

Phenotypic and Genetic Characterization of Three Different Types of Dangyooza (*Citrus grandis*), Korean Landrace Citrus

Kyung Uk Yi¹, Kook Lhim Zhin², Eun Ui Oh², Sang Suk Kim³, Ho Bang Kim¹, and Kwan Jeong Song^{2,4*}

¹Life Sciences Research Institute, Biomedic Co., Ltd., Bucheon 14548, Korea,

²Faculty of Bioscience and Industry, SARI, Jeju National University, Jeju 63243, Korea,

³Citrus Research Institute, National Institute of Horticultural & Herbal Sciences, RDA, Jeju 63607, Korea

⁴Research Institute for Subtropical Agriculture & Biotechnology, Jeju National University, Jeju 63243, Korea

*Corresponding author: Kwansong@jejunu.ac.kr

Abstract

Dangyooza (DY), a Korean landrace citrus similar to pomelo (*Citrus grandis* L. Osbeck), has been consumed as a fruit and used in folk remedies to treat colds for centuries, and it is still used as a garden tree. DY has two natural variants, the buk-daengyooza (BDY) and the seol-daengyooza (SDY) based on distinguishable phenotypic fruit characteristics. However, there is little scientific analysis available not only on the morphological and chemical properties but also on the genetic basis of these natural variants. To gain a better understanding of DY, several morphological, phytochemical, chromosomal, and genetic traits were analyzed in DY and its natural variants, BDY and SDY. Morphological characteristics such as leaf and fruit shape, fruit hardness, and peel thickness were used to discriminate SDY from DY and BDY. Notably, SDY produced smaller fruit with thinner peels than those of DY and BDY. The major flavanones occurring in citrus were also markedly higher in SDY than those of DY and BDY. However, chromosomal configuration and genetic diversity analysis using random amplified polymorphic DNA, simple sequence repeat, and plastid *trnL/F* barcoding markers were unable to clearly discriminate the phylogenetic relationships among the DY types. This suggests that SDY might have arisen from somatic mutation, perhaps as a nucellar seedling.

Additional key words: genetic resources, karyotype, morphology, plastid DNA, RAPD

Introduction

Citrus is an economically important fruit crop through the world, and accounted for about 17% of world fruit crop production in 2018 (FAO, 2018). In Jeju, Korea, citrus is the most important commercial agricultural product in terms of area and production. Moreover, citrus plays a part in the local economy and social aspect of Jeju. In the 18th century, landrace citrus fruits, including dongjeongkyul (*Citrus erythrosa* Hort. ex Tan.), yoogam (*C. suavissima* Hort. ex Tan.), and dangyooza (DY; *C. grandis* L. Osb.) were utilized as offerings for the king, and at least 20 species or natural

Received: August 17, 2020

Revised: September 3, 2020

Accepted: September 14, 2020

 OPEN ACCESS



HORTICULTURAL SCIENCE and TECHNOLOGY
39(1):96-105, 2021
URL: <http://www.hst-j.org>

pISSN : 1226-8763
eISSN : 2465-8588

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

Copyright©2021 Korean Society for Horticultural Science.

This study was supported by Korea Institute of Planning and Evaluation for Technology in Food, Agriculture, Forestry and Fisheries (IPET) through Golden Seed Project (No. 213007-05-3-SBQ10) funded by Ministry of Agriculture, Food and Rural Affairs (MAFRA).

variants were reported in historic literature (Kim, 1988; Kim et al., 2001; Moon et al., 2007). However, only 12 landrace species are preserved in present-day germplasm collections (Kim et al., 2001).

Among the Korean landrace citrus types, DY is still preserved as a garden tree planted in home, gardens and backyards. It is valued by locals because of its large, edible fruits and for use in folk remedies to treat colds. In Jeju, DY has also been produced commercially and been sold for use as a nutritional supplement, tea, homeopathic medicine, and traditional food for ritualistic rites to honor ancestors. Citrus peel, especially dried citrus peel, is an essential homeopathic medicine in Oriental medicine (Kang et al., 2005). The chemical properties of *Citrus* spp. may account for its several medicinal uses. About 60 flavonoids are found in *Citrus* spp. In particular, citrus peel contains many flavanones, flavones, and polymethoxyflavones (PMFs) (Yang et al., 2019). It is well reported that PMFs in citrus are detected in the peel of citrus fruits (Nogata et al., 2006; Gattuso et al., 2007; Yang et al., 2019). The medicinal effects of flavonoids extracted from citrus include antioxidant activity (Jeong et al., 1997; Lim et al., 2006; Mokbel and Hashinaga, 2006), antimicrobial activity (Han and You, 1988), and anticancer activity (Hertog et al., 1992; Yoshimizu et al., 2004; Benavente-Garcia and Castillo, 2008). Despite the high potential of *Citrus* for use in medicines, particularly Korean landraces or endemic citrus, there have been few studies conducted on the taxonomy, genetics, and breeding of Korean landrace citrus as a modern breeding resource.

The phylogenetic relationships of *Citrus* are highly complicated and ambiguous. Early taxonomic studies of the *Citrus* genus were mainly based on morphological and biochemical properties (Tatum et al., 1974; Barrett and Rhode, 1976; Scora, 1988). Initial molecular characterization included isozyme- (Rahman and Nito, 1994) and DNA- (Federici et al., 1998; Nicolosi et al., 2000; Abkenar et al., 2004) based analyses, which were used to more clearly elucidate the citrus taxa. Studies on genetic diversity using random amplified polymorphic DNA (RAPD) and simple sequence repeat (SSR) markers revealed a narrow genetic base within the mandarins (*Citrus reticulata*) (Machado et al., 1996; Coletta-Filho et al., 1998; El-Mouei et al., 2011). Furthermore, karyotype analysis of chromosomes has been widely used for determining phylogenies and speciation of *Citrus* spp. (Guerra, 1993; Miranda et al., 1997; Befu et al., 2000; Yamamoto and Tominaga, 2003; Carvalho et al., 2005; Yi et al., 2018a, 2018b). However, there have been few studies tracing the origin and genetic background of landrace citrus in Korea as well as Japan, neighboring the center of citrus origin.

