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Karyotyping and Genetic Diversity Analysis of Korean Landrace Citrus

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(Supervised by Professor **Kwan Jeong Song**, Ph.D)

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy in Agriculture

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Abstract

Citrus is one of the most important fruit crops widely grown in the world. Cultivation of citrus in Jeju, Korea has a long history more than a millennium and citrus is the leading agricultural produce, in terms of the area and production, in Korea as well as Jeju. Landrace citrus species represent broad and natural genetic variability, which are important and valuable as genetic material. In this study, metaphase chromosomes of eleven Korean landrace citrus were analyzed to understand the phylogenetic relationship among them and to compare these characteristics with those of other *Citrus* species at a cytogenetic level using CMA banding patterns and rDNA loci. Chromosomes were categorized into six types according to the distribution and number of heterochromatic CMA-positive bands; type A chromosomes have two telomeric and one proximal bands, type B have one telomeric and one proximal bands, type C have two telomeric bands, type D have one telomeric band, type E have no band, and type F has one subtelomeric band. Gamza mandarin (*C. benikoji*) displayed the 1A/45S + 2B/45S + 2C + 5D + 1D/45S + 1D/5S-45S + 6E pattern, without a solitary 5S rDNA locus that differentiated the gamza mandarin from the other accessions. A solitary 5S rDNA locus was observed in the chromosomes of byungkyul mandarin (*C. platymamma*) (1A/45S + 2B/45S + 1C

+ 6D + 1D/45S + 1D/5S-45S + 5E + 1E/5S) and cheongkyul mandarin (*C. nippokoreana*) (1A/45S + 1B/45S + 1C + 7D + 1D/5S + 1D/45S + 1D/5S-45S + 5E). The chromosome composition of byungkyul mandarin based on CMA banding pattern and rDNA loci suggests that byungkyul mandarin may be related to pummelo, sweet orange, and members of the *Citrus* subgenus *Papeda* during its evolution. Cheongkyul mandarin possessed a distinct marker chromosome (D/45S) that can be used to distinguish cheongkyul madrarin from the other Korean landrace mandarins. Jinkyul mandarin (*C. sunki*) (1A/45S + 1B/45S + 1C + 10D + 2D/5S-45S + 3E) may be related to pummelos. The karyotype of pyunkyul mandarin (*C. tangerina*) (3B/45S + 2C + 7D + 1D/5S-45S + 5E) suggests that the pyunkyul mandarin is a hybrid between *C. grandis* and *C. reticulata*. Binkyul mandarin (*C. leiocarpa*) (1A/45S + 1C + 6D + 2D/45S + 2D/5S-45S + 6E) seemed to be related to mandarin and pummelo. Heterogeneous karyotypes of the six accessions separated and differentiated each of the six Korean landrace mandarins and potential marker chromosomes were identified. The CMA banding patterns of the rest Korean landrace citrus were 1A+2B+2C+6D+7E in dongjeongkyul (*C. erythrosa*), 3B+1C+7D+5E+2F in hongkyul (*C. tachibana*), 2A+1B+3C+4D+8E in sadoogam (*C. pseudogulgul*), 1A+3B+1C+7D+6E in dangyooza (*C. grandis*), 1A+1B+1C+9D+6E in jigak (*C. aurantium*). Type A

chromosome is absent in hongkyul, but two of type F chromosomes were observed. The numbers of type A, B, and C chromosomes were lower in all accessions. In contrast, the type D and E chromosomes were remarkably constant and predominantly observed in all accession. The distributions of 5S and 45S rDNA loci by FISH were heterogeneous among all accessions. All accessions possessed one D/5S-45S chromosome. All 45S rDNA loci were homotopic to CMA-positive regions. Every type A and B chromosomes possessed at least one 45S rDNA locus in the proximal region of the chromosomes. There was no type C chromosome with rDNA observed. The chromosome configurations of Korean landrace citrus analyzed here suggest that all accessions in this study are hybrids that have relationships more or less with mandarin and pummelo. This study provides high resolution of chromosome configurations, which could complement previous studies, and elucidated phylogenetic relationships of Korean landrace citrus at the cytogenetic level.

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Introduction

Citrus is an immensely important fruit crop for its juice and pulp, with more than 30% of production from the five most produced fruit crops in the world (FAO, 2014). None the more, citrus is the leading agricultural produce, in terms of the area and production, in Korea, especially in Jeju. It has a profound ripple effect throughout the economy as well as the community in Jeju.

The genus *Citrus* L. is evergreen shrubs or trees with small to medium size which belongs to the tribe Citreae within the subfamily Aurantioideae of the Rutaceae family. Studies on the history and geographical origin of *Citrus* say that the genus originated in the tropical and subtropical areas such as southeastern Asia, northeastern India, southern China, the Indochinese peninsula, and northern Australia, and then spread to other continents (Chapot, 1975; Webber, 1967; Harley et al. 2006). The relationships between the species within the genus *Citrus* is complicated and has remained ambiguous due to wide cross compatibility, a long history of cultivation, apomixes, and dispersion in the world (Scora, 1975; Nicolosi et al, 2000). According to the most widely accepted taxonomic systems proposed by Swingle and Reece (1967) and Tanaka (1977), *Citrus* can be classified into 16 or 162 species, respectively depending on taxonomic system is used (Nicolosi, 2007).

Citrus has long history of selection and vegetative propagation, which mainly focused on superior genotypes. Such breeding strategy has often resulted in the loss of wild types, landraces, or local cultivars. The genetic diversity within *Citrus* has been studied using different RAPD and SSR markers (Baig et al., 2009; El-Mouei et al., 2011; Nematollahi et al., 2013) and these studies have identified a narrow genetic base within mandarins (Machado et al., 1996; Coletta-Filho et al., 1998; El-Mouei et al., 2011). Based on these data, Ogwu et al. (2014) pointed out a significant threat of genetic erosion in *Citrus* and emphasized the importance of using landraces as valuable genetic material for modern plant breeding. In Korea, more than 20 landrace species or cultivars have been reported in old literatures (Kim, 1988). However, as of now only 12 species have been conserved in germplasm collections. To promote the utilization of landrace species and conserve their genetic lineage, it is essential and necessary to learn more about the genetic origins, their genetic characteristics, and phylogenic relationships of the various remaining Korean landrace citrus.

Most members of the genus *Citrus* are diploid ($2n$ = 18 chromosomes) and their chromosomes are similar in morphology and size (Guerra et al., 1997; Krug, 1943). Chromosome staining using guanine-cytosine (GC) specific fluorochrome

chromomycin A_3 (CMA) combined with DAPI, which has an affinity for adenine-thymine (AT), has revealed the existence of an interspecific banding pattern in *Citrus* species (Guerra, 1993). Karyotype analysis using CMA/DAPI banding patterns has been proven to be a very useful technique for cytogenetically characterizing *Citrus* species (Miranda et al., 1997a; Befu et al., 2000; Yamamoto and Tominaga 2003). Moreover, fluorescence *in situ* hybridization (FISH) has been applied to more detailed chromosomal studies. The combined CMA/DAPI banding pattern with the distribution of rDNA loci using the FISH technique has been used to distinguish the heterozygosity of some *Citrus* species (Moraes et al., 2007) and to clarify the phylogenetic relationship among some *Citrus* species (Carvalho et al., 2005; Brasileiro-Vidal et al., 2007).

As of now only few cytological and phylogenetic studies of Korean landrace citrus have been reported in the literature (, 2001; Kang et al., 2008; Jin et al., 2016). Therefore, this study was conducted to understand Korean landrace citrus at the cytogenetic level, to gain insight on the phylogenetic relationship among them, and compare these data with data from other *Citrus* species by karyotyping and identifying their chromosomes using CMA/DAPI banding patterns and FISH using 5S and 45S rDNA as probes.

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Literature Review

Taxonomy and Classification of the Genus *Citrus*

The taxonomic classification system of *Citrus* species and its related genera is very complicated and controversial, mainly due to high sexual compatibility, frequent bud mutation, the long cultivation history, and their dispersed habitat. Since the first description of *Citrus* species and varieties in the 17th century, Linneus introduced the genus *Citrus* for the first time in *Genera plantarum* in 1737 (Nicolosi, 2007) and later he added two species and three varieties; *C. medica* L. (the citron) with var. *limon* L. (the lemon) and *C. aurantium* L. (orange) with var. *grandis* L. (pummelo) and var. *sinensis* L. (sweet orange) (Mabberley, 1997). Pummelo was separated from *C. aurantium* L. as *C. grandis* L. and *C. limonoa* and *C. sinensis* were added by Linneus (1767) who collaborated with Osbeck. The following year, the lomon was raised to species as *C. limon* (Burmann, 1768). Decade later, *C. nobilis* (King mandarin), *C. madurensis* (calamondin), and *C. margarita* (presently known as *Fortunella margarita* Lour. (Swing.)) were discovered (De Loureiro, 1790). In 19th century, *C. hystrix* and *C. reticulata* were newly classified by De Candolle (1813) and Blanco (1837), respectively. Linneus and Osbeck classified the genus *Citrus* L. in the family Rutaceae, which consists of three subfamilies such as Rutoideae,

Aurantioideae, and Spathelioideae, with 155 genera (Thorne, 2000). Within the subfamily Aurantiodae, many tribes and subtribes were identified during the last century (Engler, 1931; Tanaka, 1936; Swingle, 1943; Swingle and Reece, 1967). The classification of genera and delimitation of species has been controversial and confusing. Of the many taxonomic systems of the genus *Citrus* elaborated in the past, the most conceded taxonomic systems were suggested by Swingle and Reece (1967) and Tanaka (1977). Swingle and Reece (1967) divided the genus *Citrus* into two subgenera, *Citrus* and *Papeda*, according to their edibility with total 16 species. While, Tanaka (1977) proposed more detailed classification with 162 species. Among them, pummelo (*C. maxima* L. Osbeck), citron (*C. medica* L.), and mandarin (*C. reticulata* Blanco) were defined as the true species of *Citrus* (Barrett and Rhodes, 1976; Scora, 1975, 1988). Other genotypes such as *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), and *C. limon* (lemon) derived from hybridization between the true species were referred to as hybrid origins (Barrett and Rhodes, 1976). In addition to morphological and geographic data that were used for the early studies on phylogenic relationships of the genus *Citrus*, various biochemical techniques have been employed for taxonomic studies. Some of biochemical components are as in the following. Long chain hydrocarbon profiles (Nagy and Nordby, 1972),

flavonoids (Tatum et al., 1974), rind oil (Malik et al., 1974), isozymes (Button et al, 1976), and fraction I protein (Handa et al., 1986). For the last decades, prodigiously progressed molecular techniques have been applied to disambiguate taxonomy of the genus *Citrus*. Previous taxonomy and classifications of the genus *Citrus* were confirmed or supplementary revised using molecular techniques, including DNA amplified fingerprinting (Luro et al., 1995), microsatellites (Fang and Roose, 1997), RFLP, RAPD, and SSR marker analysis (Asadi Abkenar et al., 2004; Federici et al., 1998; Nicolosi et al., 2000), and whole genome sequencing analysis using next generation sequencing (NGS) (Curk et al., 2014).

Cytogenetic Studies in *Citrus*

Karyotype analysis could provide such fundamental but valuable information by identifying particular genomic variants or detecting true hybrids (Guerra et al, 1997). Most members of the genus *Citrus* are diploids of 18 chromosomes (Guerra, 1984). Despite the small chromosome number ($2n$ = 18), the small chromosome sizes in metaphase (1.0-4.0 μm) and close morphological resemblances between chromosomes made the karyotype analysis difficult (Krug, 1943). Chromosomal analysis first have been used for polyploid studies (Nakamura, 1934; Krug and

Bacchi, 1943). Traditional ways of chromosome preparation is paraffin section or the squash method. The enzymatic maceration method was developed by Kurata and Omura (1978). With the aid of this method, Ito et al. (1992) could observe good *Citrus* chromosomes. An improved Karyotype analysis using guanine-cytosine (GC) specific fluorochrome chromomycin A_3 (CMA) combined with 4', 6-diamidino-2-phenylindole (DAPI), which has an affinity for adenine-thymine (AT), has been employed several decades ago and CMA/DAPI banding patterns in *Citrus* were identified (Guerra, 1993). Based on the distribution and number of the heterochromatic CMA positive bands, chromosomes were classified into seven types (Miranda et al., 1997; Befu et al., 2000; Yamamoto and Tominaga, 2003). Type A: two telomeric and one proximal bands, type B: one telomeric and one proximal bands, type C: two telomeric bands, type D: one telomeric band, type E: no band, type F: one proximal or subtelomeric band, and type Dst: type D with a satellite chromosome (Fig. 1). Such studies substantiated the existence of characteristic CMA+/DAPI- banding patterns with a high level of diversity and heterozygosity in *Citrus* chromosomes and have proven to be very useful for determining the phylogenic relationships of *Citrus* species. Type A and B chromosomes observed in mandarins are presumed to be derived from the lime-lemon-citron-pummelo (Guerra, 1993; Befu et al., 2001). Type C

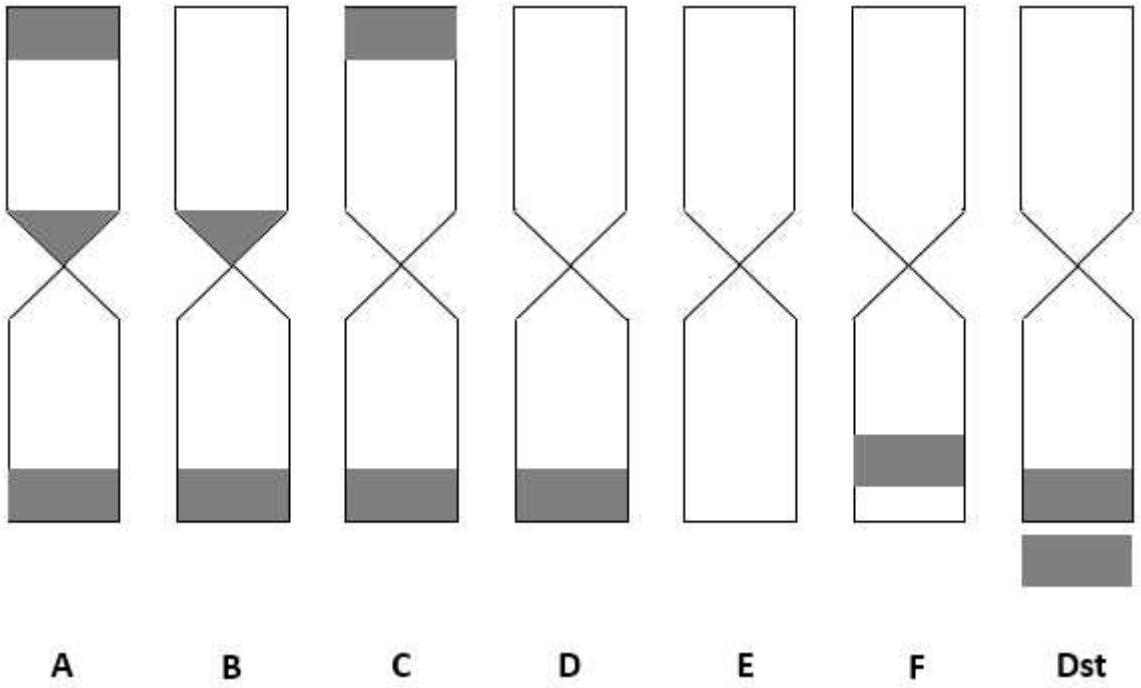

Fig. 1. Representative idiogram of *Citrus* chromosomes according to the number and distribution of CMA positive bands. A: two telomeric and one proximal bands, B: one telomeric and one proximal bands, C: two telomeric bands, D: one telomeric band, E: no band, F: one proximal or subtelomeric band, and Dst: type D with a satellite chromosome. Adapted from Yamamoto and Tominaga (2003).

chromosome may be a characteristic chromosome type in the mandarin karyotype (Cornélio et al., 2003). It is thought that type A and B chromosomes arose from *C. maxima* and *C. medica*, respectively. Type D and E chromosomes are considered as the basic chromosome types in *Citrus* (Befu et al., 2001). Type F chromosomes are found only in *C. tachibana* and some mandarins originating in Japan (Yamamoto and Tominaga, 2003). The subgroup *papeda* is the only group that possessed type Dst chromosome. And it is presumed that this type of chromosome arose exclusively from *papeda* (Yamamoto et al., 2007). Based on the karyotype analysis of almost 100 *Citrus* species, it is proposed that homozygous CMA positive banding pattern represents non-hybrid origins or true species, whereas hybrid species possess heteromorphic CMA banding patterns (Guerra et al., 2000; Carvalho et al., 2005; Brasileiro-Vidal et al., 2007; Moraes et al., 2007). Fluorescence *in situ* hybridization (FISH), which localizes any specific gene in native context of chromosomes is a very powerful tool for elucidating cyto-evolutionary events in *Citrus*. Gall and Pardue (1969) succeeded hybridizing radiolabeled RNA with DNA *in situ*. Non-radioactive probes labelled directly with fluorochromes, such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) were developed (Bauman et al., 1980). Cytogenetic studies using FISH technique have been conducted in

Citrus. Heterochromatic regions and TGG repeated-sequences in 'Trovia' orange (*C. sinensis* Osb.) were characterized and detected (Matsuyama et al., 1996, 1999). Miranda et al. (1997b) and Roose et al. (1998) also localized rDNA loci in relation to heterochromatic regions using FISH in *Citrus*. Phylogenetic relationships were elucidated based on karyotype analysis using FISH combined with CMA banding pattern in some *Citrus* species (Pedrosa et al., 2000; Carvalho et al., 2005; Brasileiro-Vidal et al., 2007; Moraes et al., 2007;). However, there have been few cytogenetic studies on Korean landrace citrus conducted so far.

Studies on Korean Landrace Citrus

More than 20 landrace species or cultivars had been reported in old literature (Kim et al., 2001; Moon et al., 2007). But, as of now, only twelve cultivars are preserved as genetic resources (Kim et al., 2001). The old literatures documented that *Citrus* in Jeju island were grown wildly since prehistoric times, but a definite origin of Korean landrace citrus still remains unknown (Jung et al., 2005). From ancient times, the Korean landrace citrus have been used as medical herbs, which was referred to dried-orange-peel (Kim et al., 1979). Lee et al. (2005) reported multidrug-resistance reversing activity of the Korean landrace citrus, especially in

dongjeongkyul, byungkyul, cheongkyul, and hongkyul, which contained chemosensitivity that potentiated vincristine cytotoxic effect in drug-resistant cancer cells. Quantitative analysis of flavonoids extracted from the peel (Kim et al., 2001) and juice (Kim et al., 2009) of Korean landrace citrus during maturation revealed that flavonoid contents are abundant mostly in dangyooza, hongkyul, jigak, and pyunkyul among Korean landrace citrus. The studies indicated that flavonoid contents were the highest at the early maturation and decreased rapidly during ripening. Antioxidant activity (Lim et al., 2006; Kim et al., 2009; Yu et al., 2014; Hyun et al., 2015) and anti-inflammatory effect (Yang et al., 2009; Shin et al., 2011; Kim et al., 2012; Hyun et al., 2015) of Korean landrace citrus extracts from the peel or juice was reported. Kim et al. (2009a) reported that the polyphenolic contents are rich in jigak, which has reactive oxygen species (ROS) scavenging activity. The investigation of anti-metastasis effect of flavonoids extracted from jigak showed that cancer cells in NOD/SCID mice were inhibited metastasis and induced apoptosis (Park et al., 2014). Similar effects of flavonoids from jinkyul were reported to include ROS scavenging activity (Kang et al., 2005), antiobesity effect (Kang et al., 2012), and protective effect against CoCl2 induced neuronal injury (Ko and Lee, 2015).

