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CMA Banding Patterns of Chromosomes in Major *Citrus* Species

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Fluorochrome staining with chromomycin A_3 (CMA) was used to characterize and compare the CMA banding patterns of chromosomes in 14 accessions of 12 species of major *Citrus* species. All accessions had 2n = 18 chromosomes. These chromosomes were classified into seven types based on the number and 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, and Dst: type D with a satellite chromosome. Each accession possessed two to six types of chromosomes and unique CMA banding patterns. The CMA banding patterns were 2B+8D+8E in *C. medica*, 1B+1C+8D+8E in *C. limon*, 2B+9D+7E in *C. aurantifolia*, 1A+1B+1C+7D+8E in *C. aurantium*, 2B+2C+7D+7E in *C. sinensis*, 3A+3C+4D+8E in *C. maxima*, 2A+3C+6D+7E in *C. paradisi*, 2B+2C+12D+2E in *C. ichangensis*, 2A+5C+8D+3E in *C. latipes*, 1B+11D+4E+2Dst in *C. micrantha*, 2B+1C+11D+3E+1F in *C. macroptera*, and 3B+1C+8D+3E+2F+1Dst in *C. hystrix*.

Key Words: chromomycin A_3 (CMA), chromosome, citrus, karyotype, papeda.

Introduction

Citrus is a major fruit crop with a complex taxonomy; Swingle (1943) identified 16 species, and Tanaka (1977) proposed 162 species. Recent phylogenic studies revealed that *C. medica* (citron), *C. maxima* (pummelo), and *C. reticulata* (mandarin) are the basic species of *Citrus*. Other species such as *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), and *C. limon* (lemon) are of hybrid origin (Barrett and Rhodes, 1976; Handa and Oogaki, 1985; Handa et al., 1986; Nicolosi et al., 2000). In addition, papedas, non-edible citrus, have played an important role in the development of edible *Citrus* species (Federici et al., 1998; Hirai and Kajiura, 1987; Nicolosi et al., 2000).

Chromosome analysis using guanine-cytosine (GC) specific fluorochrome chromomycin A_3 (CMA) has been found to be useful for determining the phylogenic relationships of citrus (Befu et al., 2000, 2001, 2002; Carvalho et al., 2005; Cornelio et al., 2003; Guerra, 1993; Miranda et al., 1997; Yamamoto and Tominaga, 2003; Yamamoto et al., 2005). These studies demonstrated the existence of characteristic CMA banding patterns with a high level of diversity and heterozygosity in citrus chromosomes. The results also revealed CMA banding patterns of important species, such as *C. sinensis*, *C. reticulata*, *C. paradisi*, *C. maxima*, and *C. medica*, and similar patterns among related species and cultivars.

However, CMA banding analysis of papedas has not been reported, and their patterns have not been clarified. In this study, we clarified the variability of CMA chromosome banding patterns in various *Citrus* species including papedas and discuss the phylogenic relationships.

Materials and Methods

In this study, 14 accessions belonging to 12 species of Swingle systematics (Swingle, 1943) were used (Table 1). Although Swingle (1943) identified 16 species, *C. indica* and *C. celebica* could not be obtained. The results of *C. reticulata* and *C. tachibana* have been already reported (Yamamoto and Tominaga, 2003).

The *Citrus* species used in this study were preserved at Kagoshima University, Saga University, and the Department of Citrus Reserach, National Institute of Fruit Tree Science. In principle, roots of young seedlings and young leaves from adult trees were used as materials from

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Table 1. *Citrus* species used in this study.

Latin name	Common or accession name	Embryony	Material[z]	No. of seedlings examined	Source[y]
Subgenus *Citrus*					
Citrus medica L. var. ethrog Engl.	Etrog citron	Mono	R	9	CO
C. medica L. var. *sarcodactylis* (Hoola van Nooten) Swingle	Fingered citron	Mono	L	—	KU
C. limon (L.) Burm. f.	Allen Eureka (lemon)	Poly	L	—	CO
C. aurantifolia (Christm.) Swingle	Mexican lime	Poly	L	—	KU
C. aurantium L.	Common sour orange	Poly	R	6	CO
	Bouquet	Poly	R	6	CO
C. sinensis (L.) Osbeck	Comuna (sweet orange)	Poly	R	4	CK
C. maxima (Burm.) Merr.	Hayasaki (pummelo)	Mono	L	—	KU
C. paradisi Macfad.	Marsh (grapefruit)	Poly	L	—	KU
Subgenus *Papeda*					
C. ichangensis Swingle	Ichang papeda	Mono	L	—	SU
C. latipes (Swingle) Tanaka	Khasi papeda	Mono	L	—	KU
C. micrantha Wester	Biasong	Mono	L	—	SU
C. macroptera Montr.	Melanesian papeda	Mono	L	—	KU
C. hystrix DC.	Purutt	Mono	L	—	KU