DY has two natural variants, buk-daengyooza (BDY) and seol-daengyooza (SDY), which are distinguished according to differences in fruit characteristics. However, scientific evaluation of differences between morphological and chemical properties and the genetic differences of these natural variants has not been performed. In this study, morphological and genetic properties were evaluated among common, commercially-produced DY and its natural variants, BDY and SDY. We employed traditional classification methods and molecular phylogenetical methods, such as comparison of morphological and chemical properties of variants, chromosome karyotyping analysis, and genotyping using DNA markers, to identify genetic diversity and relationships. A better understanding of DY genetics may give some clue as to the origin of the different variants and identify potential genetic resources useful for systemic and targeted breeding programs.

Materials and Methods

Plants Materials

Three different types of DY (*Citrus grandis*) referred to as DY (common type), BDY (puffy type), and SDY (non-puffy type) were used in this experiments. DY was maintained at the Jeju Special Self-Governing Province Agricultural

Research & Extension Service. BDY and SDY were collected from the backyards of several farmhouses in Aewol, Jeju, Korea.

Physical and Chemical Properties

To characterize physical properties, leaf length and width and the weight, length, diameter, and peel thickness of the fruit were measured. Hardness was measured three times at the equator of fruits using a penetrometer (FHM-5, Takemura, Japan). Juice was squeezed out of the fruit, and soluble solids content and acidity were measured using a soluble solids-acidity meter (GMK-707R, G-Won Hitech Co., Ltd., Seoul, Korea). Flavonoids in fruit flesh and peels were analyzed using a high-performance liquid chromatography system (e2695 Separation module) equipped with a UV-VIS detector (Waters 2489, USA). A YMC-Triart C18 column (250x4.6 mm, S-5 μm , 8 nm) was used for chromatographic separation. Ten microliters of sample was injected and delivered with the mobile phase (acetonitrile: 20mM phosphoric acid, 2:8, v/v) at the rate of 1.0 mL \cdot min⁻¹ and detected at 280 nm. Peak areas were quantitated with a Waters Empower System.

Karyotype Analysis

The chromosome preparation was performed according to Dutt et al. (2010) with minor modifications. Seeds were collected and germinated at 25°C in the dark, and root tips about 1 cm long were used. The root tips were excised and pretreated in 2 mM 8-hydroquinolin at 4°C for 12 h in the dark. Subsequently, the root tips were fixed in a methanol:acetic acid (3:1, v/v) solution. The fixed specimens were washed with distilled water and digested 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) at 37°C for 1 h. The digested specimens were mounted on slide glasses, scattered with a drop of fixing solution using fine-pointed forceps, and air-dried. After air-drying, the preparations were counterstained with chromomycin A₃ (CMA) and DAPI as described by Befu et al. (2000) with modifications. The preparations were sequentially treated with McIlvaine's buffer (pH 7.0) containing 5 mM MgCl₂ for 30 min, 0.5 mg \cdot mL⁻¹ CMA for 1 h, and then rinsed with McIlvaine's buffer for 10 min. Soon after rinsing, the preparations were mounted with coverslips using Vectashield mounting medium containing 1.5 $\mu\text{g}\cdot$ mL⁻¹ DAPI (Vector Laboratories, Burlingame, CA). Samples were observed with an epifluorescence microscope (OLYMPUS BX51, Japan) with a BV filter cassette. The images were captured using an Olympus DP71 CCD camera, analyzed using DP manager software (Olympus, Japan), and constructed using Adobe Photoshop 7.0. Five to ten cells were compared with each other for each accession and the banding patterns were determined on the basis of the number and position of CMA positive bands.

Assessment of Genetic Diversity using RAPD and SSR Marker Analyses

Total genomic DNA was extracted from fresh leaves using a Biomedic Plant gDNA Extraction Kit (Biomedic Co., Ltd., Korea; www.ibiomedic.co.kr) and quantified using a DeNovix DS-11+ spectrophotometer (DeNovix Inc., Wilmington, DE, USA). Operon primers for RAPD analysis were selected and PCR reactions were performed according to the method described in Yun (2001). Analysis of SSR markers was performed using the M13-tailed PCR method and PCR reaction and cycling conditions were performed as previously described in Schuelke (2000) and Woo et al. (2019). Fragment

analysis of PCR products was performed as described previously in Kim et al. (2012). Calling of allele sizes was performed using GeneMapper software (ver. 4.0; Applied Biosystems). The shared allele frequency was calculated using PowerMarker software (v. 3.25) (Liu and Muse, 2005).

Analysis of Genetic Variation using the Plastid *trnL-trnF* Marker

Chloroplast DNA was extracted and the plastid *trnL-trnF* intergenic region was amplified using universal primers as described by Jung et al. (2005). PCR amplification was performed using an ABI 2720 thermal cycler (Applied Biosystems, Foster City, CA, USA) in a total volume of 10 mL, containing 20 ng genomic DNA, 5 mL 2x HS Taq mix (Dongsheng Biotech, Guangzhou, China), and 0.2 mL of each 10 pmol forward and reverse primer. The conditions for PCR amplification were as follows: 5 min for initial denaturation at 95°C, 30 cycles of 30 s at 95°C, 30 s at 55°C, and 1 min at 72°C, concluding with 1 cycle of 10 min at 72°C. The PCR products were purified using Biomedic Gel & PCR Purification Kit (Biomedic Co., Ltd., Korea). The nucleotide sequences of the purified PCR product were determined using dGTP BigDye Terminator v3.0 Ready Reaction Cycle Sequencing Kit (Applied Biosystems, CA, USA).

