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CMA Staining Analysis of Chromosomes in *Citrus* Relatives, *Clymenia*, *Eremocitrus* and *Microcitrus*

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Fluorochrome staining with chromomycin A_3 (CMA) was used to characterize and compare the CMA banding patterns of chromosomes of *Clymenia*, *Eremocitrus*, and *Microcitrus*, which belong to true citrus fruit trees (*Citrinae*, Rutaceae). All species had 2n=18 chromosomes. These chromosomes were classified into six 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, and Dst: type D with a satellite chromosome. Each species possessed three or four types of chromosomes and unique CMA banding patterns. The CMA banding patterns were 2C+8D+8E in *Cl. polyandra*, 2C+9D+7E in *E. glauca*, 1C+11D+6E in *M. australis*, 1B+2C+10D+5E in *M. australasica*, 8C+7D+2E+1Dst in *M. inodora*, 2A+14D+2Dst in *M. warburgiana*, and 2C+9D+7E in Sydney hybrid. Chromosome configurations of *Cl. polyandra*, *E. glauca*, *M. australis*, and Sydney hybrid resembled each other. This may indicate a common ground of chromosome configuration in the true citrus fruit trees. On the other hand, variability in *Microcitrus* chromosomes was also demonstrated.

Key Words: chromomycin A_3, karyotype, phylogenic relationship, true citrus fruit trees.

Introduction

The true citrus fruit trees group of the subtribe *Citrinae* (Rutaceae) includes six genera, i.e., *Citrus*, *Fortunella*, *Poncirus*, *Clymenia*, *Eremocitrus*, and *Microcitrus*. The first four genera and the last distributed mainly in Asia and Australia, respectively. Despite the wide distribution of these genera, cross compatibility of several combinations within this group has been reported (Barrett, 1985; Iwamasa et al., 1988). Not only is *Citrus* one of the most important fruit trees of the world, but the other five genera are also important for industry because of the characteristics of the fruits as well as the utilization as rootstock and resistance to biotic and abiotic stresses. Phylogenic relationships of these genera have been elucidated by isozyme (Herrero et al., 1996; Rahman and Nito, 1994a, b; Rahman et al., 1994), Fraction I protein (Handa et al., 1986), and DNA (Asadi Abkenar et al., 2004a; Federici et al., 1998; Nicolosi et al., 2000) analyses.

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*, *Poncirus*, and *Fortunella* (Befu et al., 2000, 2001, 2002; Carvalho et al., 2005; Cornelio et al., 2003; Guerra, 1993; Kunitake et al., 2005; Miranda et al., 1997; Yamamoto and Tominaga, 2003; Yamamoto et al., 2005, 2007). These studies demonstrated the existence of characteristic CMA banding patterns with a high level of diversity and heterozygosity in the chromosomes of the above genera. The results also demonstrated CMA banding patterns of important species, which provide useful information on phylogenic relationships among these genera and species; however, CMA banding analysis of the other three genera has not progressed, although Guerra et al. (2000) demonstrated CMA banding patterns of one species each of *Eremocitrus* and *Microcitrus*.

In this study, we clarified the variability of CMA

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chromosome banding patterns in several species belonging to *Clymenia*, *Eremocitrus*, and *Microcitrus* and discussed their phylogenic relationships.

Materials and Methods

In this study, *Clymenia polyandra*, *Eremocitrus glauca*, *Microcitrus australis*, *M. australasica*, *M. inodora*, *M. warburgiana*, and Sydney hybrid (*M. australis* × *M. australasica*) were used (Table 1). The materials used in this study were conserved at the Faculty of Agriculture, Saga University, Japan.

Young leaves about 3–5 mm long from adult trees were used as materials. 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 young leaves were washed in distilled water to remove the fixative and macerated in an enzyme mixture containing 2% Cellulase Onozuka RS, 1.5% Macerozyme R200 (Yakult, Japan), 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 $g \cdot L^{-1}$ CMA according to Hizume (1991), and observed under a fluorescence microscope with a BV filter cassette.

Results and Discussion

All materials had $2n = 18$ chromosomes. Chromosomes were classified into the following six types based on the number and position of CMA-positive bands (Befu et al., 2000; Miranda et al., 1997; 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 and Dst: type D with a satellite chromosome (Fig. 1). Each species examined in this study exhibited high chromosomal variability with characteristic banding patterns. A light CMA-positive band was observed in the satellite position of five chromosomes in *M. warburgiana* (Fig. 2). That type of chromosome was included as type D in this study.

The CMA banding patterns were 2C + 8D + 8E in *Cl. polyandra* and 2C + 9D + 7E in *E. glauca*. In species belonged to *Microcitrus*, 1C + 11D + 6E in *M. australis*, 1B + 2C + 10D + 5E in *M. australasica*, 8C + 7D + 2E + 1Dst in *M. inodora*, 2A + 14D + 2Dst in *M. warburgiana*, and 2C + 9D + 7E in Sydney hybrid (Fig. 2, Table 1).