[z] L: Young leaves of adult trees. R: Root tips of seedlings.
[y] CO: Department of Citrus Research Okitsu, NIFTS, KU: Kagoshima University, CK: Department of Citrus Research Kuchinotsu, NIFTS, SU: Saga University.

polyembryonic species and a monoembryonic one, respectively. However, young leaves were used in polyembryonic *C. limon*, *C. aurantium*, and *C. paradisi* because of the low embryo number of *C. limon* and *C. aurantium* and low seed number of *C. paradisi*. Both roots of young seedlings and young leaves from adult trees were used as materials from *C. medica*. In the polyembryonic species and *C. medica* Etrog, seeds were collected from open-pollinated fruits. Young leaves about 3–5 mm long from adult trees were used in *C. limon*, *C. aurantium*, *C. paradisi*, and all monoembryonic species. Seeds were germinated in Petri dishes at 25°C in the dark. Roots tips about 1 cm long 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. Nucellar and zygotic seedlings were not distinguished before the observation of chromosomes.

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 macerated in an enzyme mixture containing 1 or 2% Cellulase Onozuka RS, 0.75 or 1.5% Macerozyme R200 (Yakult, Japan), 0.15 or 0.3% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd, Japan), and 1 mM EDTA, pH 4.2, at 37°C for 45–60 min.

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 position on the slide glass, the chromosomes were de-stained with 70% methanol.

Chromosomes were also stained with 0.1 g·L^{-1} CMA according to Hizume (1991), and observed under a fluorescence microscope with a BV filter cassette.

Results

All accessions had 2n = 18 chromosomes, and no variation in CMA banding patterns was found within the seedlings of any species. Chromosomes were classified into the following seven types based on the number and position of CMA-positive bands (Befu et al., 2000; Miranda et al., 1997; Yamamoto and Tominaga, 2003): 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 (Fig. 1). The *Citrus* species used in this study exhibited a high

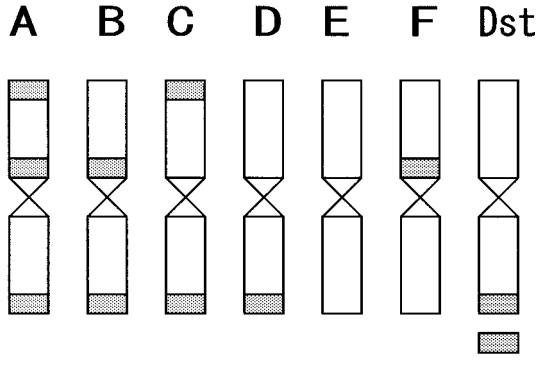

Fig. 1. Schematic representation of chromosome types in citrus according to the position of CMA-positive bands. A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, F: one proximal band, Dst: type D with a satellite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