Results and Discussion

Measurements of phenotypic characteristics of fruit and leaves among DY (common type), BDY (puffy type), and SDY (non-puffy type) are shown in Fig. 1, Tables 1 and 2. There was no significant difference in the morphological characteristics of leaves and fruit between DY and BDY. However, the leaf and fruit shape, fruit hardness, and peel thickness of SDY were significantly different. DY and BDY had an elliptical and ovate leaf shape with an acutinate apex, while SDY had an ovate-shaped leaf with an acute apex. SDY had a petiole wing wider than those of DY and BDY. In addition, SDY produced smaller fruits than those of DY and BDY. The peel (including the rind and albedo) of SDY fruit



Fig. 1. Phenotypes of leaves and fruit from three different types of dangyooza (DY, dangyooza; BDY, buk-daengyooza; SDY, seol-daengyooza).

Table 1. Morphological leaf characteristics in three different types of dangyooza (DY, dangyooza; BDY, buk-daengyooza; and SDY, seol-daengyooza)

Varietal type	Leaf length (mm)	Leaf width (mm)	Leaf shape	Leaf apex shape	Size of petiole wings
DY	10.4 ± 0.2 a ^{z,y}	5.1 ± 0.1 b	Elliptical	Acutinate	Small
BDY	10.2 ± 0.5 a	4.8 ± 0.3 b	Elliptical	Acutinate	Small
SDY	10.5 ± 0.4 a	6.5 ± 0.4 a	Ovate	Acute	Medium

^zData presented are in mean ± SE (n = 10).

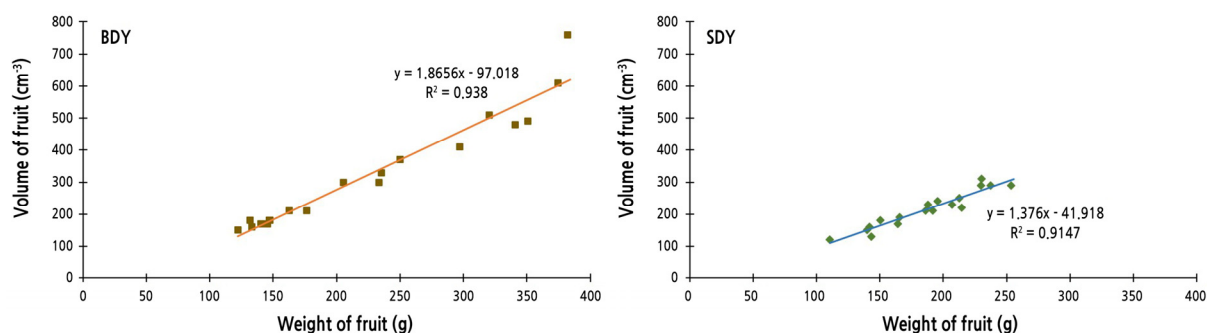
^yMean separation within columns by Duncan's multiple range test at the 5% level.

Table 2. Morphological characteristics of fruit in three different types of dangyooza (DY, dangyooza; BDY, buk-daengyooza; SDY, seol-daengyooza)

Varietal type	Fruit weight (g)	Fruit length (mm)	Fruit diameter (mm)	Fruit firmness (kg·cm ⁻²)	Peel thickness (mm)	SSC (°Brix)	Acidity (%)
DY	323.0 ± 47.0 a ^{z,y}	94.5 ± 8.1 a	93.9 ± 4.5 a	3.16 ± 0.15 b	12.5 ± 0.6 a	10.8 ± 0.3 a	3.1 ± 0.1 a
BDY	336.4 ± 12.9 a	98.7 ± 3.0 a	96.2 ± 1.3 a	3.52 ± 0.08 b	11.8 ± 0.7 a	9.0 ± 0.3 b	3.2 ± 0.3 a
SDY	240.8 ± 7.5 b	84.0 ± 2.0 a	79.6 ± 2.7 b	4.33 ± 0.09 a	8.9 ± 0.3 b	10.5 ± 0.4 a	3.5 ± 0.2 a

^zData presented are in means ± SE (n = 5).

^yMean separation within columns by Duncan's multiple range test at the 5% level.

**Fig. 2.** Relationship between volume of fruit and fruit weights of buk-daengyooza (BDY) and seol-daengyooza (SDY).

was significantly thinner than those of DY and BDY; however, it was much harder than those of DY and BDY.

For each fruit, actual fruit volume was measured by water displacement, and weight was recorded. Linear regression equations and correlation coefficients between fruit weight and volume were calculated (Fig. 2). The value of the correlation coefficient of BDY was higher than that of SDY, indicating that fruit of BDY tends to be bigger than SDY fruit of the same weight. One of the most distinguishable characteristics of BDY and SDY is the puffiness of fruit. In fact, BDY (buk-daengyooza; “buk” means inflation or puffy in Korean) is the pseudonym or alias of DY, because the puffiness is recognizable when the fruit is pounded or pressed. In addition, the albedo layer is conspicuously thicker in DY and BDY than in SDY.

Morphological traits of citrus varieties, including fruit color, size, and weight, and leaf and fruit shape, are highly diverse. In this study, morphological traits of leaves and fruit distinguished SDY from DY and BDY, suggesting that SDY may have a different genetic background compared with DY and BDY.

Chemical properties, such as total soluble solids and acidity of citrus fruit, have been used as criteria for the classification of fruit as well, especially since these traits tend to show high heritability (Ahmed et al., 2018). Individual flavonoid concentrations in the pulp and peel (flavedo and albedo) of DY, BDY, and SDY are shown in Table 3. Most flavonoid components, except narirutin, showed higher concentrations in the peel compared with the pulp. Rutin, a commonly found flavone in citrus (Nogata et al., 2006), was detected at concentrations of about 1.8- to 2.5-times higher in the peel of DY accessions than in the pulp. The pulp of SDY contained a 55% higher concentration of rutin than those of DY and BDY. Furthermore, the highest concentration of rutin was detected in the peel of SDY, followed by DY and BDY.