Availability of Korean landrace citrus as a citrus rootstock was investigated.

Dangyooza and jinkyul showed dwarf effect on scion growth whereas hongkyul and binkyul are invigorous compared to trifoliate orange trees (Koh et al., 2013). Moon et al. (2014) also ascertained that hongkyul might be a suitable citrus rootstock.

Taxonomic relation of Korean landrace citrus was studied based on their morphological characters, enzymatic browning, and coagulation of young shoot homogenates (Kim, 1988). The study indicated that the byungkyul could be easily distinguished from other Korean landrace citrus because it has a unique collared fruit shape. Jung et al. (2005) investigated the phylogenetic relationships within Korea landrace citrus and divided them into 8 different clusters based on plastid *trn*L-*trn*F sequences analysis. DNA polymorphism by random amplified polymorphic DNA (RAPD) revealed that gamza is phylogenetically distinct from the other Korean landrace citrus (Oh et al., 1996). Sequence analysis of the internal transcribed spacer (ITS) region of nuclear ribosomal DNA was consistent with the previous studies by Oh et al. (1996) (Sun et al., 2015; Jin et al., 2016).

As of now, there have been reported few cytogenetic studies on *Citrus* in Korea. Since the chromosome preparation method using root tip for counting *Citrus* chromosomes was reported by Kim et al. (1994), chromosome counting was

employed to verify ploidy levels of some *Citrus* in Korea (Lee et al., 2008; Song et al., 2011). However, up to now only one study was reported on identifying 45S rDNA loci and distribution of satellite repeat DNA in chromosomes of some Korean landrace citrus using FISH (Kang et al., 2008).

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CHAPTER I
Karyotype Diversity of Korean Landrace Mandarins by CMA Banding Pattern and rDNA Loci

Abstract

Mandarin is the major type of citrus grown in Jeju, Korea and has a long history of cultivation there. Landrace citrus species represent broad and natural genetic variability, which are important and valuable as genetic material. In this study, metaphase chromosomes of six Korean landrace mandarins were analyzed to understand the phylogenetic relationship among them and to compare these characteristics with those of other *Citrus* species at a cytogenetic level using CMA banding patterns and rDNA loci. Chromosomes were categorized into five types according to the distribution and number of heterochromatic CMA-positive bands; type A chromosomes have two telomeric and one proximal bands, type B have one telomeric and one proximal bands, type C have two telomeric bands, type D have one telomeric band, and type E have no band. Gamza mandarin (*C. benikoji*) displayed the 1A/45S + 2B/45S + 2C + 5D + 1D/45S + 1D/5S-45S + 6E pattern, without a solitary 5S rDNA locus that differentiated the gamza mandarin from other accessions. A solitary 5S rDNA locus was observed in the chromosomes of

byungkyul mandarin (*C. platymamma*) (1A/45S + 2B/45S + 1C + 6D + 1D/45S + 1D/5S-45S + 5E + 1E/5S) and cheongkyul mandarin (*C. nippokoreana*) (1A/45S + 1B/45S + 1C + 7D + 1D/5S + 1D/45S + 1D/5S-45S + 5E). The chromosome composition of byungkyul mandarin based on CMA banding pattern and rDNA loci suggests that byungkyul mandarin may be related to pummelo, sweet orange, and members of the *Citrus* subgenus *Papeda* during its evolution. Cheongkyul mandarin possessed a distinct marker chromosome (D/45S) that can be used to distinguish cheongkyul madrarin from the other Korean landrace mandarins. Jinkyul mandarin (*C. sunki*) (1A/45S + 1B/45S + 1C + 10D + 2D/5S-45S + 3E) may be related to pummelos. The karyotype of pyunkyul mandarin (*C. tangerina*) (3B/45S + 2C + 7D + 1D/5S-45S + 5E) suggests that the pyunkyul mandarin is a hybrid between *C. grandis* and *C. reticulata*. Binkyul mandarin (*C. leiocarpa*) (1A/45S + 1C + 6D + 2D/45S + 2D/5S-45S + 6E) seemed to be related to mandarins and pummelos. Heterogeneous karyotypes of six accessions separated and differentiated each of the six Korean landrace mandarins and potential marker chromosomes were identified.

Introduction

Citrus is one of the most important and widely grown fruit crops in the world. *Citrus* spp., especially in the Jeju area of Korea, has been cultivated for more than a millennium and is the leading fruit crop in terms of cultivated area and amount produced. Mandarin is the major type of citrus grown in Jeju, covering approximately 90% of the total area cultivated with citrus (Korea Rural Economic Institute, 2017). The taxonomy of the genus *Citrus* is complicated and ambiguous due to wide cross compatibility, a long history of cultivation, repeated cross hybridizations, apomixis, and dispersion in the wild. The genetic diversity within *Citrus* has been studied using different RAPD and SSR markers (Baig et al., 2009; El-Mouei et al., 2011; Nematollahi et al., 2013); these studies have identified a narrow genetic base within mandarins (Machardo et al., 1996; Coletta-Filho et al., 1998; El-Mouei et al., 2011). Based on these data, Ogwu et al. (2014) pointed out a significant threat of genetic erosion in citrus and emphasized the importance of using landraces as valuable genetic material for modern plant breeding. To promote the utilization of landrace mandarins and conserve their genetic lineage, it is essential and necessary to learn more about the genetic origins of the various remaining Korean landrace citrus species, their genetic characteristics, and phylogenic relationships.

Chromosome staining [using guanine-cytosine (GC) specific fluorochrome chromomycin A_3 (CMA)] combined with DAPI, which has an affinity for adenine-thymine (AT), has revealed the existence of an interspecific banding pattern in *Citrus* species (Guerra, 1993). Karyotype analysis using CMA/DAPI banding patterns has been proven to be a very useful technique for cytogenetically characterizing *Citrus* species (Miranda et al., 1997a; Befu et al., 2000; Yamamoto and Tominaga 2003). Moreover, fluorescence *in situ* hybridization (FISH) has been applied to more detailed chromosomal studies. The combined CMA/DAPI banding pattern with the distribution of rDNA loci (using the FISH technique) has been used to distinguish the heterozygosity of some *Citrus* species (Moraes et al., 2007) and to clarify the phylogenetic relationship among some species (Carvalho et al., 2005; Brasileiro-Vidal et al., 2007).

Only few cytological and phylogenetic studies of Korean landrace mandarins have been reported in the literature (Yun, 2001; Kang et al., 2008; Jin et al., 2016). This study was conducted to understand Korean landrace mandarins at the cytogenetic level, gain insight on the phylogenetic relationship among Korean landrace mandarins, and compare these data with data from other *Citrus* species by examining six Korean mandarin species by karyotyping and identifying their

chromosomes using CMA/DAPI banding patterns and FISH using 5S and 45S rDNA as probes.

Materials and Methods

Plant Materials

Six mandarin species, recognized as landraces in Korea, were used in this study (Table 1). The mandarins conserved at the Jeju Special Self-Governing Province Agricultural Research and Extension Service. Befu et al. (2000) reported that polymorphisms of CMA banding patterns have been observed in monoembryonic seedlings. Conventionally, monoembryonic accession studies use young leaves (approximately 2-4 mm long) from adult trees, while polyembryonic accession studies use seedling root tips (approximately 1-3 mm long).

Table 1. Korean landrace mandarins (*Citrus* spp.) used in this study.

Scientific name	Common name	Embryony	Material[z]	Source[y]
C. benikoji Hort. ex Tan.	Gamza	Mono	L	CRS
C. platymamma Hort. ex Tan.	Byungkyul	Poly	R	CRS
C. sunki Hort. ex Tan.	Jinkyul	Poly	R	CRS
C. nippokoreana Tan.	Cheongkyul	Poly	R	CRS
C. tangerina Hort. ex Tan.	Pyunkyul	Mono	L	CRS
C. leiocarpa Hort. ex Tan.	Binkyul	Poly	L	CRS

[z] L: Young leaves of adult trees, R: Root tips of seedlings.

[y] Citrus Research Station, National Institute of Horticultural & Herbal Science, Seogwipo-si, Jeju-do, Korea.

Chromosome Preparation

Chromosome preparation was performed according to Dutt et al. (2010), with minor modifications. Twenty fresh young leaves for monoembryonic accessions and twenty-five root tips for polyembryonic accessions of sampled mandarins were excised and pretreated in 2 mM 8-hydroquinoline at 4℃ for 8 h in the dark. Then, samples were fixed in an ethanol : acetic acid (3:1, v/v) solution. The fixed specimens were washed with distilled water and digested at 37℃ for 1 h with an enzyme mixture containing 2% Cellulase from *Trichoerma viribe* (Sigma, Japan), 1% Macerozyme R-200 (Yakult, Japan), and 0.3% Pectolyase Y-23 (Kyowa Chemical Products Co., Ltd, Japan). Digested specimens were mounted on glass slides, scattered with a drop of fixed solution using a pair of fine pointed forceps, and air dried.

CMA/DAPI Staining

Metaphase chromosomes were stained with CMA and counterstained with DAPI as described by Schweizer and Ambros (1994), but with modifications. The preparations were sequentially treated for 30 min with McIlvaine's buffer (pH 7.0) containing 5 mM $MgCl_2$, for 1 h with 0.5 $mg \cdot mL^{-1}$ CMA, and then for 10 min with

McIlvaine's buffer. The preparations were counterstained with Vectashield mounting medium containing 1.5 μg·mL^{-1} DAPI (Vector Laboratories, Burlingame, CA, USA). Samples were observed using an epifluorescence microscope (Leica DMRBE, Germany) with an E4 filter cassette and then the image captured using a CCD camera (INFINITY 3, Lumenera, Canada). Subsequently, slides were de-stained for 30 min in the fixed solution and then left overnight in absolute ethanol at ambient temperature. Then, the slides were air dried.

DNA Probes and Labeling

DNA probes were provided by the Life Sciences Research Institute (Biomedic Co., Ltd., Korea). 5S and 45S rDNA probes (Genebank accession numbers: KF156926 and MF171086) were amplified from the genomic DNA of *C. clementina* using primers 5′-CATCAGAACTCCGCAGTTAAGCG-3′ and 5′-CTGCAATCTACTTAACTCGTGC-3′ for 5S rDNA, and primers 5′-CCTTAACGAGGATCCATTG-3′ and 5′CCGTCTCTTAGGATCGACTAAC-3′ for 45S rDNA. Each DNA probe was labelled with tetramethyl rhodamine-5-dUTP and fluorescein-12-dUTP (Roche, Switzerland) using the nick translation DNA labeling system (Enzo Life Sciences Inc., USA) according to the manufacturer's instruction.

Fluorescent *in situ* Hybridization

The procedure and conditions for FISH were based on the method described by Miranda et al. (1997), but with some modifications. Slides with metaphase chromosomes were treated at 37℃ for 1 h with RNase A (100 μg·mL^{-1}) in 2X saline-sodium citrate (SSC) buffer, washed three times in 2X SSC for 2 min each, followed by dehydration in a series of ice-cold ethanol treatments (70%, 80%, and 100%), and then air dried. Chromosomes were denatured at 70℃ for 2 min in 70% formamide in 2X SSC, dehydrated again, and then air dried. They were then denatured at 85℃ for 10 min in a hybridization mixture containing 50% formamide (v/v), 10% dextran sulfate (w/v), 200 ng·μL^{-1} sheared salmon sperm DNA, and 10 ng·μL^{-1} for each rDNA probe in 2X SSC . Then, 10 μL of the mixture was applied on each slide. Slides were covered with glass coverslips which were sealed with rubber cement. After overnight incubation at 37℃, the coverslips were removed and slides were washed at 37℃ for 20 min with agitation every 5 min in 60% formamide in 2X SSC, and then rinsed twice in 2X SSC at 37℃ for 5 min each. Slides were counterstained with DAPI and then FISH images were acquired using I3 and N2.1 filter cubes for FITC and TRITC, respectively. All images were analyzed and optimized using Fiji (Schindelin et al., 2012) and Adobe Photoshop CS6 (Adobe System Inc., CA, USA).

Results

Six Korean landrace mandarins examined in this study were all diploids (2n = 18). Chromosomes were categorized into five types according to the distribution and number of heterochromatic CMA-positive bands, following procedures by Befu et al. (2000) and Yamamoto et al. (2007). Type A chromosomes have two telomeric and one proximal bands, type B have one telomeric and one proximal bands, type C have two telomeric bands, type D have one telomeric band, and type E have no band (Fig. 1). More than 100 cells for each accessions of six Korean landrace mandarins were analyzed and showed some analogy in the CMA banding patterns. However, instantly recognizable heteromorphic karyotypes were evident with no polymorphism of chromosome configurations within the accessions (Table 2). In the gamza (*C. benikoji*), byungkyul (*C. platymamma*), jinkyul (*C. sunki*), and cheongkyul (*C. nippokoreana*) mandarins, one or two of each type of A, B, and C chromosomes were observed (Table 2 and Fig. 2A1, B1, C1, and D1). The pyunkyul mandarin (*C. tangerina*) possessed three type B chromosomes, but a type A chromosome was absent (Table 2 and Fig. 2E1). The binkyul mandarin (*C. leiocarpa*) displayed a CMA banding pattern without any evident type B chromosome (Table 2 and Fig. 2F1).

Table 2. CMA banding patterns and distribution of 5S and 45S rDNA loci of Korean landrace mandarins.

Common name	CMA banding pattern	rDNA locus		
		5S	45S	5S-45S
Gamza	1A+2B+2C+7D+6E	-	1A+2B+1D	1D
Byungkyul	1A+2B+1C+8D+6E	1E	1A+2B+1D	1D
Jinkyul	1A+1B+1C+12D+3E	-	1A+1B	2D
Cheongkyul	1A+1B+1C+10D+5E	1D	1A+1B+1D	1D
Pyunkyul	3B+2C+8D+5E	-	3B	1D
Binkyul	1A+1C+10D+6E	-	1A+2D	2D

The distributions of 5S and 45S rDNA loci by FISH were heterogeneous among the six accessions (Fig. 2 and Table 2). In all accessions, the 5S rDNA loci were detected in a subterminal region adhered to a telomeric CMA-positive band of type D chromosomes in all accessions. However in the byungkyul mandarin one (of two 5S rDNA loci) was located in a subterminal region of a type E chromosome. Gamza and pyunkyul mandarins possessed one 5S rDNA locus, wheras byungkyul, jinkyul, cheongkyul, and binkyul mandarins displayed two 5S rDNA loci. All 45S rDNA loci were homotopic to CMA-positive regions. Every type A and B chromosomes possessed one 45S rDNA locus in the centromere near the proximal region of the chromosomes. In a type D chromosome, the 45S rDNA locus was observed in the telomeric terminal region. Gamza, byungkyul, and binkyul mandarins displayed five 45S rDNA loci, whereas jinkyul, cheoungkyul, and pyunkyul mandarins displayed four 45S rDNA loci. At least one 5S rDNA locus was always observed adjacent to the edge of the 45S rDNA locus toward the centromere in type D chromosomes. One or two type D chromosomes, bearing co-localized 5S and 45S rDNA loci, were observed in all accessions. There was no type C chromosome with rDNA. Some type D chromosomes looked as if they were a type D chromosome with a satellite chromosome, but these chromosomes were characterized as type D.

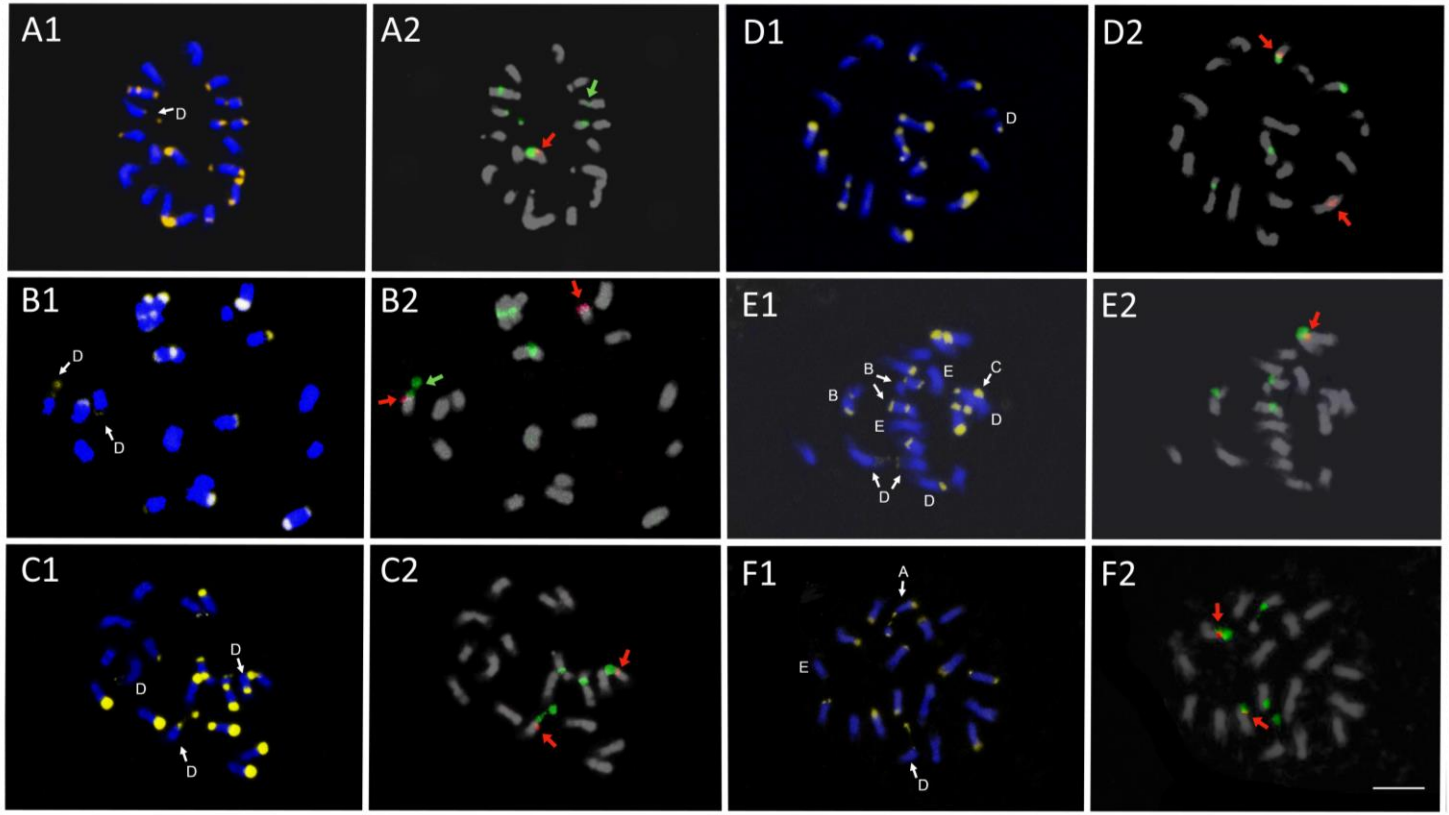

Fig. 2. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of six Korean landrace mandarins. A: gamza, B: byungkyul, C: jinkyul, D: cheongkyul, E: pyunkyul, and F: binkyul. Blue: DAPI positive region, yellow: CMA positive region, red: 5S rDNA locus, green: 45S rDNA locus, and grey: DAPI region. Scale bar = 5 ㎛.