E. glauca and the Sydney hybrid showed identical CMA banding patterns. Chromosome configurations of *Cl. polyandra*, *M. australis* and the aforementioned two species (accessions) resembled each other; the numbers of types C, D, and E chromosomes were similar. *M. australasica* possessed types B, C, D, and E chromosomes. All these chromosomes are observed commonly in *Citrus*, and types D and E chromosomes are also predominant in *Citrus* and *Poncirus* (Befu et al., 2000, 2001; Carvalho et al., 2005; Cornelio et al., 2003; Guerra, 1993; Miranda et al., 1997; Yamamoto and Tominaga, 2003; Yamamoto et al., 2005, 2007). However, the chromosome configuration of *M. inodora*

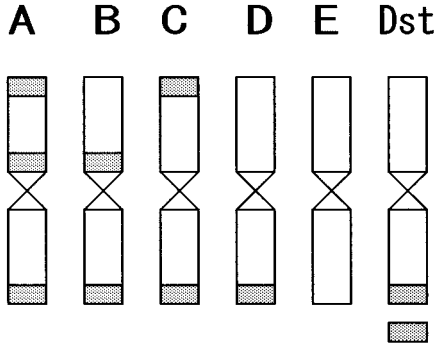

Fig. 1. Schematic representation of chromosome types according to the position of CMA-positive bands. A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands, Dst: type D with a satellite chromosome. The gray regions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

Table 1. Species belonging to the true citrus fruit trees used in this study and their CMA banding patterns of somatic chromosomes.

Latin name	Distribution	CMA banding pattern[z]
Clymenia polyandra (Tan.) Swing.	Bismarc Archipelago	2C + 8D + 8E
Eremocitrus glauca (Lindl.) Swing.	Australia	2C + 9D + 7E
Microcitrus australis (Planch.) Swing.	Australia	1C + 11D + 6E
M. australasica (F. Muell.) Swing.	Australia	1B + 2C + 10D + 5E
M. inodora (F. M. Bail.) Swing.	Australia	8C + 7D + 2E + 1Dst
M. warburgiana (F. M. Bail.) Swing.	South eastern New Guinea	2A + 14D + 2Dst
Sydney hybrid (*M. australis* × *M. australasica*)		2C + 9D + 7E

[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, Dst: type D with a satellite chromosome.