The major flavanones in citrus, including naringin, hesperidin, and neohesperidin, were also higher in both the pulp and peel of SDY than in DY and BDY. Narirutin was higher in only the peel of SDY. The concentrations of hesperidin and neohesperidin in the pulp and peel of SDY were considerably higher than those of DY and BDY. Moreover, the pulp of SDY contained almost twice as much hesperidin and neohesperidin. The two major PMFs in citrus, nobiletin and tangeretin, were only detected in the peel of all three types of DY, and there were no significant differences in their concentrations.

Chemical composition studies, in addition to morphological characteristics, were applied to the taxonomic classification of citrus, and it has resulted in remarkable progress in citrus taxonomic studies (Barrett and Rhodes, 1976). For instance, nucellar seedlings could be distinguished from zygotic seedlings by methoxy flavonoid compounds (Tatum et al., 1974).

The somatic metaphase chromosomes of BDY and SDY were all diploid ($2n = 18$). Banding patterns from CMA staining of chromosomes were typed based on the number and position of CMA positive bands according to Befu et al. (2000) and Miranda et al. (1997) (Fig. 3). The chromosome configurations of both BDY and SDY were $1A + 3B + 1C + 7D + 6E$, which is identical to that of DY (Yi et al., 2018b). Both BDY and SDY possessed a total of five A-, B- and C-type chromosomes. Such large numbers of A, B, and C chromosomes are typical for CMA banding patterns commonly found in *C. maxima* (Guerra, 1993; Miranda et al., 1997; Befu et al., 2001). Due to its self-incompatibility and broad cultivation worldwide, pomelo (*C. maxima*) can be easily hybridized to produce diverse cultivars, varieties, and strains. However, the result of this study revealed that there was no variation in chromosome composition among DY, BDY, and SDY. This suggests that they are not distinct hybrids or strains resulting from interspecific hybridization.

Table 3. Individual flavonoid concentration in three different types of dangyooza (DY, dangyooza; BDY, buk-daengyooza; SDY, seol-daengyooza)

Flavonon	Pulp			Peel		
	DY	BDY	SDY	DY	BDY	SDY
Rutin	914.5 ± 36.0 b ^{2y}	825.2 ± 32.6 b	1553.4 ± 56.5 a	2320.3 ± 120.2 b	1605.5 ± 65.7 c	2814.4 ± 129.3 a
Narirutin	165.3 ± 6.9 b	213.8 ± 7.7 a	231.8 ± 9.2 a	191.1 ± 11.0 b	242.9 ± 9.3 a	188.3 ± 8.9 b
Naringin	2126.3 ± 95.6 b	1644.3 ± 61.6 c	2896.0 ± 111.2 a	3234.5 ± 166.7 a	3028.6 ± 112.8 a	3532.7 ± 159.2 a
Hesperidin	32.7 ± 2.7 a	36.8 ± 1.4 a	77.5 ± 3.6 b	108.7 ± 6.2 a	104.7 ± 4.6 a	155.1 ± 10.3 b
Neohesperidin	1225.7 ± 59.5 b	1134.2 ± 44.1 b	2595.7 ± 105.6 a	3445.1 ± 181.8 b	2654.9 ± 104.9 c	4547.2 ± 210.2 a
Nobiletin	-	-	-	113.2 ± 4.2 a	113.5 ± 4.6 a	117.5 ± 3.9 a
Tangeretin	-	-	-	68.8 ± 1.9 a	62.5 ± 1.4 b	57.4 ± 1.0 b

²All data presented are in mg·100 mg⁻¹ FW and mean ± SE (n = 3).

³Mean separation within columns by Duncan's multiple range test at the 5% level.

The genetic variation and diversity among DY, BDY, and SDY was analyzed to distinguish them at a molecular level using RAPD, SSR, and *trnL-trnF* barcoding markers. However, there was no polymorphism found among the three accessions. No polymorphic bands were revealed by the RAPD and SSR data (Suppl. Fig. 1s and Suppl. Table 1s). Nucleotide sequences of the amplified *trnL-trnF* intergenic region were identical among the three accessions without any different SNPs (Fig. 4). Given these results, DY, BDY, and SDY are all very closely related.

DNA barcoding markers, such as *trnL-trnF*, are considered powerful tools for identifying field gametophytes (Chen et al., 2013). These markers have been widely used for studying phylogenetic relationships in *Citrus* (Jung et al., 2005; Yingzhi et al., 2007; Jena et al., 2009; Li et al., 2010; Lu et al., 2011). Genetic diversity and phylogenetic relationships among many citrus cultivars have been analyzed using RAPD markers (Machado et al., 1996; Coletta-Filho et al., 1998; Federici et al., 1998; Abkenar and Isshiki, 2003; Baig et al., 2009) and SSR markers (Biswas et al., 2011; Shrestha et al.,

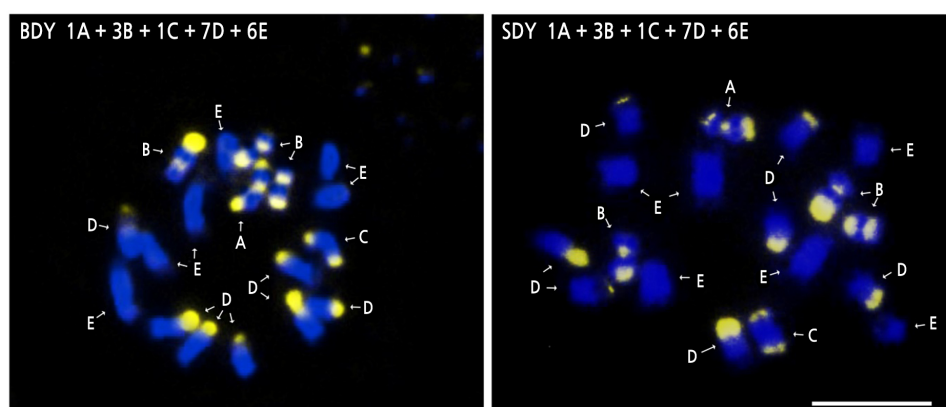


Fig. 3. CMA/DAPI-stained somatic chromosomes in buk-daengyooza (BDY) and seol-daengyooza (SDY). Letters indicate the number and distribution of CMA positive bands as follows; A, two telomeric and one proximal band; B, one telomeric and one proximal band; C, two telomeric bands; D, one telomeric band; and E, no band. The gray regions signify CMA positive bands. Scale bar indicates 5 µm.