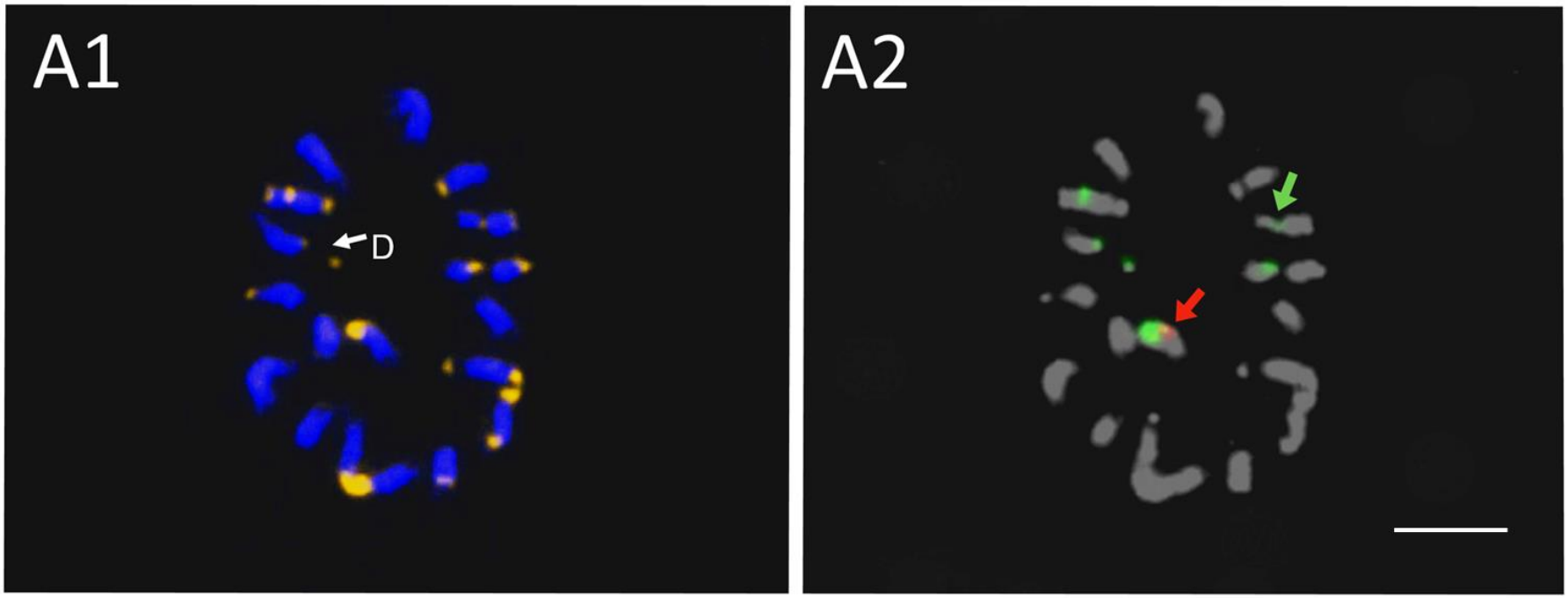

Fig. 3. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of gamza. Scale bar = 5 ㎛.

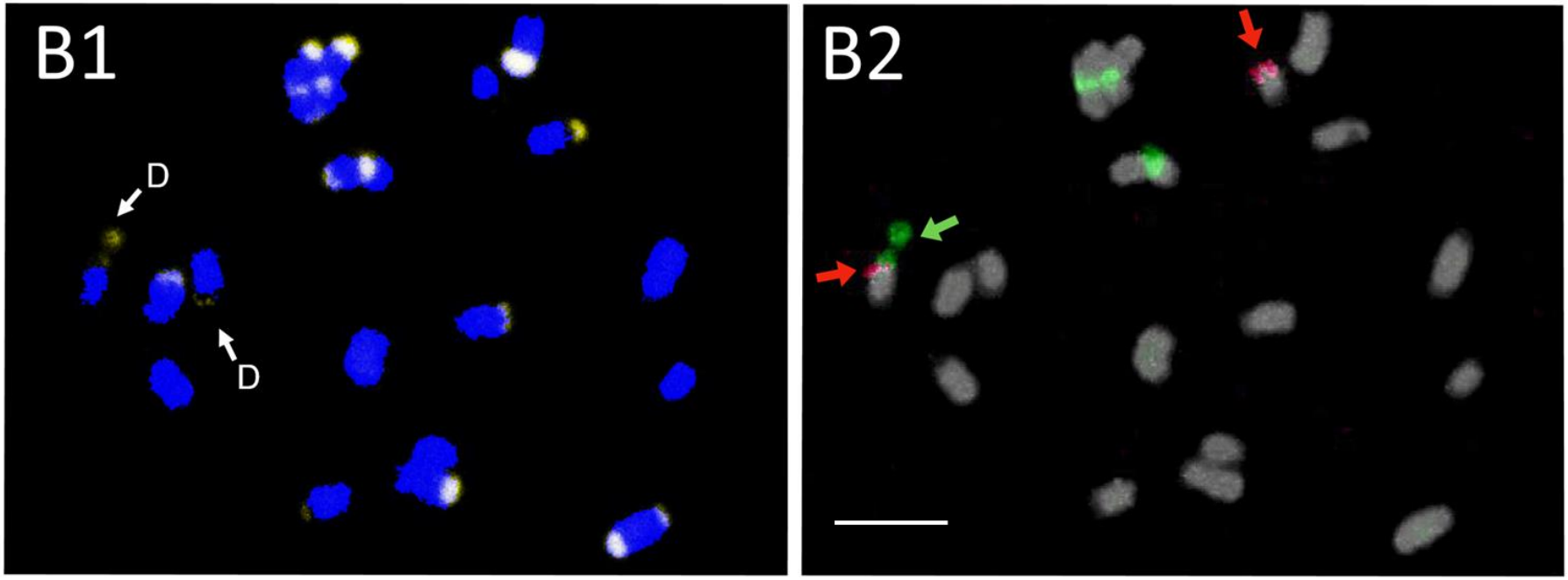

Fig. 4. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of byungkyul. Scale bar = 5 ㎛.

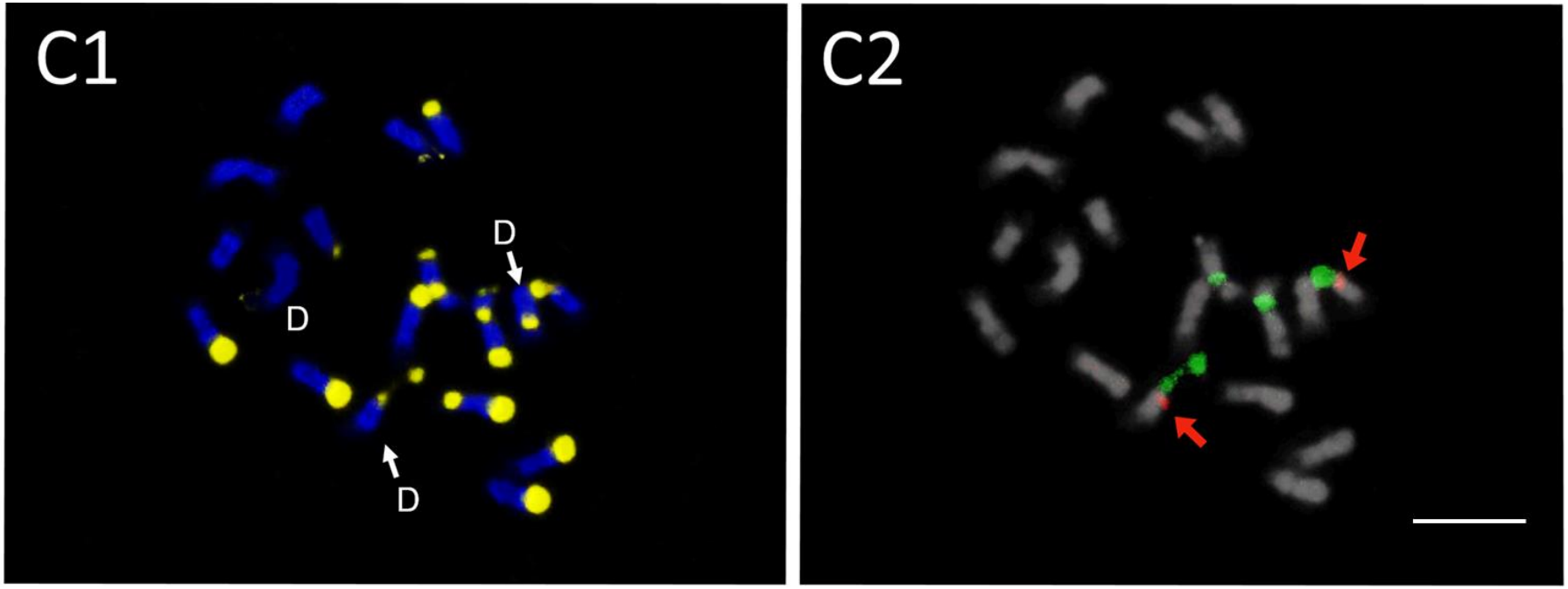

Fig. 5. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of jinkyul. Scale bar = 5 ㎛.

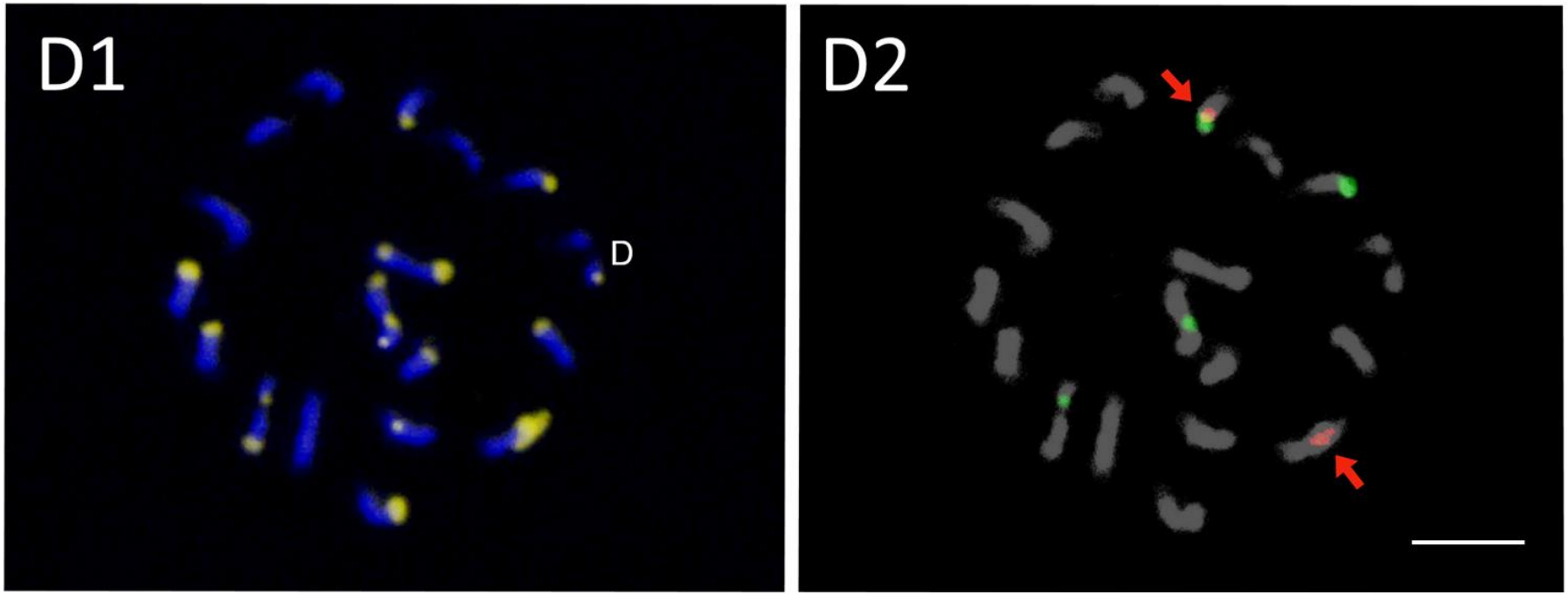

Fig. 6. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of cheongkyul. Scale bar = 5 ㎛.

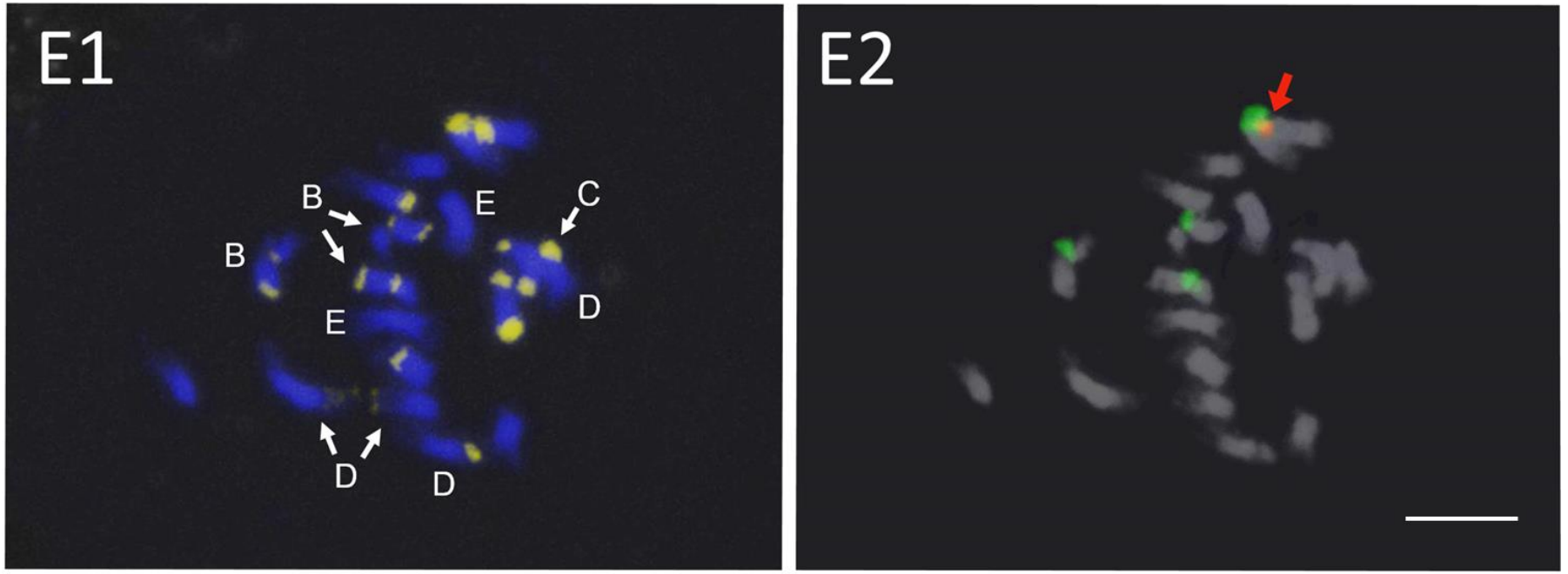

Fig. 7. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of pyunkyul. Scale bar = 5 ㎛.

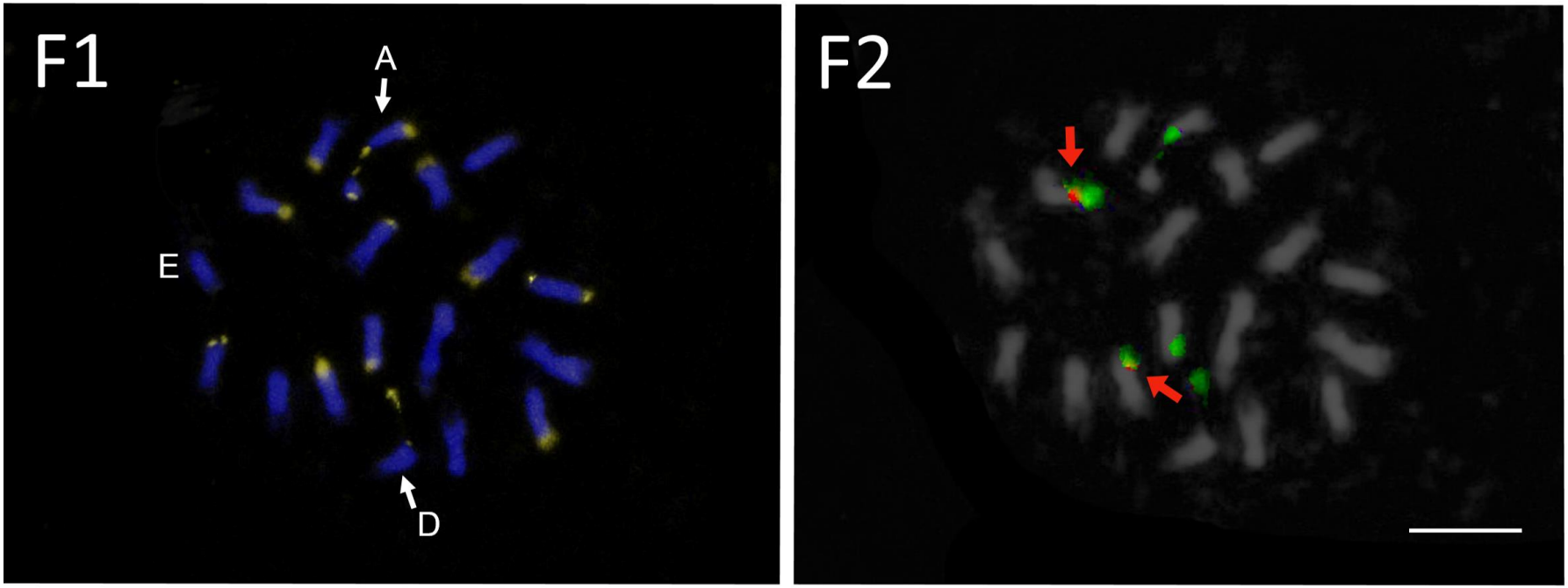

Fig. 8. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of binkyul. Scale bar = 5 ㎛.