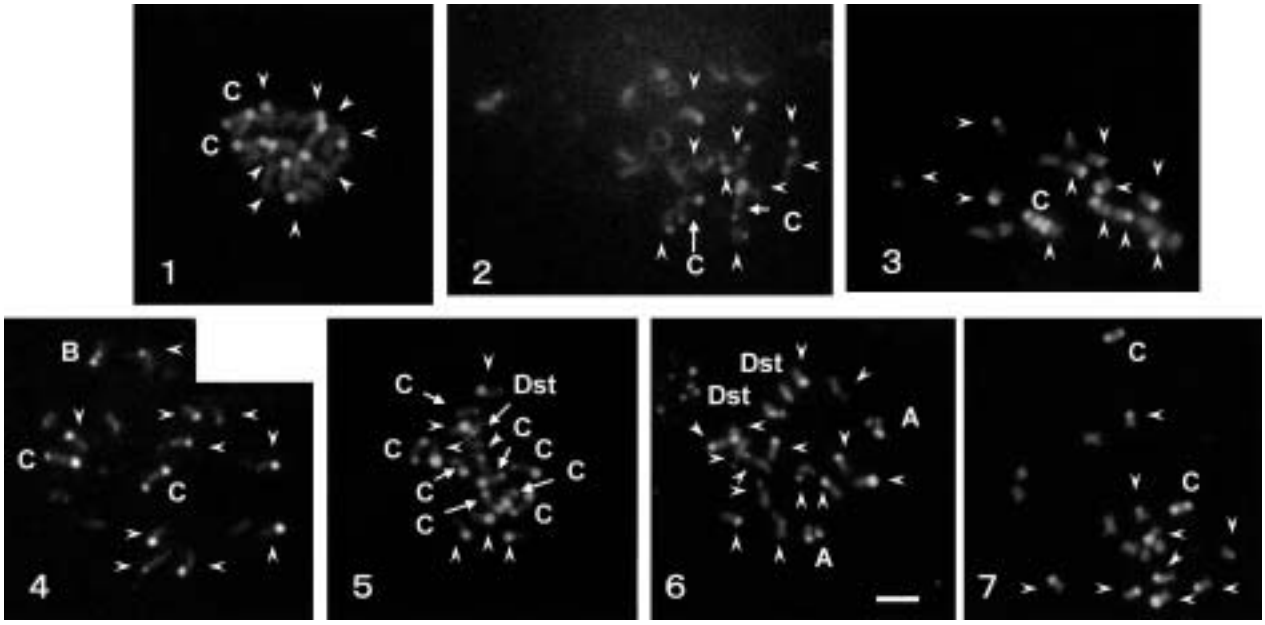

Fig. 2. CMA staining of somatic chromosomes in *Clymenia*, *Eremocitrus* and *Microcitrus*. 1: *Cl. polyandra*, 2: *E. glauca*, 3: *M. australis*, 4: *M. australasica*, 5: *M. inodora*, 6: *M. warburgiana* and 7: Sydney hybrid. Arrowhead indicates type D chromosome. A, B, C, D, and Dst: See Figure 1. Bar in 6 represents 5 μm for all figures.

and *M. warburgiana* did not fall into the same category as *Citrus*, *Poncirus*, and *Fortunella*. *M. warburgiana* possessed five characteristic chromosomes which had a light CMA-positive band in the satellite position. *M. inodora* possessed eight type C chromosomes, the largest number in true citrus fruit trees.

Previous studies (Asadi Abkenar et al., 2004a; Katayama et al., 1994; Rahman and Nito, 1994a) reported genetic diversity among species of *Microcitrus*. The present study also demonstrated the variability of chromosome configuration of *Microcitrus*. Since *M. warburgiana* is the only species of the genus distributed outside of Australia, the characteristics of the fruits and leaves are distinct from those of other species of *Microcitrus* (Swingle and Reece, 1967). *M. inodora* is considered to be adapted to tropical rain forests (Swingle and Reece, 1967) and clearly distinguished from other *Microcitrus* species by the composition of essential oil in the mature leaves (Katayama et al., 1994). The present findings agree with those concepts and previous findings because the chromosome configurations of those two species are distinct from those of other species of *Microcitrus* and each other. Resemblance was found in the CMA banding patterns of *M. australis* and *M. australasica*, although the latter possessed type B chromosome and the former did not; however, these two species were not considered very similar to each other based on morphological traits (Swingle and Reece, 1967) and DNA analysis (Asadi Abkenar et al., 2004a). Differentiation of the *Microcitrus* species is the result of millions of years of slow evolution from the primitive ancestral type (Swingle and Recce, 1967). The chromosome configuration of *M. australis* and *M. australasica* may remain that of the ancestral type. The Sydney hybrid is considered to be a hybrid between *M. australis* and *M. australasica* (Swingle and Reece, 1967), and its female parent is probably *M. australis* (Asadi Abkenar et al., 2004b). The chromosome configuration of the Sydney hybrid resembled those of *M. australis* and *M. australasica*.

The chromosome configuration of *Clymenia polyandra* (2C + 8D + 8E), considered the most primitive of all the genera of true citrus fruit trees, and *Eremocitrus glauca* (2C + 9D + 7E), a genus very similar to *Microcitrus* (Swingle and Reece, 1967), was quite similar. Moreover, these chromosome configurations resembled that of *M. australis* (1C + 11D + 6E). *Citrus medica* is considered a primitive type of *Citrus* (Handa et al., 1986) and showed a 2B + 8D + 8E CMA banding pattern (Befu et al., 2001; Yamamoto et al., 2007). The CMA banding pattern of *Poncirus trifoliata* was 2B + 10D + 6E (Miranda et al., 1997) and 4B + 8D + 6E (Befu et al., 2000). Although *Clymenia*, *Eremocitrus*, and *M. australis* possessed type C chromosome instead of type B chromosome in *C. medica* and *Poncirus*, their chromosome configurations were similar. These results may indicate the existence of an ancestral type of true citrus fruit trees. The chromosome configuration seems to have maintained the pattern of few type B or C chromosomes and predominant type D and E chromosomes.

The CMA banding patterns of *Cl. polyandra*, *M. australis*, *M. inodora*, *M. warburgiana*, and Sydney hybrid were reported for the first time in this study; those of *E. glauca* and *M. australasica* have been previously reported (Guerra et al., 2000). Although the

high reproducibility of CMA chromosome configuration is well known (Yamamoto, 2007), CMA banding patterns of these two species in the present study were not identical but were similar to those in studies by Guerra et al. (2000). There is variability within species at least in *M. australasica* (Swingle and Reece, 1967). These differences may result from using different accessions in the present and previous studies.

This study demonstrated the cytogenetical characterization of *Clymenia*, *Eremocitrus*, and *Microcitrus* species, because every species exhibited a characteristic CMA banding pattern. There seems to be a common ground of chromosome configuration in true citrus fruit trees; however, variability in *Microcitrus* chromosomes was also demonstrated. Further CMA staining analysis using more genera and species in the subfamily Aurantioideae should be conducted to clarify the phylogenic relationships among true citrus fruit trees and related genera.

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