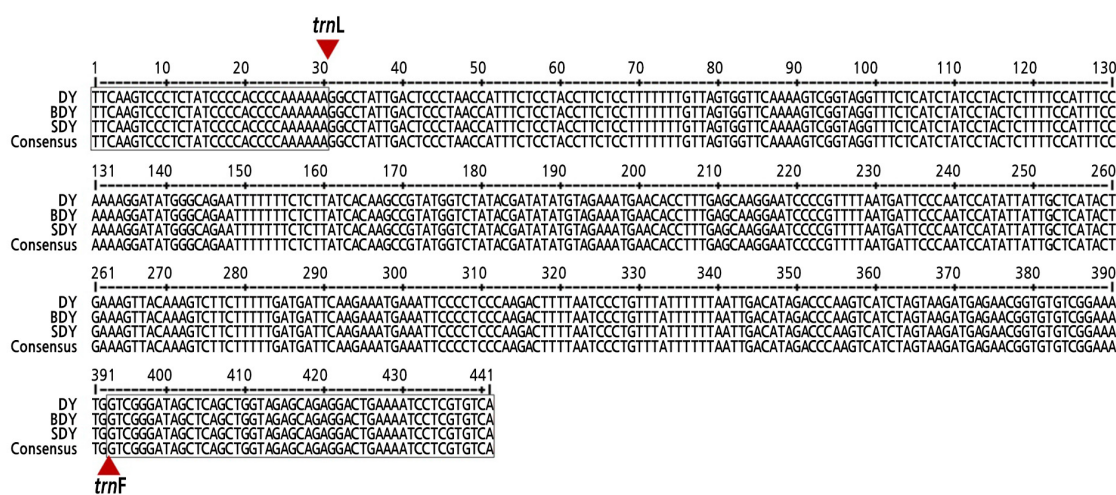


Fig. 4. Nucleotide sequence alignment and amplified *trnL-trnF* DNA barcoded region of three different types of dangyooza (DY, dangyooza; BDY, buk-daengyooza; and SDY, seol-daengyooza) based on the sequence alignment of the chloroplast *trnL-trnF* region.

2012; Nematollahi et al., 2013; Ahmed et al., 2018). SSR markers have also been applied for citrus germplasm collection (Barkley et al., 2006) and identification of zygotic and nucellar seedling in citrus (Yildiz et al., 2013; Woo et al., 2019).

In this study, we analyzed phenotypic, phytochemical, cytogenetic, and genotypic characteristics of DY, BDY, and SDY to elucidate their phylogenetic relationship. Morphological and phytochemical traits of leaves and fruit distinguished SDY from DY and BDY. The karyotype analysis revealed that there was no polymorphism among the CMA/DAPI banding patterns for the accessions tested. Despite morphological and phytochemical differences among DY, BDY, and SDY, chromosomal configuration and DNA marker analyses were unable to clearly discriminate their phylogenetic relationships. This suggests that SDY and BDY may have differentiated from DY with highly similar genetic backgrounds. The distinct morphological and phytochemical traits of SDY may have also arisen from a somatic mutation, such as a nucellar seedling.