Discussion

From the karyotype analysis of Korean landrace mandarins based on CMA banding patterns, a very few number of type A or B chromosomes were present in most accession tested in this study; this is contrast to banding patterns frequently exhibited in sour orange (Yamamoto et al., 2007), kumquat (Barros e Silva et al., 2010), and pummelo and its relatives (Yamamoto et al., 2005; Befu et al., 2000, 2001). The type C chromosome has been found in a variety of *Citrus* species, including mandarin hybrids (Moraes et al., 2007). Hence, type A, B, and C chromosomes have been adopted as marker chromosomes to differentiate pummelo citrus from most mandarins. A large number of type D and E chromosomes have been consistently found in *Citrus* species and so are considered to be the basic type of chromosome associated with citrus (Guerra, 1993; Miranda et al., 1997a; Befu et al., 2000, 2001; Cornélio et al., 2003; Yamamoto and Tominaga, 2003; Carvalho et al., 2005; Brasileiro-Vidal et al., 2007; Moraes et al., 2007a; Yamamoto et al., 2007;). All six Korean landrace mandarins analyzed in this study exhibited the characteristic CMA banding pattern typical of mandarin hybrids, that is, they possessed fewer than three chromosomes of type A or B chromosomes, at least two type C chromosomes, and a predominant number of type D and E chromosomes (Table 2. And Fig. 2.),

which agrees with the classification of *Citrus* species provided by Cottin (2002). Among the six accessions, gamza (1A + 2B + 2C + 7D + 6E), byungkyul (1A + 2B + 1C + 8D + 6E), jinkyul (1A + 1B + 1C + 12D + 3E), and cheongkyul (1A + 1B + 1C + 10D + 5E) mandarins revealed a similar chromosomal composition relative to CMA banding patterns. However, the FISH of 5S and 45S rDNA loci showed clear cytological differences among these four accession patterns (Table 2).

The gamza mandarin displayed the 1A/45S + 2B/45S + 1D/45S + 1D/5S-45S pattern (Fig. 3.), with no chromosome bearing a solitary 5S rDNA locus. The existence of a type D/45S locus and absence of a solitary 5S rDNA locus enabled us to differentiate the gamza mandarin from other accessions possessing similar CMA banding patterns. In addition, the distribution of rDNA loci among mandarins, such as among byungkyul, jinkyul, and cheongkyul types (Table 2) allowed us to further differentiate the gamza mandarin from the others. Kim (1988) reported that gamza and pyunkyul mandarins shared similar morphological characteristics, except for fruit size. However, their heteromorphic karyotypes indicate the extent of their heterogeneity. The results were supported by Jung et al. (2005), who used analysis of plastid *trn*L-*trn*F sequences to determine that the gamza mandarin is phylogenetically distant from other Korean landrace accessions.

The byungkyul mandarin displayed rDNA loci of 1A/45S + 2B/45S + 1D/45S + 1E/5S + 1D/5S-45S (Fig. 4). The most conspicuous characteristic karyotype in byungkyul is the presence of the type E/5S chromosome, which is a strong candidate for use as a chromosomal marker that could distinguish byungkyul from the rest of Korean landrace mandarins. Inconsistencies exist among researchers in their characterizations of chromosome types based on the distribution of CMA bands. For instance, the chromosome without band was classified as a type E chromosome or a type F chromosome (Yamamoto et al., 2007). The type E (or F) chromosome bearing a 5S rDNA locus has been reported for tangor cv. Murcott, a synthetic hybrid between *C. sinensis* (L.) Osb. × *C. reticulata* Blanco (Moraes et al., 2007). Although the byungkyul mandarin was classified as a member of the mandarin group by Tanaka (1961), it was clustered in the pummelo cluster after determining the *mat*K sequences of *Citrus* species and its relatives (Penjor et al., 2013). Jung et al. (2005) reported that the byungkyul mandarin can be phylogenically segregated from other Korean landrace citrus species after conducting a sequence analysis using the *trn*L-*trn*F intergenic spacer. Jung et al. (2005)'s result is in strong agreement with morphological traits of the byungkyul mandarin, with a tapered neck that is unique among Korean landrace citrus,

described by Kim (1988). The CMA banding patterns and FISH with rDNA probes of the byungkyul mandarin showed characteristic chromosomal markers for pummelo, mandarin, sweet orange, and the *Citrus* subgenus *Papeda*. This suggests that the byungkyul mandarin may have evolutionary relation to these species during its phylogenetic history.

For the jinkyul mandarin, twelve type D and three type E chromosomes were observed, and one each of type A, B, and C chromosomes (Fig. 5). This chromosomal configuration is quite different from configurations obtained in previous studies, where they karyotyped as 14D + 4E (Cornélio et al., 2003; Moares et al., 2007; Barros e Silva et al., 2010) and 12D + 6E (Yamamoto and Tominaga, 2003). Differences in the numbers of type D and E chromosomes among previous studies could be due to different staining intensity. Moreover, the distribution of the rDNA loci in this study (1A/45S + 1B/45S + 2D/5S-45S) was not in agreement with the previous studies, where 2D/5S-45S configuration was obtained by Moares et al. (2007) and 2D/45S was identified by Barros e Silva et al. (2010). The high polymorphic karyotype between the jinkyul mandarin and *C. sunki* may be due to the use of accessions cultivated in different geographic regions, such as Brazil, Japan, and Korea, and karyotype variation may have been occurred. The jinkyul mandarin and *C. sunki* fruits used in

the above two studies did not differ much morphologically. Molecular genetic studies, such as those using *mat*K analysis, chloroplast DNA analysis, and RFLP analysis, reveal that the jinkyul mandarin and *C. sunki* belong to the mandarin group (Federici et al., 1998; Jung et al., 2005; Lu et al., 2011; Penjor et al., 2013). Based on these studies and the result of karyotype analysis in this study, it is proposed that the jinkyul mandarin may be an intraspecific hybrid, possibly, related to pummelo.

The chromosomal compositions of cheongkyul and jinkyul mandarins, based on CMA banding patterns, were very similar (Table 2, Fig. 6). That is, the numbers of type A, B, and C chromosomes were identical in both accessions. Although the numbers of type D and E chromosomes were differed slightly, both type dominated in both accessions. However, the distribution of rDNA loci identified using FISH differentiated the cheongkyul mandarin from other Korean landrace mandarins. For the cheongkyul mandarin (1A/45S + 1B/45S + 1D/5S + 1D/45S + 1D/5S-45S), one solitary 5S rDNA locus in a type D chromosome (D/5S) was observed (Fig. 6 and 9). In *Citrus*, a 5S rDNA locus in a type D chromosome has been reported to be adjacent to a 45S rDNA locus (Pedrosa et al., 2000; Brasileiro-Vidal et al., 2007) or in the euchromatic terminal region (Carvalho et al., 2005). On the other hand, a 5S rDNA locus in the cheongkyul mandarin was detected in the CMA-positive region of a

type D chromosome. This single, unique type of chromosome may be the only marker chromosome that can be used to karyotypically distinguish cheongkyul mandarin from the other Korean landrace mandarin accessions, given the similarity in karyotype patterns among Korean landrace mandarins. This could be explained by RFLP and RAPD data obtained Federici et al. (1998), which showed equivocal clustering of *C. nippokoreana* with other mandarins.

The CMA banding pattern and distribution of rDNA loci of pyunkyul mandarin (3B + 2C + 8D + 5E, 3B/45S + 1D/5S-45S) (Fig. 7) were similar to those of *C. sinensis* (L.) Osbeck (2B + 2C + 7D + 7E, 2B/45S + 1D/5S-45S + 1E/5S) and supposedly their evolutionary paths would be similar as well, which suggests that the pyunkyul mandarin is a hybrid between *C. grandis* and *C. reticulata* (Pedrosa et al., 2000). This is supported by the results of analysis of plastid *trn*L-*trn*F sequences that clustered pyunkyul mandarin with pummelo (Jung et al., 2005). The existence of 3B/45S chromosomes and the absence of type A chromosome are noteworthy. The pyunkyul mandarin may be closely related to *Citron*, which displayed a 2B + 8D + 8E pattern (Yamamoto et al., 2007), and is weakly related to *C. grandis*.

Binkyul mandarin displayed CMA banding pattern of 1A + 1C + 10D + 6E and 1A/45S + 2D/45S + 2D/5S-45S rDNA loci in this study (Fig. 8), which is inconsistent

with other studies, none of which performed FISH analyses of rDNA (Befu et al., 2001; Yamamoto and Tominaga, 2003). The polymorphic karyotypes were found may be a result of using accessions from different populations. For zygotic embryos, the use of apical shoot tips or young leaves provide more reliable results for studying chromosomes (Befu et al., 2000). The chromosome type A/45S was considered to be a chromosomal marker that characterizes *C. grandis* (Moraes et al., 2007a). The binkyul mandarin, based on the chromosomal configuration using CMA banding patterns and FISH with rDNA probes, seemed to be closely related to mandarins and pummelos. This relationship is supported by Jung et al. (2005), who found that binkyul and *C. grandis* were clustered together in a phylogenetic tree based on the *trn*L-*trn*F sequences.

The 5S rDNA loci were found only at the subterminal eukaryotic region of type D and E chromosomes, whereas the 45S rDNA loci were at the proximal regions of type A and B chromosomes and at the telomeric region of type D chromosome (Fig. 2). This result was consistent with those of previous studies (Pedrosa et al., 2000; Carvalho et al., 2005; Moraes et al., 2007; Moraes et al., 2007a;). In all accessions, at least one type D chromosome (bearing co-localized 5S and 45S rDNA loci) was observed, which has been consistently observed among mandarins (Moraes et al.,

2007). Some chromosomes that had a terminal heterochromatic segment attached by a chromatin thread were observed, which exhibited CMA-positive bands and were co-localized with 45S rDNAs (Fig. 9). It seemed to embody a secondary constriction, resembling a type D chromosome with satellite, which has been reported by Yamamoto et al. (2007) as a type Dst in *Citrus* subg. *Papeda*. In contrast, the constriction (or gaps) at the subterminals of chromosomes have been reported as fragile sites (Lan et al., 2016), which are distended 45S rDNA loci found to be hypo-methylated. Extensively methylated DNA is considered to be an attribute of heterochromatin regions in *Citrus* (Marques et al., 2011). The most common fragile site in *Citrus* has been referred to as a Df chromosome (Lan et al., 2016). Because it is still controversial, this type of chromosome was characterized as type D. If type Dst and Df chromosomes could be differentiated, they could provide very useful chromosome markers for distinguishing *Citrus* cultivars.

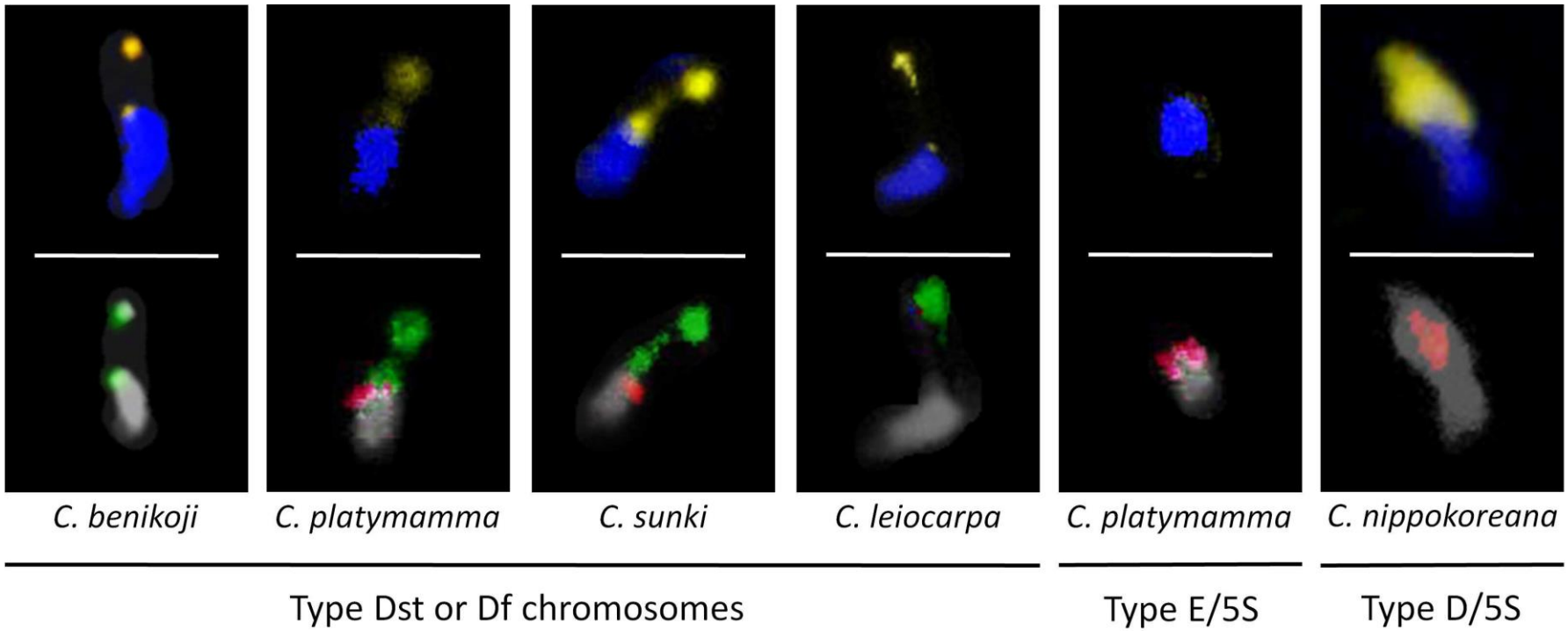

Fig. 9. Four suspicious chromosomes characterized as type D chromosomes, and noteworthy type E and type D chromosomes that possess solitary 5S rDNA locus. Blue: DAPI positive region, yellow: CMA positive region, red: 5S rDNA locus, green: 45S rDNA locus, and grey: DAPI region.

D/5S-45S chromosomes were consistently observed in all Korean landrace accessions. Previous studies suggested that co-localized 5S-45S rDNA loci are conserved through evolution in geographic origin species (Barros e Silva et al., 2013; Zhang et al., 2016). Most *Citrus* species are hybrid origins, of which karyotypes are characterized as displaying heterogeneous chromosome compositions based on CMA banding patterns and odd numbers of rDNA loci (Marques et al., 2011; Moraes et al., 2007). Furthermore, it has been suggested that CMA-positive heterochromatin has expanded through evolutionary processes in *Citrus* (Guerra et al., 2000; Yamamoto, 2007; Yamamoto et al., 2008; Yamamoto et al., 2009). These phenomena were consistently observed in this study. Therefore, the six Korean landrace mandarins examined in this study might be hybrids and may be phylogenetically distinct varieties derived from their long history of cultivation in Korea.

This study provides the first karyotypes of Korean landrace mandarins by using the combination of CMA banding patterns and the distribution of 5S and 45S rDNA loci (Fig. 10).

Some karyotypes in this study differed from those of previous studies. This may be a consequence of using different plant materials in this study than the other previous studies. The plant materials used in this study have many centuries of domesticated history in Jeju, Korea, which has provided geographical separation with the other populations of the species. The different environmental conditions with limited mating patterns may have caused karyotype variation or genetic variation within the spices. The results of this study hypothesizes that Korean landrace mandarins may be homonymous to the species sharing the same Latin

name. Sequence analysis of rDNA internal transcribed spacer (ITS) regions of Korean landrace citrus and those species with the same Latin names (this study has been recently conducted at Citrus Research Institute, National Institute of Horticultural and Herbal Science (NIHHS), Rural Development Administration (RDA), Republic of Korea and preparing for publication) supported the hypothesis and thus they may be distinguished species or subspecies within the species. Further studies, such as diversity analysis using simple sequence repeat (SSR) marker, chloroplast barcoding marker, and sequence analysis of rDNA internal transcribed spacer (ITS) regions, may be required to more precisely elucidate the phylogenetic relationships among them.

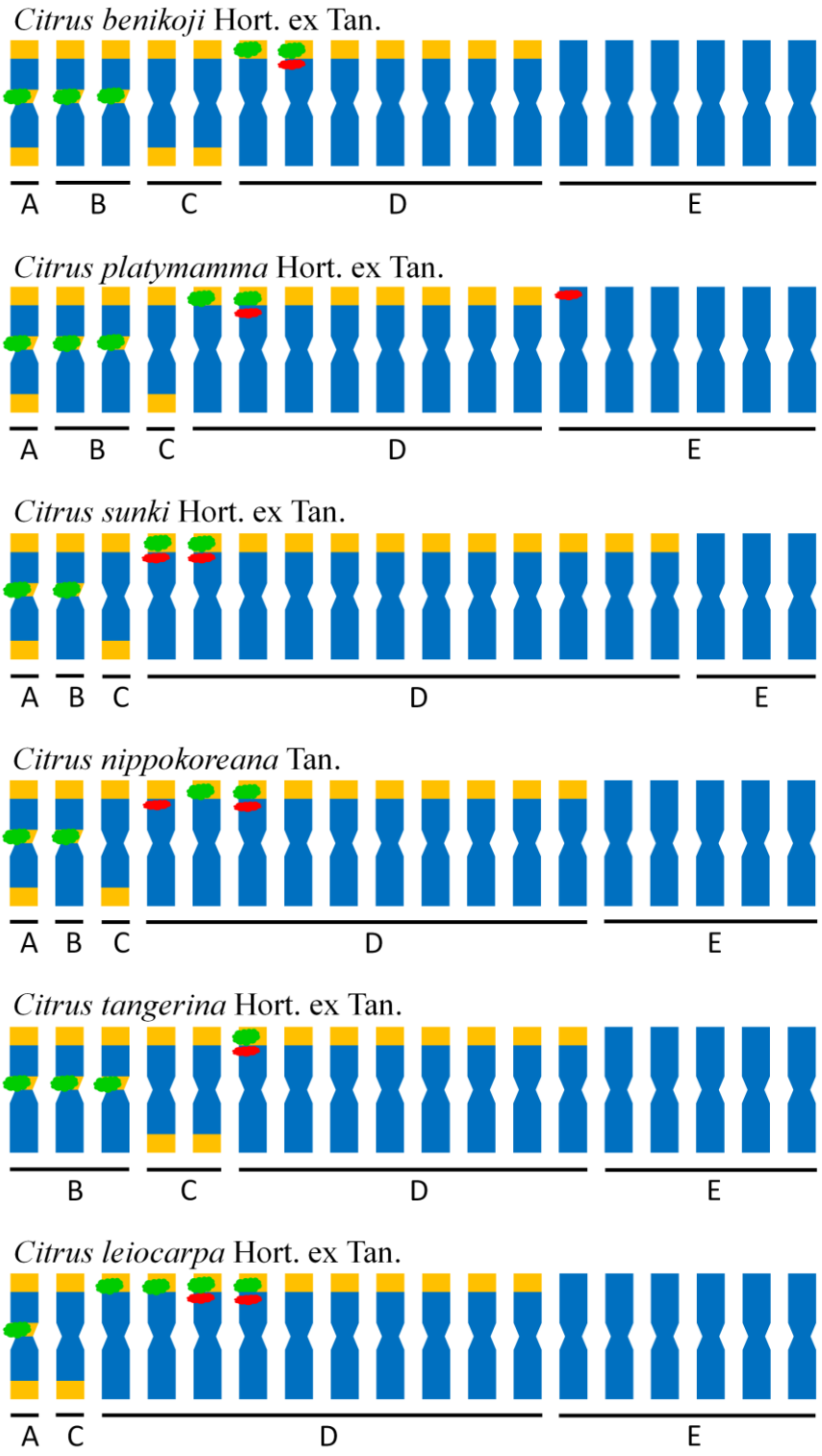

Fig. 10. Schematic representative idiograms of six Korean landrace mandarins showing the distribution of CMA positive regions (in yellow), DAPI stained regions (in blue), 5S rDNA loci (in red), and 45S rDNA loci (in green). Alphabet letters under lines represent chromosome types.