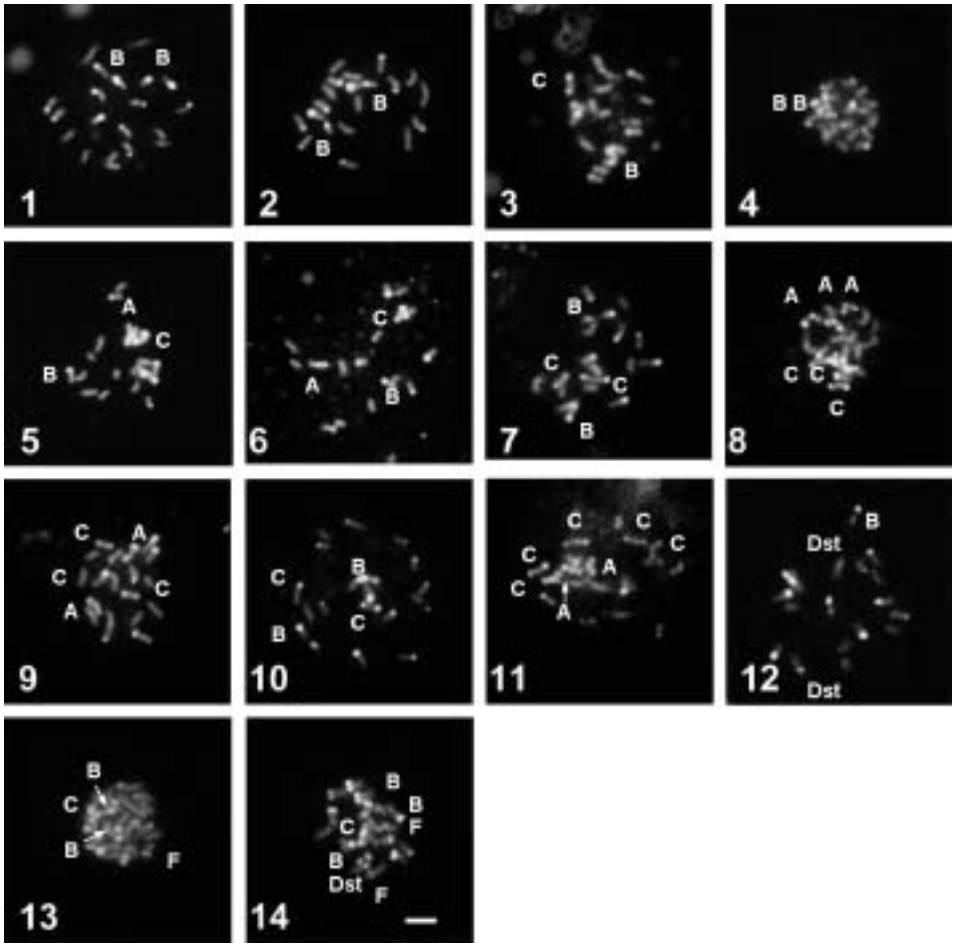

Fig. 2. CMA staining of somatic chromosomes in *Citrus* species. 1: *C. medica* Etrog, 2: *C. medica* Fingered citron, 3: *C. limon* Allen Eureca, 4: *C. aurantifolia* Mexican lime, 5: *C. aurantium* Common sour orange, 6: *C. aurantium* Bouquet, 7: *C. sinensis* Comuna, 8: *C. maxima* Hayasaki, 9: *C. paradisi* Marsh, 10: *C. ichangensis*, 11: *C. latipes*, 12: *C. micrantha*, 13: *C. macroptera*, 14: *C. hystrix*. A, B, C, F, and Dst: See Figure 1. Bar in 14 represents 5 μm for all figures.

Table 2. CMA banding patterns of somatic chromosomes of *Citrus* species.

Genotype	CMA banding pattern[z]
Citrus medica Etrog	2B + 8D+8E
C. medica Fingered citron	2B + 8D+8E
C. limon Allen Eureca	1B+1C+ 8D+8E
C. aurantifolia Mexican lime	2B + 9D+7E
C. aurantium Common sour orange	1A+1B+1C+ 7D+8E
C. aurantium Bouquet	1A+1B+1C+ 7D+8E
C. sinensis Comuna	2B+2C+ 7D+7E
C. maxima Hayasaki	3A +3C+ 4D+8E
C. paradisi Marsh	2A +3C+ 6D+7E
C. icahngensis	2B+2C+12D+2E
C. latipes	2A +5C+ 8D+3E
C. micrantha	1B +11D+4E +2Dst
C. macroptera	2B+1C+11D+3E+1F
C. hystrix	3B+1C+ 8D+3E+2F+1Dst

[z] 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.

chromosomal variability with characteristic banding patterns (Fig. 2).

The CMA banding patterns of subgenus *Citrus* were 2B+8D+8E in *C. medica*, 1B+1C+8D+8E in *C. limon*, 2B+9D+7E in *C. aurantifolia*, 1A+1B+1C+7D+8E in *C. aurantium*, 2B+2C+7D+7E in *C. sinensis*, 3A+3C+4D+8E in *C. maxima*, and 2A+3C+6D+7E in *C. paradisi*. Two accessions belonging to *C. medica* and *C. aurantium* exhibited identical banding patterns. The CMA banding patterns of the subgenus *Papeda* were 2B+2C+12D+2E in *C. ichangensis*, 2A+5C+8D+3E in *C. latipes*, 1B+11D+4E+2Dst in *C. micrantha*, 2B+1C+11D+3E+1F in *C. macroptera*, and 3B+1C+8D+3E+2F+1Dst in *C. hystrix* (Fig. 2 and Table 2).

Type D and E chromosomes were predominant in all species investigated. In contrast, type A and F chromosomes were observed in only four and two of the species, respectively. In particular, *C. micrantha* and *C. hystrix* of the subgenus *Papeda* possessed type Dst chromosomes. Nine species carried type B and C chromosomes.