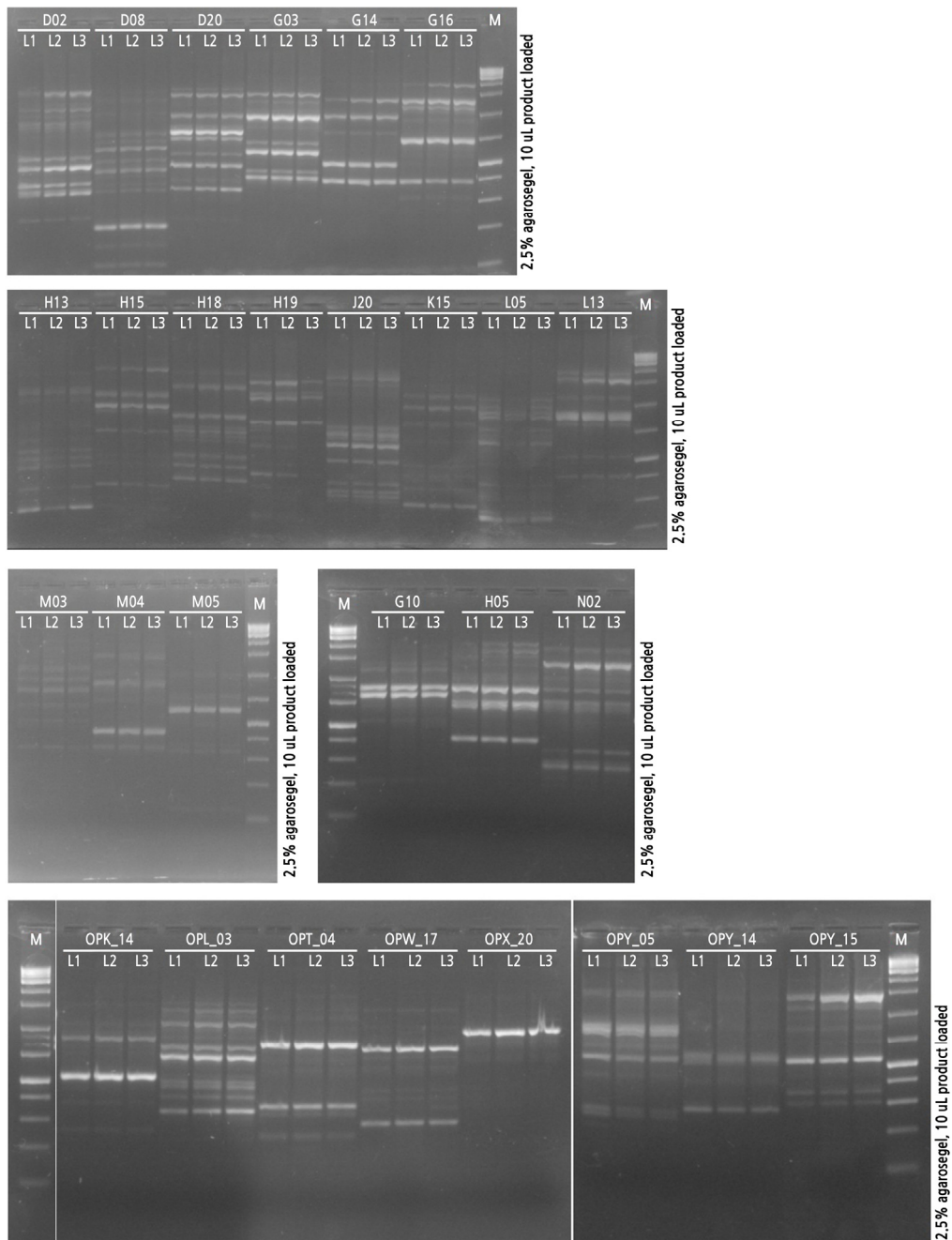
The data and results from this study lay groundwork for future study of DY. By having a better understanding of morphological, chemical, and genetic differences among DY, BDY, and SDY, continued experiments can be designed and used to identify potential genetic resources useful for systemic and targeted breeding programs. Furthermore, these findings give some preliminary clues to the origin of the different variants.

Literature Cited

- Abkenar AA, Isshiki S (2003) Molecular characterization and genetic diversity among Japanese acid citrus (*Citrus* spp.) based on RAPD markers. *J Horticult Sci Biotechnol* 78:108-112. doi:10.1080/14620316.2003.11511574
- Abkenar AA, Isshiki S, Tashiro Y (2004) Phylogenetic relationships in the "True Citrus Fruit Trees" revealed by PCR-RFLP analysis of cpDNA. *Sci Hortic* 102:233-242. doi:10.1016/j.scienta.2004.01.003
- Ahmed S, Rattanpal HS, Singh G (2018) Diversity assessment of grapefruit (*Citrus × paradisi*) and tangelo (*Citrus × tangelo*) under Indian conditions using physico-chemical parameters and SSR markers. *Appl Ecol Environ Res* 16:5343-5358. doi:10.15666/aeer/1605_53435358
- Baig MNR, Grewal S, Dhillon S (2009) Molecular characterization and genetic diversity analysis of citrus cultivars by RAPD markers. *Turk J Agric For* 33:375-384
- Barkley NA, Roose ML, Krueger RR, Federici CT (2006) Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theor Appl Genet* 112:1519-1531. doi:10.1007/s00122-006-0255-9
- Barrett HC, Rhodes AM (1976) A numerical taxonomic study of affinity relationships in cultured *Citrus* and its close relatives. *Syst Bot* 1:105-136. doi:10.2307/2418763
- Befu M, Kitajima A, Hasegawa K (2001) Chromosome composition of some *Citrus* species and cultivars based on the chromomycin A₃ (CMA) banding patterns. *J Japan Soc Hortic Sci* 70:83-88. doi:10.2503/jjshs.70.83
- Befu M, Kitajima A, Yang X, Hasegawa K (2000) Classification of 'Tosa-Buntan' pummelo (*Citrus grandis* [L.] Osb.), 'Washington' navel orange (*C. sinensis* [L.] Osb.), and trifoliolate orange (*Poncirus trifoliata* [L.] Raf.) chromosomes using young leaves. *J Japan Soc Hortic Sci* 69:22-28. doi:10.2503/jjshs.69.22
- Benavente-Garcia O, Castillo J (2008) Update on uses and properties of Citrus flavonoids: new findings in anticancer, cardiovascular, and anti-inflammatory activity. *J Agric Food Chem* 56:6186-6205. doi:10.1021/jf8006568
- Biswas MK, Chai L, Amar MH, Zhang X, Deng XX (2011) Comparative analysis of genetic diversity in Citrus germplasm collection using AFLP, SSAP, SAMPL and SSR markers. *Sci Hortic* 129:798-803. doi:10.1016/j.scienta.2011.06.015
- Carvalho R, dos Santos Soares Filho W, Brasileiro-Vidal AC, Guerra M (2005) The relationships among lemons, limes and citron: a chromosomal comparison. *Cytogenet Genome Res* 109:276-282. doi:10.1159/000082410
- Chen CW, Huang YM, Kuo LY, Nguyen QD, Luu HT, Callado JR, Ferrar DR, Chiou WL (2013) *trnL-F* is a powerful marker for DNA identification of field vittarioid gametophytes (Pteridaceae). *Ann Bot* 111:663-673. doi:10.1093/aob/mct004
- Coletta-Filho HD, Machado MA, Targon MLPN, Pompeu Jr J (1998) Analysis of the genetic diversity among mandarins (*Citrus* spp.) using RAPD markers. *Euphytica* 102:133-139. doi:10.1023/A:1018300900275
- Dutt M, Vasconcellos M, Song KJ, Gmitter FG, Grosser JW (2010) *In vitro* production of autotetraploid Ponkan mandarin (*Citrus reticulata* Blanco) using cell suspension cultures. *Euphytica* 173:235-242. doi:10.1007/s10681-009-0098-y
- El-Mouei R, Choumane W, Dway F (2011) Molecular characterization and genetic diversity in genus *Citrus* from Syria. *Int J Agric Biol* 13:351-356

