticultural Science* **76**, 36-40
Yamamoto M, Haji T, Yamaguchi M, Yaegaki H, Sanada T, Kudo K, Mase N (1999) Fluorescent banding pattern of peach (*Prunus persica* (L.) Batsch) chromosomes. *Journal of the Japanese Society for Horticultural Science* **68**, 471-475
Yamamoto M, Kobayashi S, Nakamura Y, Yamada Y (1993) Phylogenic relationships of citrus revealed by RFLP analysis of mitochondrial and chloroplast DNA. *Japanese Journal of Breeding* **43**, 355-365
Yamamoto M, Kubo T, Tominaga S (2005) CMA banding patterns of chromosome of mid- and late-maturing citrus and acid citrus growing in Japan. *Journal of the Japanese Society for Horticultural Science* **74**, 476-478
Yamamoto M, Tominaga S (2003) High chromosomal variability of mandarin (*Citrus* spp.) revealed by CMA banding. *Euphytica* **129**, 267-274
Yamamoto M, Tominaga S (2004a) Chromosome identification in haploid Clementine (*Citrus clementrina* hort. ex Tanaka) by fluorescent staining. *Scientia Horticulturae* **101**, 201-206
Yamamoto M, Tominaga S (2004b) CMA banding pattern of chromosomes is useful for the identification of chromosome doubling in haploid citrus. *Breeding Science* **54**, 351-354
Yang X, Kitajima A, Hasegawa K (2002) Chromosome pairing set and the presence of unreduced gamates explain the possible origin of polyploidy progenies from the diploids 'Tosa-Buntan' X 'Suisho-Buntan' pummelo. *Journal of the Japanese Society for Horticultural Science* **71**, 538-543

JAPANESE ABSTRACT

染色体は遺伝子の担体であり、その知見を深めることは、遺伝および育種研究に不可欠である。本総説ではグアニンーシトシンに特異的な蛍光色素であるクロモマイシンA_3（CMA）染色によるカンキツ染色体研究の進展について紹介する。カンキツ染色体をCMA染色すると、種特有のバンドパターン（CMAバンドパターン）が得られる。このバンドパターンは類縁関係と密接に関与しており、本法によってカンキツの進化・分類に関する新知見が得られている。CMAバンドパターンは再現性が非常に高いので、各染色体のマーカーともなり、小型で相互に形態的に似通っているカンキツ染色体の核型分析にも有効である。CMAバンドパターンによって染色体構成を明らかにすることが可能であり、これは特に倍数性育種を進める上での強力な手法の一つになっている。