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CHAPTER II
High Resolution Chromosome Configurations of Some Korean Landrace Citrus by CMA Banding and rDNA loci

Abstract

Citrus is the major agricultural crop in Korea as well as Jeju with a long history of cultivation. Despite its long cultivation history and the immense clout of agricultural and economical value, ecological, evolutional, and phylogenetical taxonomic investigations of *Citrus* based on phenotypes or genotypes are scarcely conducted. Landrace citrus species represent broad and natural genetic variability, which are important and valuable as genetic material. In this study, metaphase chromosomes of five Korean landrace citrus were analyzed to understand the phylogenetic relationship among them and to compare these characteristics with those of other *Citrus* species at a cytogenetic level using CMA banding patterns and rDNA loci. The CMA banding patterns of the five Korean landrace citrus were 1A+2B+2C+6D+7E in dongjeongkyul (*C. erythrosa*), 3B+1C+7D+5E+2F in hongkyul (*C. tachibana*), 2A+1B+3C+4D+8E in sadoogam (*C. pseudogulgul*), 1A+3B+1C+7D+6E in dangyooza (*C. grandis*), 1A+1B+1C+9D+6E in jigak (*C. aurantium*) (Table 4 and Fig. 13.). All types of chromosome bands were present in all accessions except in hongkyul (*C. tachibana*), in which type A chromosome is absent but two of type F chromosomes were observed. The numbers of type A, B, and C chromosomes were lower in all accessions. In contrast, the type D and E chromosomes were remarkably constant and predominantly observed in all accession. The distributions of 5S and

45S rDNA loci by FISH were heterogeneous among them. All accessions possessed one 5S rDNA locus except hongkyul (*C. tachibana*), which displayed two 5S rDNA loci. And they always co-localized with 45S rDNA locus. All 45S rDNA loci were homotopic to CMA-positive regions. Every type A and B chromosomes possessed one 45S rDNA locus in the proximal region of the chromosomes. There was no type C and E chromosome with rDNA observed. The chromosome configurations of Korean landrace citrus analyzed here suggest that all accessions in this study are hybrids that have relationships more or less with mandarin and pummelo. hierarchical cluster analysis and UPGMA phenogram based on CMA banding pattern combined with 5S and 45S rDNA loci of Korean landrace citrus showed the strong karyotype dissimilarity in the invested taxa. This study provides high resolution of chromosome configurations, which could complement previous studies, and elucidated phylogenetic relationships of Korean landrace citrus at the cytogenetic level.

Introduction

Citrus is an evergreen deciduous tree that belongs to the subfamily, Aurantioideae within the Rutaceae family. Citrus is the leading agricultural produce, in terms of the area and production, in Korea as well as Jeju. Despite its long cultivation history and the immense clout of agricultural and economical value, ecological, evolutional, and phylogenical taxonomic investigations of Citrus based on phenotypes or genotypes are scarcely conducted in Korea. The most widely conceded taxonomic systems are proposed by Swingle and Tanaka as 16 and 162 species, respectively (Davies and Albrigo, 1994). Among them, *C. maxima* (pummelo), *C. medica* (citron), and *C. reticulata* (mandarin) are the only basic species of *Citrus*, which are within the subgenus *Eucitrus* of Swingle's system. Other genotypes such as *C. sinensis* (sweet orange), *C. paradisi* (grapefruit), and *C. limon* (lemon) derived from hybridization between the basic species are referred to as hybrid origins (Scora, 1975; Barrett and Rhodes, 1976).

Allied species and landraces of citrus are considered as imperative resources for structured and targeted breeding programs. It is essential and necessary to learn about their genetic origins, genetic characteristics, and phylogenic relationships to promote utilization in modern breeding programs. Karyotype analysis could provide such fundamental but valuable information by identifying particular genomic variants or detecting true hybrids (Guerra et al, 1997). Most members of the genus *Citrus* are diploids of 18 chromosomes (Guerra, 1993). Although the chromosome number is relatively small ($2n$ = 18), karyotype analysis is difficult

because of the small chromosome sizes in metaphase (1.0-4.0 μm) and close morphological resemblances between chromosomes. Yamamoto et al. (2003 and 2007) and Moraes et al. (2007) classified citrus chromosomes up to seven types based on the distribution and number of the heterochromatic CMA positive bands. Each citrus species possesses its typical karyotype based on the seven types. The involvement of the basic species in the establishment of species could also be verified by karyotype analysis.

In this study, five Korean landrace citrus were examined at the cytogenetic level to gain insight on the phylogenetic relationship among Korean landrace citrus, and to compare these data with data from other citrus species by karyotyping and identifying their chromosomes using CMA/DAPI banding patterns and FISH using 5S and 45S rDNA as probes.

Materials and Methods

Plants Materials

Five accessions of citrus species referred to as Korean landrace citrus were used (Table 3). The *Citrus* species used in this investigation were preserved at the Jeju Special Self-Governing Province Agricultural Research and Extension Service. Polyembryonic seed formation is common phenomenon in *Citrus*, which gives a rise of nucellar cells and may produce seedlings that are genotypically homogeneous to the maternal plant (Koltunow et al., 1996). On the other hand, seedlings from monoembryonic seeds are zygotic. Polymorphisms of CMA banding patterns in monoembryonic seedlings have been reported by Befu et al. (2000). Therefore in this study, young leaves (approximately 2-4 mm long) from adult trees were used for monoembryonic accessions, and root tips (approximately 1-3 mm long) from germinated seeds were used for polyembryonic accessions.

Table 3. Korean landrace citrus species used in this study.

Scientific name	Common name	Type	Embryony	Material[z]	Source[y]
Citrus erythrosa Hort. ex Tan.	Dongjeongkyul	Mandarin	mono	L	CRS
C. tachibana (Mak.) Tan.	Hongkyul	Mandarin	poly	L	CRS
C. pseudogulgul Hort. ex Shirai	Sadoogam	Pummelo	mono	L	CRS
C. grandis (L.) Osb.	Dangyooza	Pummelo	poly	L	CRS
C. aurantium L.	Jigak	Sour orange	poly	R	CRS

[z]L: Young leaves of adult trees. R: Root tips of seedlings.

[y]Citrus Research Station, National Institute of Horticultural & Herbal Science, Seogwipo-si, Jeju-do, Korea.

Chromosome Preparation

Chromosome slide preparation was performed according to Waminal et al. (2012), with minor modifications. Twenty fresh young leaves for monoembryonic accessions and twenty-five root tips for polyembryonic accessions were excised. The specimens were washed thoroughly with distill water then pretreated in 2 mM 8-hydroquinoline at 4℃ for 8 h in the dark. Then, the arrested specimens were washed with distilled water and fixed in Carnoy's solution (ethanol:acetic acid, 3:1, v/v). Prior to enzymatic maceration, the fixed specimens were washed with distilled water and macerated at 37℃ for 1 h to 2 h with an enzyme mixture containing 2% Cellulase from *Trichoerma viribe* (Sigma, Japan), 1% Macerozyme R-200 (Yakult, Japan), and 0.3% Pectolyase Y-23 (Kyowa Chemical Products Co., Ltd, Japan). The macerated specimens were briefly vortexed and centrifuged to spin down the specimens. After discarding the enzyme mixture, the specimens were washed with the Carnoy's soluton. The specimens was suspended by gentle vortexing the tube briefly then centrifuged at 5000 xg for 5 min. This step was repeated twice. The pellet was resuspended in the Carnoy's solution. The suspended pellet was mounted on glass slides and allowed air dried.

CMA/DAPI Staining

CMA/DAPI staining of metaphase chromosomes were proceeded according to Schweizer and Ambros (1994), but with modifications. The prepared chromosome slides were sequentially incubated for 30 min in McIlvaine's buffer (pH 7.0) containing 5 mM $MgCl_2$, for 1 h with 0.5 $mg \cdot mL^{-1}$ CMA, and then for 10 min in McIlvaine's buffer.

The prepared chromosome slides were counterstained with Vectashield mounting medium containing 1.5 μg·mL^{-1} DAPI (Vector Laboratories, Burlingame, CA, USA). Metaphase chromosomes were observed using an epifluorescence microscope (Leica DMRBE, Germany) with an E4 filter cassette and then the image captured using a CCD camera (INFINITY 3, Lumenera, Canada). Coordinates of photographed metaphase chromosomes on the slide were recorded, and then the slides were de-stained for 30 min in the Carnoy's solution. Then, the slides were air dried.

DNA Probes and Labeling

DNA probes were provided by the Life Sciences Research Institute (Biomedic Co., Ltd., Korea). 5S and 45S rDNA probes (Genebank accession numbers: KF156926 and MF171086) were obtained from the genomic DNA of *C. clementina* by PCR using primers 5′-CATCAGAACTCCGCAGTTAAGCG-3′ and 5′-CTGCAATCTACTTAACTCGTGC-3′ for 5S rDNA, and primers 5′-CCTTAACGAGGATCCATTG-3′ and 5′CCGTCTCTTAGGATCGACTAAC-3′ for 45S rDNA. 5S and 45S rDNA fragments were directly labelled with tetramethyl rhodamine-5-dUTP and fluorescein-12-dUTP (Roche, Switzerland), respectively, using the nick translation DNA labeling system (Enzo Life Sciences Inc., USA) according to the manufacturer's instruction. The labelled rDNA probes were verified by gel electrophoresis (Fig. 11).

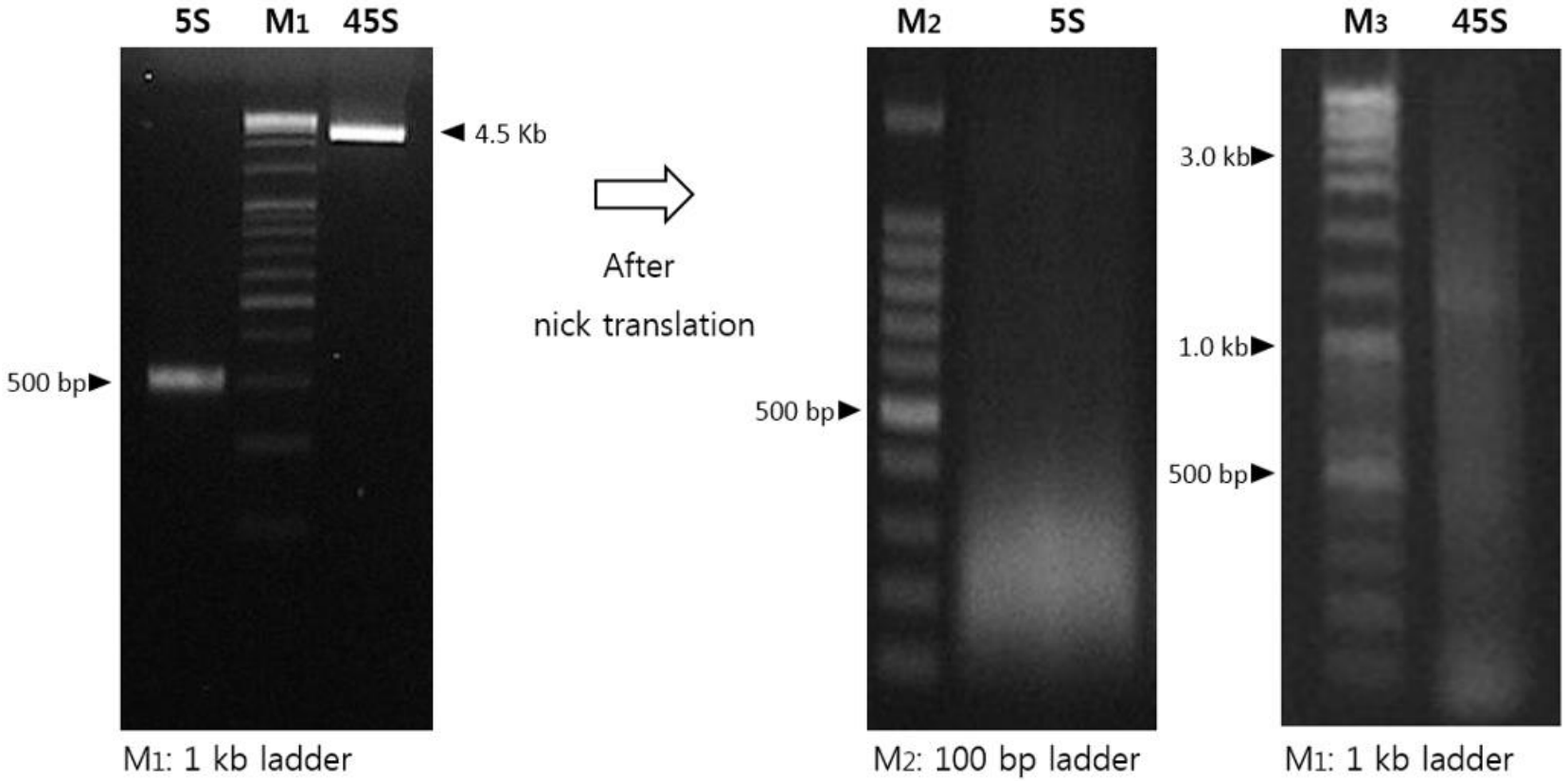

Fig. 11. 5S and 45S rDNAs isolated from *C. clementine* gDNA (left). 5S (center) and 45S (right) rDNA probes were labeled by nick translation and conjugated with TRITC and FITC, respectively.

Fluorescent *in situ* Hybridization

The procedure and conditions for FISH were based on the method described by Miranda et al. (1997), but with some modifications. The destained slides after CMA/DAPI staining were treated with RNase A (100 μg·mL^{-1}) in 2X saline-sodium citrate (SSC) buffer at 37℃ for 1 h, then washed three times in 2X SSC (for 2 min each), followed by dehydration in a series of ice-cold ethanol (70%, 80%, and 100%). The slides were air dried at least 30 min. Chromosomes on the slides were denatured at 70℃ for 2 min in 70% formamide in 2X SSC, dehydrated again in a series of ice cold ethanol and then air dried. Hybridization mixture containing 10 ng·μL^{-1} for each rDNA probes, 50% formamide (v/v), 10% dextran sulfate (w/v), and 200 ng·μL^{-1} sheared salmon sperm DNA in 2X SSC was denatured at 85℃ for 10 min. Then, 10 μL of the mixture was applied on the denatured chromosome slides. The Slides were covered with glass coverslips and sealed with rubber cement. After incubation at 37℃ overnight, the coverslips were removed and slides were washed in 60% formamide in 2X SSC at 37℃ for 20 min with agitation every 5 min, and then rinsed twice in 2X SSC at 37℃ for 5 min each. The Slides were counterstained with DAPI. FISH images were acquired using an epifluorescence microscope (Leica DMRBE, Germany) with I3 and N2.1 filter cubes for FITC and TRITC, respectively. FISH signals were analyzed and optimized using Fiji (Schindelin et al., 2012) and Adobe Photoshop CS6 (Adobe System Inc., CA, USA).

Hierarchical Cluster Analysis and Phenogram Construction

Hierarchical cluster analysis of thirty-four taxa including eleven Korean landrace citrus and twenty-three various *Citrus* species was performed based on CMA banding patterns (Yamamoto, 2007) to examine taxonomic relationship among Korean landrace citrus. A data matrix of 34 OTUs (operational taxonomic unit) X 7 variables (chromosome types based on the distribution of CMA bands) was normalized and standardized by the Euclidean distance method. Hierarchical clustering was performed using the unweighted pair-group method with arithmetic (UPGMA). Data analysis and construction of phenogram were performed using RStuio (RStudio, 2015) with ape (Paradis et al., 2004) and phangorn (Schliep, 2011) packages.

Results

The somatic metaphase chromosomes of five taxa used in this study were all diploids (2n = 18). Chromosomes were classified into six types based on the number and position of CMA positive bands according to Carvalho et al. (2005) and Moraes et al. (2007). Type A chromosomes have two telomeric and one proximal bands, type B have one telomeric and one proximal bands, type C have two telomeric bands, type D have one telomeric band, type E have no band, and type F has one subtelomeric band (Fig. 1). All *Citrus* species used in this study exhibited a high chromosomal variation with characteristic CMA banding patterns with some analogy within accessions. However, instantly recognizable heteromorphic karyotypes were evident with no variation within more than 100 cells analyzed for each accessions (Table 4). The CMA banding patterns of the five Korean landrace citrus were 1A+2B+2C+6D+7E in dongjeongkyul (*C. erythrosa*), 3B+1C+7D+5E+2F in hongkyul (*C. tachibana*), 2A+1B+3C+4D+8E in sadoogam (*C. pseudogulgul*), 1A+3B+1C+7D+6E in dangyooza (*C. grandis*), 1A+1B+1C+9D+6E in jigak (*C. aurantium*) (Table 4 and Fig. 12). All types of chromosome bands existed in all accessions except in hongkyul (*C. tachibana*), in which type A chromosome is absent but two of type F chromosomes were observed. The numbers of type A, B, and C chromosomes were lower in all accessions. In contrast, the type D and E chromosomes were remarkably constant and predominantly observed in all accession.

Table 4. CMA banding patterns and distribution of 5S and 45S rDNA loci of Korean landrace citrus.

Common name	CMA banding pattern[z]	rDNA locus		
		5S	45S	5S-45S
Dongjeongkyul	1 A + 2 B + 2 C + 6 D + 7 E	-	1 A + 2 B + 1 D	1 D
Hongkyul	3 B + 1 C + 7 D + 5 E + 2F	-	3 B	2 D
Sadoogam	2 A + 1 B + 3 C + 4 D + 8 E	-	2 A + 1 B	1 D
Dangyooza	1 A + 3 B + 1 C + 7 D + 6 E	-	1 A + 3 B	1 D
Jigak	1 A + 1 B + 1 C + 9 D + 6 E	-	1 A + 1 B	1 D

[z]A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telemetric band, D: one telemetric band, E: no band, and F: one subtelomeric band.