- Federici CT, Fang DQ, Scora RW, Roose ML (1998) Phylogenetic relationships within the genus *Citrus* (Rutaceae) and related genera as revealed by RFLP and RAPD analysis. *Theor Appl Genet* 96:812-822. doi:10.1007/s001220050807
- Food and agriculture organization of the United Nations (FAO) (2018) AOSTAT. Food and agriculture organization of the United Nations. Rome, Italy
- Gattuso G, Barreca D, Gargiull C, Leuzzi U, Caristi C (2007) Flavonoid composition of citrus juices. *Molecules* 12:1641-1673. doi:10.3390/12081641
- Guerra M (1993) Cytogenetics of Rutaceae. V. High chromosomal variability in *Citrus* species revealed by CMA/DAPI staining. *Heredity* 71:234-241. doi:10.1038/hdy.1993.131
- Han SS, You IJ (1988) Studies on antimicrobial activities and safety of natural naringin in Korea. *Korean J Mycol* 16:1-8
- Hertog MGL, Hollman PCH, Katan MB (1992) Content of potentially anticarcinogenic flavonoids of 28 vegetables and 9 fruits commonly consumed in the Netherlands. *J Agric Food Chem* 4:2379-2383. doi:10.1021/jf00024a011
- Jena SN, Kumar S, Nair NK (2009) Molecular phylogeny in Indian *Citrus* L. (Rutaceae) inferred through PCR-RFLP and *trnL-trnF* sequence data of chloroplast DNA. *Sci Hortic* 119:403-416. doi:10.1016/j.scienta.2008.08.030
- Jeong WS, Park SW, Chung SK (1997) The antioxidative activity of Korean *Citrus unshiu* peels. *Food Biotechnol* 6:292-296
- Jung YH, Kwon HM, Kang SH, Kang JH, Kim SC (2005) Investigation of the phylogenetic relationships within the genus *Citrus* (Rutaceae) and related species in Korea using plastid *trnL-trnF* sequences. *Sci Hortic* 104:179-188. doi:10.1016/j.scienta.2004.08.008
- Kang SH, Lee YJ, Lee CH, Kim SJ, Lee DH, Lee YK, Park DB (2005) Physiological activities of peel of Jeju-indigenous *Citrus sunki* Hort. Tanaka. *Korean J Food Sci Technol* 37:983-988
- Kim HB, Jeon JH, Han AR, Lee Y, Jun SS, Kim TH, Cho GH, Park PB (2012) Genetic evaluation of domestic walnut cultivars trading on Korean tree markets using microsatellite markers. *Afr J Biotechnol* 11:7366-7374
- Kim HY (1988) Distribution, taxonomy, horticultural characters of the local *Citrus* spp. in Cheju, and the genetic markers among them. PhD. Diss. Cheonnam Nat'l Univ, Korea
- Kim YC, Koh KS, Koh JS (2001) Changes of flavonoids in the peel of Jeju native *Citrus* fruits during maturation. *Food Sci Biotechnol* 10:483-487
- Li X, Xie R, Lu Z, Zhou Z (2010) The origin of cultivated citrus as inferred from internal transcribed spacer and chloroplast DNA sequence and amplified fragment length polymorphism fingerprints. *J Am Soc Hortic Sci* 135:341-350. doi:10.21273/JASHS.135.4.341
- Lim HK, Yoo ES, Moon JY, Jeon YJ, Cho SK (2006) Antioxidant activity of extracts from *Dangyuja* (*Citrus grandis* Osbeck) fruits produced in Jeju Island. *Food Sci Biotechnol* 15:312-316
- Liu KJ, Muse SV (2005) PowerMarker: an integrated analysis environment for genetic marker analysis. *Bioinformatics* 21:2128-2129. doi:10.1093/bioinformatics/bti282
- Lu ZH, Zhou ZG, Xie RJ (2011) Molecular phylogeny of the "True citrus fruit trees" group (Aurantioideae, Rutaceae) as inferred from chloroplast DNA sequence. *Agr Sci China* 10:49-57. doi:10.1016/S1671-2927(11)60306-4
- Machado MA, Coletta Filho HD, Targon MLPN, Pompeu Jr J (1996) Genetic relationship of Mediterranean mandarins (*Citrus deliciosa* Tenore) using RAPD markers. *Euphytica* 92:321-326. doi:10.1007/BF00037115
- Miranda M, Ikeda F, Endo T, Morigushi T, Omura M (1997) Comparative analysis on the distribution of heterochromatin in *Citrus*, *Poncirus* and *Fortunella* chromosomes. *Chromosome Res* 5:86-92. doi:10.1023/A:1018409922843
- Mokbel MS, Hashinaga F (2006) Evaluation of the antioxidant activity of extracts from buntan (*Citrus grandis* Osbeck) fruit tissues. *Food Chem* 94:529-534. doi:10.1016/j.foodchem.2004.11.042
- Moon DK, Kim KS, Kwon HM, Kim CM (2007) *Citrus* (*Citrus* spp., Rutaceae). In JM Lee, GW Choi, J Janick, eds, *Horticulture in Korea*. Korean Soc Hortic Sci Press, Suwon, pp 224-234
- Nematollahi AK, Golein B, Vahdati K (2013) Analysis of the genetic diversity in citrus (*Citrus* spp.) species using SSR markers. *J Plant Physiol Breeding* 3:41-49
- Nicolosi E, Deng ZN, Gentile A, La Malfa S, Continella G, Tribulato E (2000) Citrus phylogeny and genetic origin of important species as investigated by molecular markers. *Theor Appl Genet* 100:1155-1166. doi:10.1007/s001220051419
- Nogata Y, Sakamoto K, Shiratsuchi H, Ishii T, Yano M, Ohta H (2006) Flavonoid composition of fruit tissues of citrus species. *Biosci Biotechnol Biochem* 70:178-192. doi:10.1271/bbb.70.178
- Rahman MM, Nito N (1994) Phylogenetic relationships among the "True citrus fruit trees" by glutamate oxaloacetate transaminase isozymes analysis. *J Jpn Soc Hortic Sci* 62:755-760. doi:10.2503/jjshs.62.755
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nat Biotechnol* 18:233-234
- Scora RW (1988) Biochemistry, taxonomy and evolution of modern cultivated *Citrus*. *Proc Int Soc Citricult VI Congr Vol 1*, Margraf Publishers, Weikersheim, Germany, pp 277-289
- Shrestha RL, Dhakal DD, Gautum DM, Paudyal KP, Shrestha S (2012) Genetic diversity assessment of acid lime (*Citrus aurantifolia* Swingle) landraces in Nepal, using SSR markers. *Am J Plant Sci* 3:1674-1681. doi:10.4236/ajps.2012.312204
- Tatum JH, Berry RE, Hearn CJ (1974) Characterization of citrus cultivars and separation of nucellar and zygotic seedlings by thin-layer chromatography. *Proc Florida State Hortic Soc* 87:75-81
- Woo J, Park YC, Lee JW, Yun SH, Kim M, Park S, Lee Y, Song KJ, Kim HB (2019) Evaluation of polyembryony for genetic resources and efficacy of simple sequence repeat markers for the identification of nucellar and zygotic embryo-derived individuals in citrus. *Appl Biol Chem* 62:30. doi:10.1186/s13765-019-0437-1