Discussion

Numerical taxonomic studies (Barrett and Rhodes, 1976; Handa and Oogaki, 1985), biochemical studies (Handa et al., 1986) and DNA analysis (Nicolosi et al., 2000; Yamamoto et al., 1993) revealed that *C. medica*, *C. maxima*, and *C. reticulata* are the basic species of the subgenus *Citrus*. Other species, such as *C. sinensis*, *C. paradis*, and *C. limon*, are of hybrid origin.

In our results with *C. medica*, the chromosome configuration of both accessions was 2B+8D+8E and agreed with the results of Befu et al. (2001) and Carvalho et al. (2005). Although interindividual variation in the CMA banding pattern was usually found in seedlings derived from monoembryonic accession (Miranda et al., 1997), the CMA banding pattern of all seedlings was identical in *C. medica* Etrog. This might indicate that each homologous chromosome had the same CMA-positive band of *C. medica* in contrast to many *Citrus* species of which some homologous chromosomes did not exhibit the same CMA-positive band (Befu et al., 2000, 2001; Guerra, 1993; Miranda et al., 1997; Yamamoto and Tominaga, 2003; Yamamoto et al., 2005). It is considered that this result reveals the non-hybrid origin of *C. medica*. Large total numbers of type A, B, and C chromosomes have been considered a characteristic CMA configuration in *C. maxima* (Befu et al., 2001; Guerra, 1993; Miranda et al., 1997). The present results of *C. maxima* 'Hayasaki' were in agreement with those previous studies; three types of A and C chromosomes were observed, though it did not possess the type B chromosome. The same results were observed in the chromosome configuration of *C. maxima* 'Suisho Buntan' (Befu et al., 2001) and 'Shadenyu' (Miranda et al., 1997). There was wide variation in the CMA configuration of *C. reticulata*, but numbers of type A, B, and C chromosomes were generally lower (Yamamoto and Tominaga, 2003). 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. *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* (Yamamoto and Tominaga, 2003).

The ancestors of species considered to be of hybrid origin according to isozyme and DNA analyses (Gulsen and Roose, 2001; Hirai and Kajiura, 1987; Nicolosi et al., 2000) seem to be as follows: *C. limon*: *C. aurantium* and *C. medica*, *C. aurantifolia*: *C. micrantha* and *C. medica*, *C. aurantium* and *C. sinensis*: *C. maxima* and *C. reticulata*, and *C. paradisi*: *C. maxima* 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 chromosome of *C. aurantium* and large numbers of type A and C chromosomes of *C. paradisi* were considered to derive 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* (Yamamoto and Tominaga, 2003) 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 chromosome configurations suggested that *C. micrantha*, *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 RAPD, SCAR, and cpDNA 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 RFLP, RAPD, and SCAR analyses (Federici et al., 1998), its cpDNA was close to *C. reticulata* (Nicolosi et al., 2000). Hirai and Kajiura (1987) suggested the similarity of isozyme banding patterns between *C. ichangensis* and Yuzu, *C. ichangensis*

hybrid according to Swingle's system (Swingle, 1943), or *C. junos* according to Tanaka's system (Tanaka, 1977). The present result of *C. ichangensis* was 2B+2C+12D+2E. Yuzu had a 2B + 1C + 11D + 4E chromosome configuration (Yamamoto et al., 2005). Resemblance was found in the CMA banding patterns of *C. ichangensis* and Yuzu; numbers of type B, C, D, and E chromosomes are quite similar. *C. ichangensis* may therefore be very closely related to Yuzu.

The CMA banding patterns of all species belonging to the subgenus *Papeda* were reported for the first time in this study. Those of the remaining species have been previously reported. The CMA banding pattern of *C. medica* and *C. sinensis* obtained in our study is identical to the results of previous studies (Befu et al., 2000, 2001; Carvalho et al., 2005; Miranda et al., 1997; Pedrosa et al., 2000). This indicates a high reproducibility of chromosome analysis using the CMA staining method. CMA banding patterns of other species were not identical but were similar to those of previous studies (Befu et al., 2001, 2002; Cornelio et al., 2003; Guerra, 1993; Miranda et al., 1997). The differences may result from using different accessions in the present and previous studies.

This study demonstrated the variability and heterozygosity in *Citrus* chromosomes. Moreover, the CMA staining method can be used as a cytogenetical characterization of *Citrus* species because every species exhibited a characteristic CMA banding pattern. The CMA staining analysis of various *Citrus* relatives would clarify the phylogenic relationships.

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