The distributions of 5S and 45S rDNA loci by FISH were heterogeneous among the five accessions (Table 4 and Fig. 12). All accessions possessed one 5S rDNA locus except hongkyul (*C. tachibana*), which displayed two 5S rDNA loci. The 5S rDNA loci were detected only in a subterminal region adhered to a telomeric CMA-positive band of type D chromosomes in all accession. And they always co-localized with 45S rDNA locus. All 45S rDNA loci were homotopic to CMA-positive regions. Every type A and B chromosomes possessed one 45S rDNA locus in the proximal region of the chromosomes. In a type D chromosome, the 45S rDNA locus was observed in the telomeric terminal region. In dongjeongkyul, hongkyul, and dangyooza displayed five 45S rDNA loci, while sadoogam and jigak displayed four and three 45S rDNA loci, respectively. There was no type C and E chromosome with rDNA observed.

The UPGMA phenogram of hierarchical clustering based on CMA banding patterns of Korean landrace citrus and various *Citrus* species was constructed and shows five major clusters with two minor clusters (Fig. 19). Twenty three taxa including mandarin, sweet orange, sour orange, lemon, and pummelo were selected for the clustering analysis based on CMA bands from the previous study (Yamamoto, 2007) and were clustered separately. Sadoogam was found in pummelo cluster, which is one of the major cluster that apart from other clusters at level 4. Jigak, cheongkyul, and binkyul were incorporated in mandarin cluster. Byungkyul, danyooza, dongjeongkyul, gamza, and pyunkyul were clustered together and the cluster neighbored to sweet orange cluster. Mandarin, sweet orange, and sour orange clusters appeared in the same class that separated at level 3.5. *C. tachibana* and hongkyul were clustered together forming

bifolious minor cluster which was virtually isolated from other clusters at the highest level. Jinkyul classified into one of mandarin among Korean landrace species was substantially apart from *C. sunki* and also formed a bifolious minor cluster with *C. juno*. Another UPGMA phenogram of hierarchical clustering constructed based on CMA banding patterns combined with 5S and 45S rDNA loci of 11 Korean landrace citrus was constructed (Fig. 20), which shows slightly different clustering pattern among the 11 taxa. There were two bifolious clusters, one of which consisted of dongjeongkyul and gamza and the other contained cheongkyul and byungkyul. The rest taxa were completely separated and formed simplicifolious clusters.

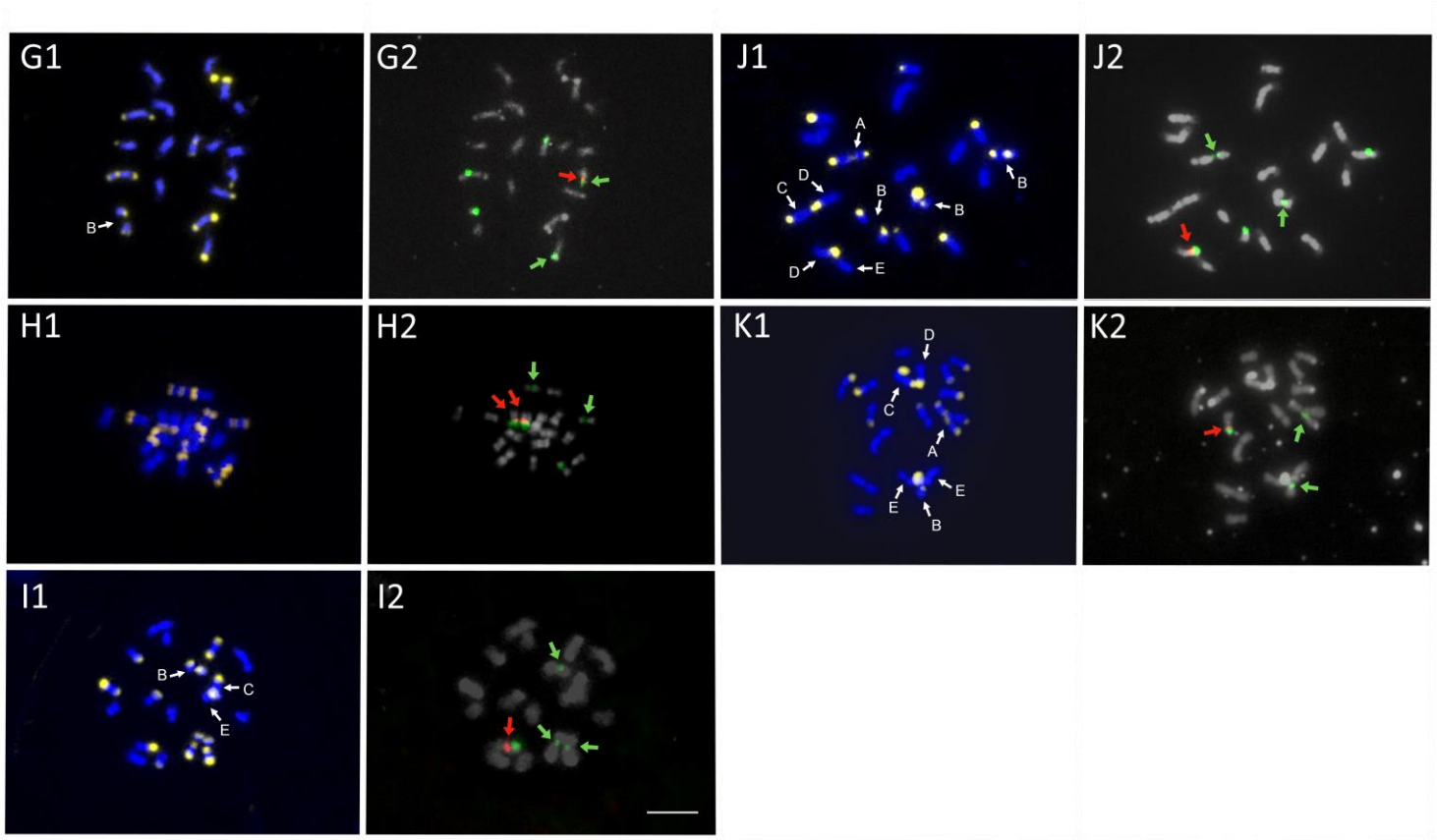

Fig. 12. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of Korean landrace citrus. G: dongjeongkyul, H: hongkyul, I: sadoogam, J: dangyooza, and K: jigak. Scale bar = 5 ㎛.

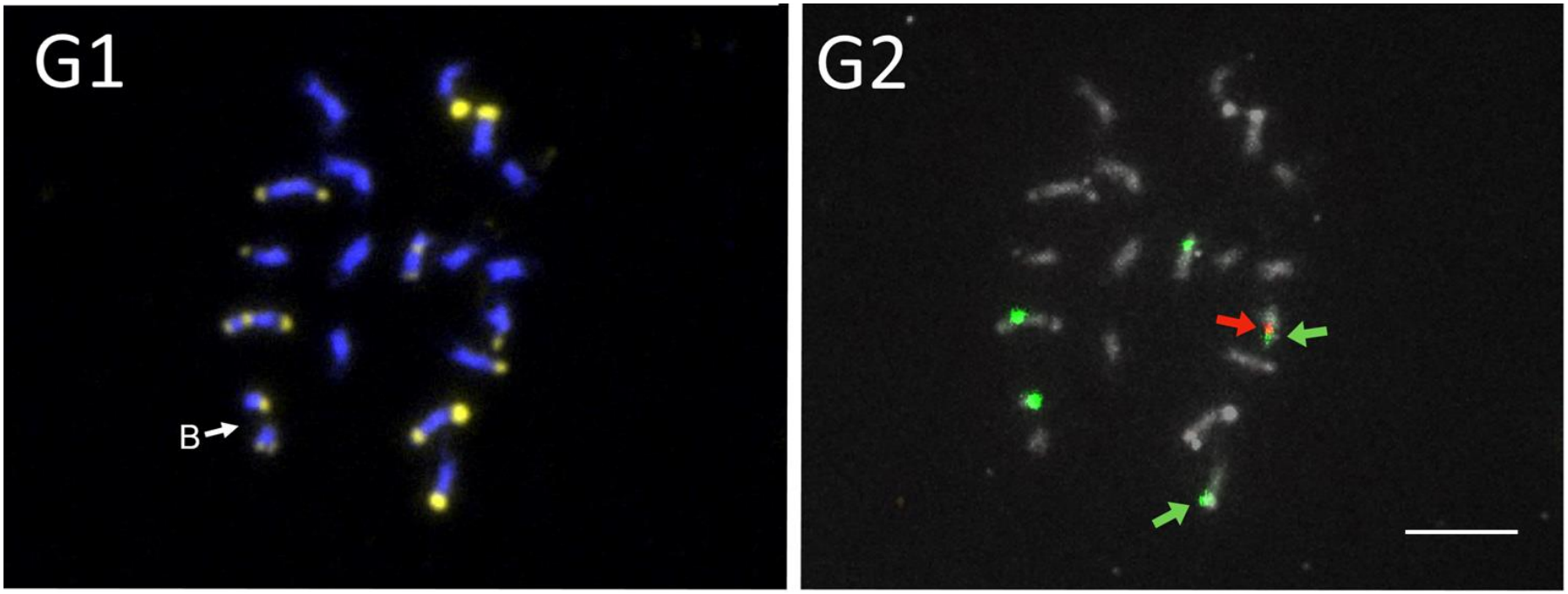

Fig. 13. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of dongjeongkyul. Scale bar = 5 ㎛.

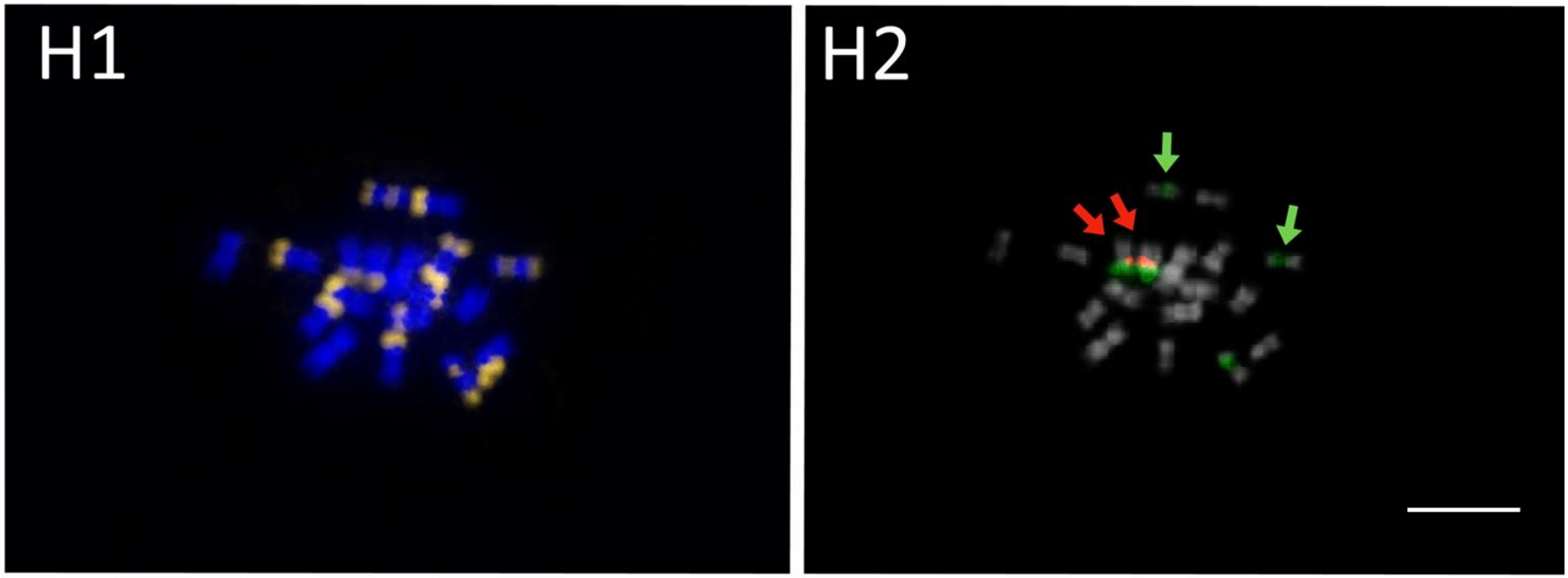

Fig. 14. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of hongkyul. Scale bar = 5 ㎛.

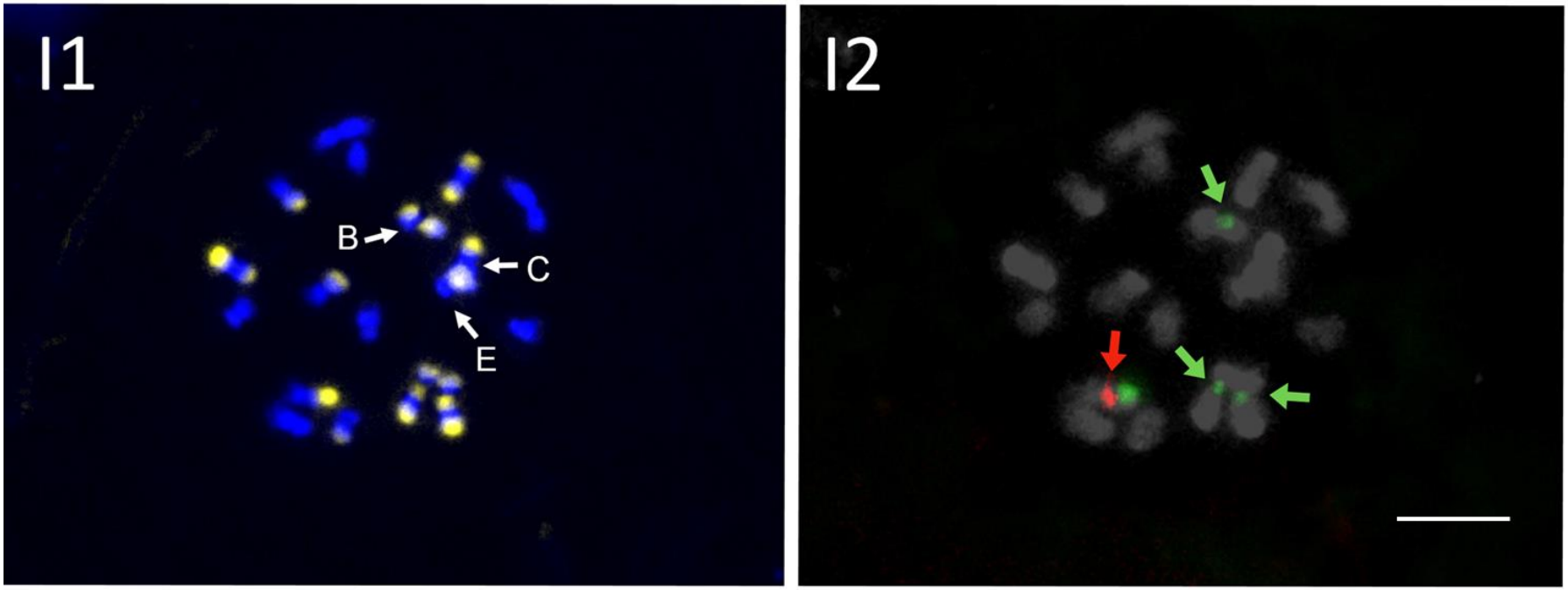

Fig. 15. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of sadoogam. Scale bar = 5 ㎛.

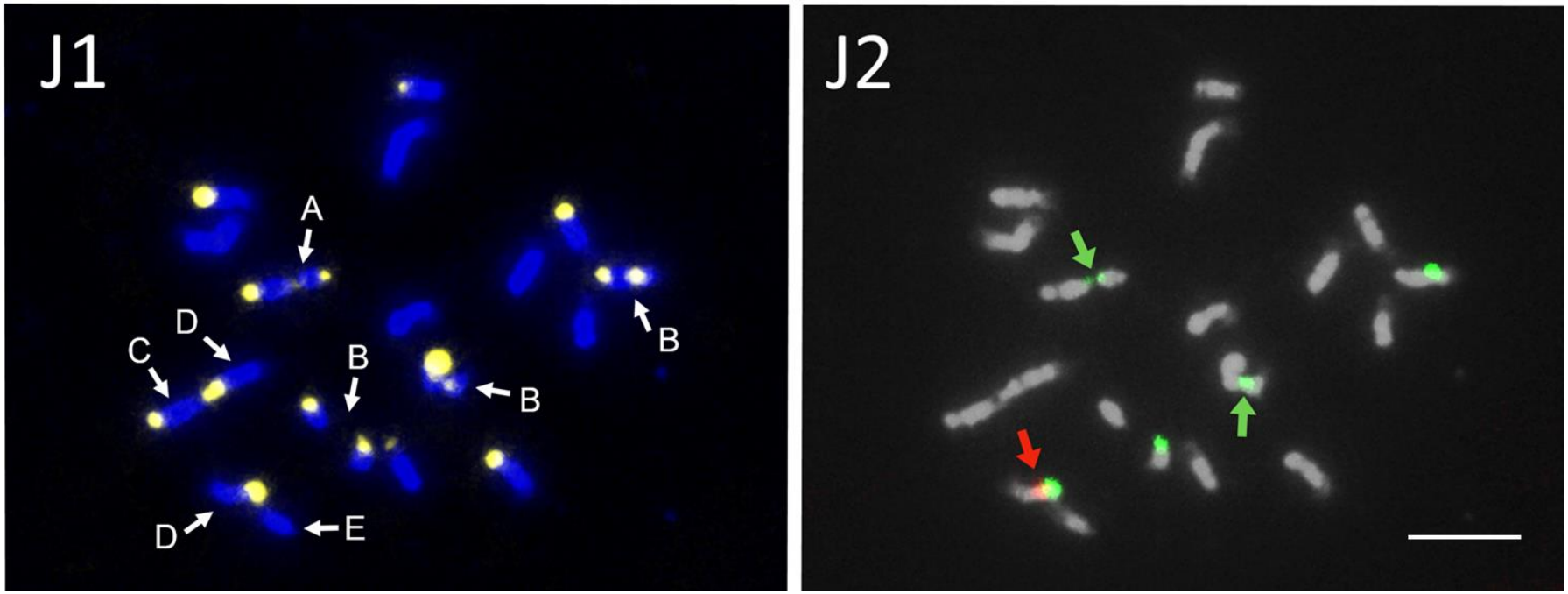

Fig. 16. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of dangyooza. Scale bar = 5 ㎛.