- Yamamoto M, Tominaga S** (2003) High chromosomal variability of mandarins (*Citrus* spp.) revealed by CMA banding. *Euphytica* 129:267-274. doi:10.1023/A:1022284908424
- Yang YT, Kim HB, Lee SH, Park YC** (2019) Composition characteristics of flavonoids in citrus juice. *Hortic Sci Technol* 37:10-19
- Yi KU, Kim HB, Song KJ** (2018a) Karyotype diversity of Korean landrace mandarins by CMA banding pattern and rDNA loci. *Sci Hortic* 228:26-32. doi:10.1016/j.scienta.2019.10.001
- Yi KU, Kim HB, Song KJ** (2018b) Cytogenetic phenogram with high resolution chromosome configurations of some Korean landrace citrus by CMA banding and rDNA loci. *Sci Hortic* 240:417-424. doi:10.1016/j.scienta.2018.06.058
- Yildiz E, Kaplankiran M, Demirköser TH, Uzun A, Toplu C** (2013) Identification of zygotic and nucellar individuals produced from several citrus crosses using SSRs markers. *Not Bot Horti Agrobo* 41:478-484. doi:10.15835/nbha4129037
- Yingzhi L, Yunjiang C, Nengguo T, Xiuxin D** (2007) Phylogenetic analysis of mandarin landraces, Wild mandarins, and related species in China using nuclear LEAFY second intron and plastid *trnL-trnF* sequence. *J Jpn Soc Hortic Sci* 132:796-806. doi:10.21273/JASHS.132.6.796
- Yoshimizu N, Otani Y, Saikawa Y, Kubota T, Yoshida M, Furukawa T, Kumai K, Kameyama K, Fujii M, et al.** (2004) Anti-tumor effects of nobiletin, a citrus flavonoid, on gastric cancer include: antiproliferative effects, induction of apoptosis and cell cycle deregulation. *Aliment Pharmacol Ther* 1:95-101. doi:10.1111/j.1365-2036.2004.02082.x
- Yun SH** (2001) Classification of genus *Citrus* and its related genera using RAPD. Master Diss. Jeju Nat'l Univ, Jeju, Korea



Supplementary Fig. 1s. PCR amplified products of 28 RAPD primers with dangyooza (DY), buk-dangyooza (BDY), and seol-dangyooza (SDY) accessions (L1; DY, L2; BDY, and L3, SDY).

Supplementary Table 1s. Allele sizes of dangyooza (DY), buk-daengyooza (BDY), and seol-daengyooza (SDY) accessions determined by 24 polymorphic SSR markers

SSR primer	DY	BDY	SDY
BM-CiSSR-201	264/264	264/264	264/264
BM-CiSSR-202	289/289	289/289	289/289
BM-CiSSR-012	177/308	177/308	177/308
BM-CiSSR-013	159/159	159/159	159/159
BM-CiSSR-217	316/316	316/316	316/316
BM-CiSSR-225	345/352	345/352	345/352
BM-CiSSR-239	408/408	408/408	408/408
BM-CiSSR-243	286/286	286/286	286/286
BM-CiSSR-246	310/326	310/326	310/326
BM-CiSSR-247	408/420	408/420	408/420
BM-CiSSR-253	348/354	348/354	348/354
BM-CiSSR-256	289/289	289/289	289/289
BM-CiSSR-258	357/373	357/373	357/373
BM-CiSSR-260	384/384	384/384	384/384
BM-CiSSR-273	344/344	344/344	344/344
BM-CiSSR-077	185/189	185/189	185/189
BM-CiSSR-082	154/154	154/154	154/154
BM-CiSSR-087	183/189	183/189	183/189
BM-CiSSR-094	271/274	271/274	271/274
BM-CiSSR-111	137/140	137/140	137/140
BM-CiSSR-115	231/231	231/231	231/231
BM-CiSSR-137	310/326	310/326	310/326
BM-CiSSR-159	382/403	382/403	382/403
BM-CiSSR-162	206/209	206/209	206/209