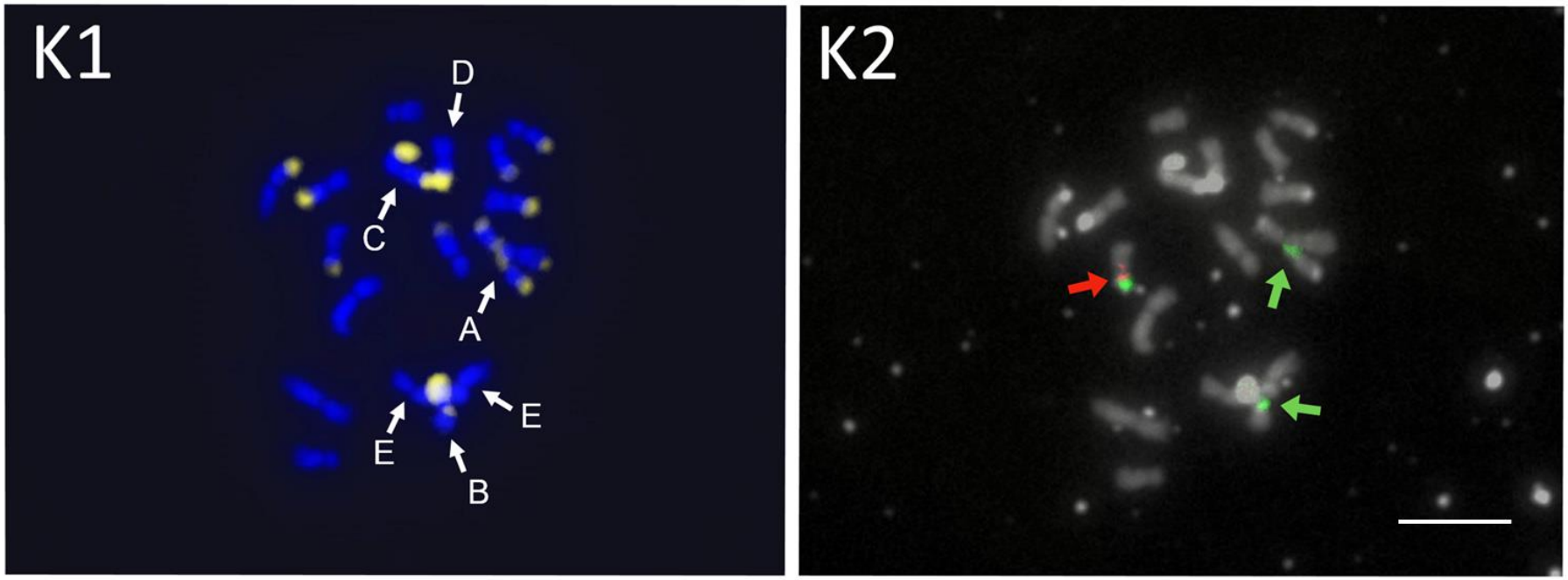

Fig. 17. CMA/DAPI stained chromosomes (denoted by 1) and 5S and 45S rDNA loci on the chromosomes by FISH (denoted by 2) of jigak. Scale bar = 5 ㎛.

Discussion

Karyotype analysis of Korean landrace citrus based on CMA banding patterns revealed that most accession tested in this study included a very few number of type A or B chromosomes. It is thought that type A and B chromosomes arose from *C. maxima* and *C. medica*, respectively (Guerra, 1993; Befu et al. 2001). Type C chromosome may be a characteristic chromosome type for mandarin (Cornélio et al., 2003). Type D and E chromosomes are considered as the basic chromosome types in *Citrus* (Guerra, 1993; Miranda et al., 1997a; Befu et al., 2000, 2001; Cornélio et al., 2003; Yamamoto and Tominaga, 2003; Carvalho et al., 2005; Brasileiro-Vidal et al., 2007; Moraes et al., 2007a; Yamamoto et al., 2007) .

The chromosome configuration of dongjeongkyul displayed the 1A/45S+2B/45S+2C+4D+1D/45S+1D/5S-45S+7E pattern, with no chromosome bearing a solitary 5S rDNA locus (Table 4 and Fig. 14). The existence of a type D/45S locus enabled to differentiate dongjeongkyul from the other accessions. The chromosome configuration of dongjeongkyul is almost identical to those of the gamza mandarin (1A/45S + 2B/45S + 2C + 5D + 1D/45S + 1D/5S-45S + 6E). Dongjeongkyul was classified as *C. erythrosa* Hort. ex Tanaka by Tanaka (1961), while Swingle (1943) included it in *C. reticulata* where mandarins and mandarin hybrids are belonged. Tanaka (1969) described that *C. erythrosa Hort. ex Tanaka* is a vermillion mandarin of Himalayan origin and a common mandarin in India and costal China. It was revealed that *C. erythrosa Hort. ex Tanaka* is phylogenetically closely related to mandarin based on RFLP, RAPD, and chloroplast DNA sequence analysis data (Federici et al., 1998; Nicolosi et al., 2000;

Lu et al., 2011), where they used Chinese accessions (also known as Fuzhu mandarin). But, dongjeongkyul was separated from mandarin groups and clustered with *C. junos* based on plastid *trn*L-*trn*F sequence (Jung et al., 2005). Also, dongjeongkyul has smooth rind in bright yellow with rugged skin (Kim et al., 2008), which are different morphological traits from Fuzhu mandarin. The CMA banding patterns of most *C. reticulata* possessed no type A chromosome and less numbers of type B and C chromosomes, but the numbers of type D and E chromosomes are larger than that of dongjeongkyul (Yamamoto, 2007). In dongjeongkyul, type A chromosome was observed with 2B + 2C chromosomes, which suggests that dongjeongkyul may be a mandarin hybrid phylogenically diverged from *C. reticulata*.

Although Swingle and Reece (1967) claimed *C. tachibana* might be a satellite species of *C. reticulate*, Tanaka (1977) classified it with a mandarin species. Recent studies using molecular techniques, including inter-simple sequence repeat (ISSR) marker analysis (Fang et al., 1998), random amplified polymorphic DNA (RAPD) sequence-characterized amplified region (SCAR) marker analysis (Nicolosi et al., 2000), and plastid *trn*L-*trn*F sequence analysis using hongkyul (Jung et al., 2005) also supported Tanaka classification. Penjor et al. (2013) also classified *C. tachibana* into the minor subcluster within the mandarin cluster based on *mat*K gene sequence analysis. The chromosome configuration of hongkyul in this study displayed the 3B/45S+1C+5D+2D/5S-45S+5E+2F pattern (Fig. 15), which is almost identical to the CMA banding pattern, 1C+10D+5E+2F (without FISH data) reported by Yamamoto and Tominaga (2003). It has been claimed that the proximal CMA band could be small with

low intensity of colour, and this could lead to misidentify type B chromosome as a type D chromosome (Brasileiro et al., 2007). It is assumed that three type D chromosomes classified by Yamamoto and Tominaga (2003) are probably type B chromosomes. Type F chromosome supplemented by Yamamoto and Tominaga (2003), which was observed only in some Japanese mandarins, was also found in this study. Hongkyul seems to be the same accession of *C.tachibana* that Yamamoto and Tominaga (2003) used for their study. Two type D chromosomes bearing 5S and 45S rDNA co-localized loci was observed in this study. Moraes et al. (2007) also observed 2D/5S-45S as well as B/45S chromosome in *C. tachibana*. However, this CMA banding pattern differs from the one described here and shown by Yamamoto and Tominaga (2003). This may be due to geographical differences of accessions used in the previous studies. Yamamoto and Tominaga (2003) presented that type F chromosome has a proximal CMA positive band. However, this study confirmed that the CAM positive band of type F chromosome is located in the subtelomeric region of chromosome. This finding was verified by Moraes et al. (2007). Kang et al. (2008) reported total four 45S rDNA loci, two in proximal and two in telomeric regions of chromosome. It seems that this study presents the most reliable chromosome configuration of hongkyul by CMA banding pattern and FISH with rDNA probes.

The CMA banding patterns of sadoogam and dangyooza displayed 2A+1B+3C+4D+8E and 1A+3B+1C+7D+6E, respectively (Fig. 16 and 17). *C. pseudogulgul* hort. ex Tanaka and *C. grandis* (L.) Osbeck are classified into pummelo subgroup (CPVO, 2014). The large numbers of type A, B, and C chromosomes, which is a typical

CMA banding patterns commonly found in *C. Maxima*, (Guerra, 1993; Miranda et al., 1997; Befu et al., 2001), were observed in sadoogam and dangyooza. They were also clustered together with other pummelo in the same group in the phylogenetic tree drawn based on *trn*L-*trn*F sequence analysis (Jung et al., 2005). With some variation, leaves, flowers, and fruits of pummelo are usually the largest of other *Citrus* (Davies and Albrigo, 1994). Morphological traits of sadoogam and dangyooza are closely in accord with those characteristics (Kim, 1988). What is noteworthy is that the CMA banding pattern of dangyooza was very similar to that of the byungkyul mandarin with a minor difference in the numbers of type B and D chromosomes. Although *C. platymamma* Hort. ex Tan. was classified into a mandarin group by Tanaka, it was clustered in the pummelo group after determining the *mat*K sequences of citrus and its relatives (Penjor et al., 2013). This may be able to explain the resemblance of the CMA banding patterns between them. However, E/5S chromosome observed only in byungkyul mandarin, could be the distinct chromosome marker to distinguish byungkyul mandarin from dangyooza as well as other Korean landrace citrus. Our CMA banding pattern of dangyooza was not accorded with those of previous studies using *C. grandis* (Miranda et al., 1997; Befu et al., 2000). Pummelos are broadly cultivated in worldwide, where many cultivars and accessions are existed. This indicated that variations have been reported in chromosome composition of *C. grandis*. The 45S rDNA loci generally co-localizes with CMA positive region (Brasileiro et al., 2007), CMA null and DAPI negative region (Carvalho et al., 2005), or hypo-methylated fragile site (Lan et al., 2016), as were observed in the present study. The proximal CMA

bands of every type A and B chromosomes found in sadoogam and dangyooza possessed a solitary 45S rDNA locus. And one of type D chromosomes in both accessions displayed a co-localized 5S-45S rDNA locus. It seems that 45S rDNA loci tend to be located in proximal region of type A and B chromosomes first and 5S rDNA locus normally co-localized with 45S rDNA locus in telomeric region of type D chromosome. This distributional pattern of rDNA loci was in agreement with the previous studies (Pedrosa et al., 2000; Moraes et al., 2007a). However, Miranda et al. (1997a) observed rDNA site in telomeric heterochromatin region of type C chromosome of *Fortunella crassifolia* Swing., and Brasileio et al. (2007) reported that co-localized 5S-45S rDNA loci on type B chromosomes in *Poncirus*. These specific types of chromosomes could be chromosome markers to differentiate those *Citrus* species. This suggests that sadoogam and dangyooza have none or very limited phylogenetic relationship with kumquats and *Poncirus*. However, the existence of D/5S-45S chromosome, which is homogeneous among mandarins and considered as chromosome marker for this group (Moraes et al., 2007), suggests that sadoogam and dangyooza might be originated from a cross between pummelo and mandarin.

The chromosome composition of jigak was 1A+1B+1C+9D+6E (Fig. 18), which is very similar to the CMA banding pattern of *C. aurantium* L. reported previously with 1A+1B+1C+7D+8E (Yamamoto et al., 2007). Determination of chromosome type D was difficult because of its abundance in numbers and its varied size in many accessions. Sporadically, type D chromosome bands were seemingly absent and appear like type E chromosomes. Moreover, perceptibility of CMA-stained bands is often affected by

staining intensity (Cornelio et al., 2003). Hence, this study provides the latest update and more reliable CMA banding pattern for common *C. aurantium*. Three 45S rDNA loci, one each in the proximal region of type A and B chromosomes, and one in the telomeric region of type D chromosome, were observed. This was in accord with the previous report by Kang et al. (2008) in terms of the number of 45S rDNA loci, but not with their positions which they showed polymorphism. One 5S rDNA locus co-localized with the 45S rDNA on type D chromosome was observed. Moraes et al. (2007) suggested that D/5S-45S chromosome could be a marker chromosome for mandarins because this chromosome was homogeneous in all mandarin accessions they studied. And it was proposed that sour orange derived from hybridization between pummelo and mandarin (Nicolosi et al., 2000). Jung et al. (2005) also reported close relationship between *C. pesudogulgul*, *C. grandis*, *C. aurantium*, and *C. tachibana* by clustering them together based on plastid *trn*L-*trn*F sequence analysis. These previous findings could explain the resemblance of chromosome configurations between accessions in this study. In this study, the 45S rDNA loci showed numerical variation and positional conservation, whereas conserved number and localization of 5S rDNA loci were observed.

In this study, two UPGMA phenogram of hierarchical clustering were constructed based on karyotypical phenetic characteristics of analyzed taxa. The phenogram constructed only based on CMA banding patterns of 11 Korean landrace citrus and 23 various *Citrus* species (Fig. 19) shows interspecific differentiation of analyzed 34 taxa, and it seemed that the major clustering tendency resembled Swingle's taxonomic

system. It has been suggested that cytogenetic characteristics including karyotype features could be used to differentiate some species taxonomically (Yamamoto et al., 1984; Murray et al, 1992; Seijo and Fernandez, 2003; Badr, 2007; Sheidai et al., 2011). Mandarin, sweet orange, sour orange, lemon, and pummelo taxa used in this study for the hierarchical clustering analysis based on CMA bands were clustered separately (Fig. 19). Furthermore, most of Korean landrace species were grouped together in a cluster and distinguished from those other clusters. This is in accord with the previous report based on the *trn*L-*trn*F sequence analysis (Jung et al., 2005), where Korean landrace citrus were clustered together distanced from other citrus taxa. Among Korean landrace citrus, hongkyul is the only species that possessed type F chromosome (Table 1 and 2). This obtrusive karyotype characteristics differentiated hongkyul from the other Korean landrace citrus as well as other citrus tax analyzed in this study (Fig. 19). Hongkyul was clustered with *C. tachibana* and their bifolious clade split away from the others at high level of class in the UPGMA phenogram. This result suggested that these two taxa might have different phylogenetic history from other *Citrus*. Sadoogam was clustered with pummelo and this result correspond with the karyotype of sadoogam, which displayed two chromosomes of type A (Table 4 and Fig. 15). The karyotype difference between jinkyul and *C. sunki* was discussed in Chapter I (Fig. 5) and it was confirmed by UPGMA hierarchical clustering analysis here. Jinkyul was separated apart from *C. sunki* as well other Korean landrace citrus (Fig. 19). Jung et al. (2005) pointed out that *C. aurantium* and *C. grandis* are closely related each other than other taxa. In the second UPGMA phenogram (Fig. 20) constructed based on CMA banding

patterns combined with 5S and 45S rDNA loci of 11 Korean landrace citrus, the two accessions were clustered together forming a bifolious clade. Another bifolious clade contains dongjeongkyul and gamza, while others were simplicifolious. Overall the second UPGMA phenogram shows a simplicifolious type of dendrogram (Fig. 20), which implies the strong dissimilarity of karyotype among Korean landrace citrus. Hongkyul, sadoogam, and jinkyul that were disconnected from the other Korean landrace citrus clusters (Fig. 19) were separated in serial order and formed individual simplicifolious clusters (Fig. 20).

This study demonstrated high levels of heterogeneity and variation in Korean landrace citrus by CMA/DAPI staining and FISH with rDNA probes. It showed the existence of large numbers of type D and E chromosomes which are remarkably constant in all *Citrus* (Miranda et al., 1997) and are considered to be the basic type chromosomes in *Citrus* (Guerra, 1993; Miranda et al., 1997; Befu et al., 2000, 2001; Cornelio et al., 2003; Yamamoto and Tominaga, 2003; Carvalho et al., 2005; Yamamoto et al., 2005, 2007). Also, type A, B, and C chromosomes are thought to be originated from *C. maxima* and *C. medica*, respectively (Guerra, 1993; Befu et al., 2001), while type C chromosome being a characteristic chromosome type in mandarins (Cornélio et al., 2003). Therefore, chromosome configurations of Korean landrace citrus analyzed here suggest that all accessions in this study might be hybrids that have blood or phylogenetic relationships more or less with mandarin and pummelo.

This is the first study representing the CMA banding pattern and physical map of 5S and 45S rDNA loci using FISH in dongjeongkyul (*C. erythrosa* hort. ex. Tanaka) and

sadoogam (*C. pseudogulgul* hort. ex Shirai). Furthermore, this study provides high resolution of chromosome configurations, which could be complementary to previous studies, and elucidated phylogenetic relationships of some Korean landrace citrus at the cytogenetic level.

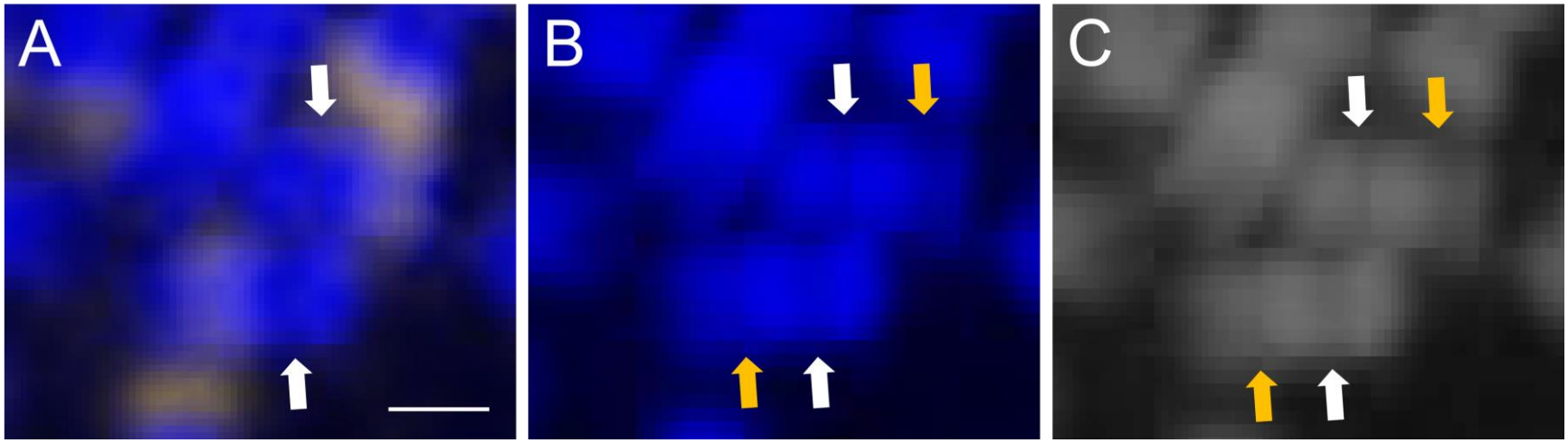

Fig. 18. Type F chromosomes observed in hongkyul. A: CMA/DAPI stained chromosomes; B: DAPI stained chromosomes; C: grey scale of DAPI stained chromosomes. Scale bar = 1㎛.

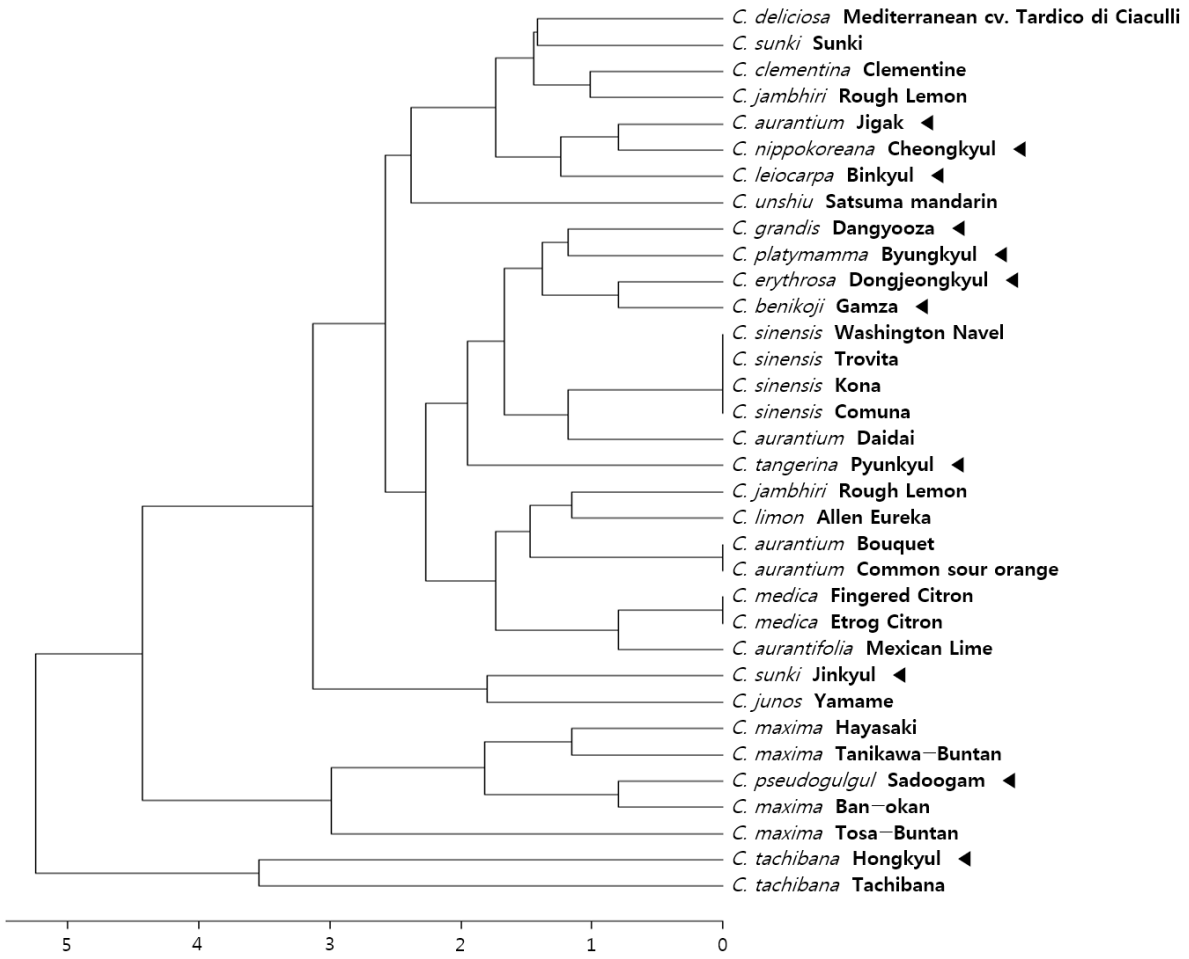

Fig. 19. UPGMA phenogram of hierarchical clustering constructed based on CMA banding patterns of Korean landrace citrus and 23 various *Citrus* species. ◀ indicates Korean landrace citrus.

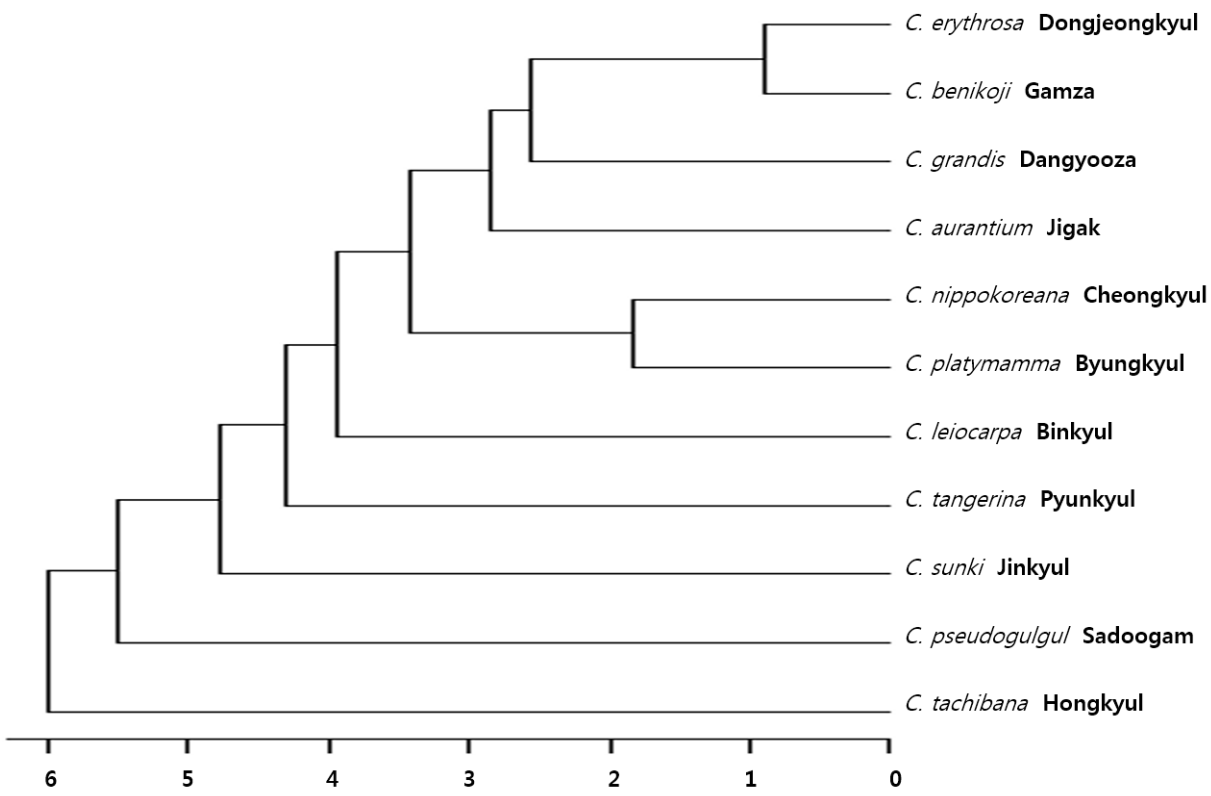

Fig. 20. UPGMA phenogram of hierarchical clustering constructed based on CMA banding patterns combined with 5S and 45S rDNA loci of 11 Korean landrace citrus.

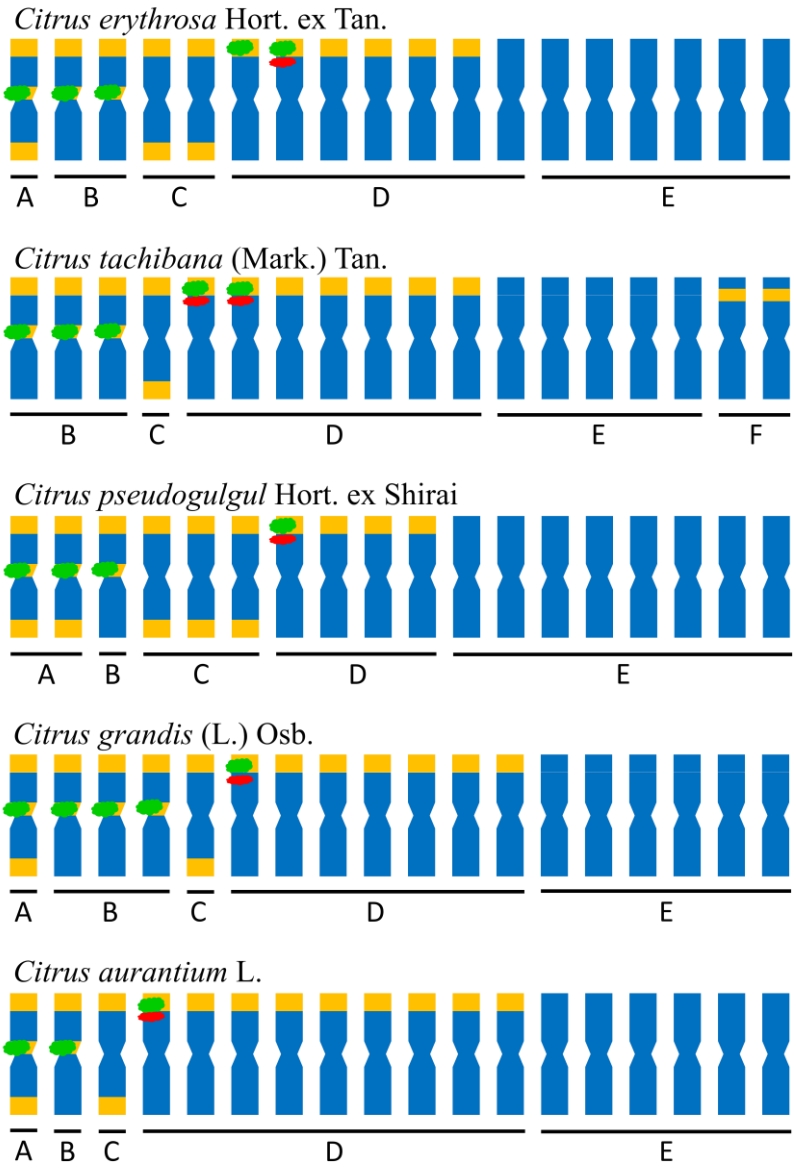

Fig. 21. Representative idiograms of Korean landrace citrus showing the distribution of CMA positive regions (in yellow), DAPI stained regions (in blue), 5S rDNA loci (in red), and 45S rDNA loci (in green). Alphabet letters under lines represent chromosome types.

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Conclusions

Karyotype analysis of eleven Korean landrace citrus were carried out using CMA banding patterns and rDNA loci by FISH. The results revealed heterogeneous CMA banding patterns and distribution of 5S and 45S rDNA loci among the eleven accessions. Type A chromosome, which is considered to be driven from *C. maxima* (Guerra, 1993; Befu et al., 2001), were observed in all accessions except pyunkyul and hongkyul. Sadoogam displayed CMA banding pattern with two chromosomes of type B, while others possessed only one chromosome of the type. This suggests that Korean landrace citrus possessed type A chromosome are phylogenetically related to pummelo. Except binkyul, all accessions displayed type B chromosome in their CMA banding patterns. They might be in relation to *C. medica* during their evolution (Guerra, 1993; Befu et al., 2001), especially pyunkyul and hongkyul which possessed three chromosomes of type B, might have more citron blood compared to the other accessions. Type C, D, and E chromosomes were commonly found in all accessions. Type C chromosome is commonly found in mandarin and mandarin hybrids (Cornélio et al., 2003). Type D and E chromosomes are thought to be basic chromosome type in *Citrus* (Guerra, 1993; Miranda et al., 1997a; Befu et al., 2000, 2001; Cornélio et al., 2003; Yamamoto and Tominaga, 2003; Carvalho et al.,

2005; Brasileiro-Vidal et al., 2007; Moraes et al., 2007a; Yamamoto et al., 2007). Therefore Korean landrace citrus analyzed in this study are presumed to be mandarin hybrids or phylogenetically related to mandarins. Type F chromosome has been only observed in *Citrus* that were claimed to be Japanese native mandarin (Yamamoto and Tominaga, 2003). In this study, two chromosomes of type F were observed in hongkyul. However the position of CMA bands are in subtelomeric region of the chromosomes, whereas the previous study reported that they are found in proximal region. Type F chromosome having CMA band in subtelomeric region verified by Moraes et al. (2007). Brasileiro et al. (2007) reported that type B chromosome could be misidentified as a type D chromosome. In consideration of these previous findings, it seems that this study presents the most updated and reliable CMA banding pattern of hongkyul. Moreover, this study presents CMA banding patterns of byungkyul (*C. platymamma*), cheongkyul (*C. nippokoreana*), dongjeongkyul (*C. erythrosa*), and sadoogam (*C. pseudogulgul*) for the first time. 45S rDNA loci identified in this study were found in type A, B, D and E chromosomes, but not type C chromosome. All of type A and B chromosomes bear one 45S rDNA locus in only proximal region of the chromosome. In type D chromosome, 45S rDNA loci were detected in telomeric region. Byungkyul possessed type E

chromosome bearing a solitary 5S rDNA locus in telomeric region, which could be a notable karyotype characteristic as a chromosome marker. In all accessions, one or two chromosomes of type D bearing co-localized 5S-45S rDNA loci were observed. In this case, 5S rDNA locus is adjacent to 45S rDNA locus and positioned toward to proximal region. Another solitary 5S rDNA locus was observed in cheongkyul, which could clearly distinct this species from other Korean landrace citrus. All rDNA loci found in this study were homotopic to CMA positive region, except type E/5S chromosome.

This is the first study representing the CMA banding pattern and physical map of 5S and 45S rDNA loci using FISH in Korean landrace citrus. In addition to that, UPGMA phenograms of hierarchical clustering based on karyotypical phenetic characteristics of Korean landrace citrus and other *Citrus* were constructed. The phenogram shows that most of Korean landrace species were grouped together and distinguished from other *Citrus*. Another UPGMA phenogram for 11 Korean landrace citrus constructed based on CMA banding patterns combined with 5S and 45S rDNA loci together shows that most Korean landrace citrus were clustered separately in a simplicifolious manner. Chromosome configurations of Korean landrace citrus analyzed here suggest that all accessions might be hybrids that have

blood or phylogenetic relationships more or less with mandarin and pummelo. Furthermore, this study provides high resolution of chromosome configurations, which could be complementary to previous studies, and elucidated phylogenetic relationships of some Korean landrace citrus at the cytogenetic level. The karyotype results in this study are not always in accord with previous reports and Korean landrace citrus were clustered distinguishingly apart from other *Citrus* in the UPGMA phenogram of hierarchical clustering. This study, therefore, suggests that Korean landrace citrus and *Citrus* species, which share the same species epithet, might be homonymous species. Further studies including diversity analysis using simple sequence repeat (SSR) marker, chloroplast barcoding marker, sequence analysis of rDNA internal transcribed spacer (ITS) regions, and whole genome sequencing using next generation sequencing (NGS) may be required to more precisely elucidate the phylogenetic relationships among them.

Abstract in Korean

감귤은 세계적으로 널리 재배되고 있으며, 가장 중요한 과수 작물 중의 하나이다. 국내에서 감귤은 천년 이상의 긴 재배역사를 갖고 있으며, 제주도에서는 현재 그 재배면적과 생산량 측면에서 가장 중요한 농작물이다. 재래종 감귤은 다양한 유전적 변이성을 갖고 있어서 유전적 침식의 방지를 위한 유전자원으로써 중요한 가치를 갖는다. 본 연구는 11개의 한국 재래 감귤에 대하여 CMA 염색형태와 rDNA loci를 이용한 핵형 분석을 통하여 그들의 근연관계를 이해하고자 수행하였다. 감귤 염색체는 CMA 염색 밴드의 수와 위치에 따라 각각 여섯 개의 유형으로 분류되었다. A형 염색체는 두개의 말단부 밴드와 하나의 중심절 밴드; B형 염색체는 하나의 말단부 밴드와 하나의 중심절 밴드; C형 염색체는 두개의 말단부 밴드; D형 염색체는 하나의 말단부 밴드; E형 염색체는 밴드 없음; F형 염색체는 하나의 말단부에 가까이 위치한 밴드를 갖는다. 감자(*C. benikoji*)의 핵형은 1A/45S + 2B/45S + 2C + 5D + 1D/45S + 1D/5S-45S + 6E 으로 관찰되었다. 독립적인 5S rDNA는 병귤(*C. platymamma*) (1A/45S + 2B/45S + 1C + 6D + 1D/45S + 1D/5S-45S + 5E + 1E/5S) 과 청귤(*C. nippokoreana*) (1A/45S + 1B/45S + 1C + 7D + 1D/5S + 1D/45S + 1D/5S-45S + 5E) 에서 관찰되었다. 병귤의 CMA 염색형태와 rDNA loci로 미루어 보아 병귤은 그 계통 발생과정에서 문단, 스위트 오렌지, *Papeda* 등과 관련이 있는 것으로 판단된다.

청귤을 다른 재래귤과 구별하는 특징적인 D/45S형 염색체는 염색체 마커로 이용 가능할 것이다. 진귤(*C. sunki*) (1A/45S + 1B/45S + 1C + 10D + 2D/5S-45S + 3E) 은 문단과 연관이 있는 것으로 사료된다. 편귤(*C. tangerina*) (3B/45S + 2C + 7D + 1D/5S-45S + 5E) 의 핵형으로 보아 *C. grandis* 와 *C. reticulata* 간의 잡종으로 판단되며, 빈귤(*C. leiocarpa*) (1A/45S + 1C + 6D + 2D/45S + 2D/5S-45S + 6E) 은 만다린 및 문단과 유전적으로 관계가 있는 것으로 사료된다. 서로 다른 핵형들에 의하여 여섯 개의 재래귤을 분리하였다. 나머지 재래귤의 CMA 염색형태는 각각 다음과 같다: 동정귤(*C. erythrosa*), 1A+2B+2C+6D+7E; 홍귤(*C. tachibana*), 3B+1C+7D+5E+2F; 사두감(*C. pseudogulgul*), 2A+1B+3C+4D+8E; 당유자(*C. grandis*), 1A+3B+1C+7D+6E; 지각(*C. aurantium*), 1A+1B+1C+9D+6E. 홍귤은 A형 염색체가 관찰되지 않은 반면, 두 개의 F형 염색체가 관찰되었다. 조사된 모든 품종에서 A, B, C형 염색체의 수가 상대적으로 적었다. 반면, D형과 E형 염색체의 수는 두드러지게 많았으며 그 차가 거의 없었다. FISH법에 의한 5S와 45S rDNA loci의 분포는 품종간에 뚜렷한 차이를 보였다. 모든 품종들이 하나씩의 D/5S-45S형 염색체를 갖고 있었다. 관찰된 모든 45S rDNA loci는 CMA 염색 밴드와 위치가 일치 하였다. 모든 A와 B형 염색체들은 그 중심절에 45S rDNA locus가 존재하였다. C형 염색체에서는 rDNA locus가 관찰되지 않았다. 본 연구 결과, 한국 재래 감귤은 모두 잡종이며, 만다린 및 문단과 유전적 근연관계에 있음을

알 수 있었다. 한국 재래귤의 핵형분석 결과를 이용하여 계층 군집화 분석(HCA: hierachical cluster analysis)을 하고 UPGMA 방법에 의한 계통수를 그려본 결과, 재래귤 품종 간에 개별적인 군을 형성하는 것을 확인하였다. 본 연구 결과는 고해상도의 염색체 핵형을 제시하여 기존에 보고된 핵형 분석 결과를 보완하였으며, 한국 재래귤의 근연관계를 세포유전학적 관점에서 제시하였다.

But by the grace of God I am what I am

1 Corinthians 15:10

Thus will I bless thee all my life long and in thy name I will lift up my hands

Psalm 63:4

나의 사랑하는 가족들

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