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TAXONOMY AND PHYLOGENY OF THE GENUS *CITRUS* BASED ON THE NUCLEAR RIBOSOMAL DNA ITS REGION SEQUENCE

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Abstract

The genus *Citrus* (Aurantioideae, Rutaceae) is the sole source of the citrus fruits of commerce showing high economic values. In this study, the taxonomy and phylogeny of *Citrus* species is evaluated using sequence analysis of the ITS region of nrDNA. This study is based on 26 plants materials belonging to 22 *Citrus* species having wild, domesticated, and cultivated species. Through DNA alignment of the ITS sequence, ITS1 and ITS2 regions showed relatively high variations of sequence length and nucleotide among these *Citrus* species. According to previous six-tribe discrimination theory by Swingle and Reece, the grouping in our ITS phylogenetic tree reconstructed by ITS sequences was not related to tribe discrimination but species discrimination. However, the molecular analysis could provide more information on citrus taxonomy. Combined with ITS sequences of other subgenera in the "true citrus fruit tree" group, the ITS phylogenetic tree indicated subgenera *Citrus* was monophyletic and nearer to *Fortunella*, *Poncirus*, and *Clymenia* compared to *Microcitrus* and *Eremocitrus*. Abundant sequence variations of the ITS region shown in this study would help species identification and tribe differentiation of the genus *Citrus*.

Key words: *Citrus*; Phylogenetic relationship; ITS region; Genetic diversity.

Introduction

Citrus is one of the most important fruit crops in the world. It is widely grown in the tropical, subtropical, and borderline subtropical areas of the world, with total global production reaching 7.4 million metric tons in 2009-2010 (The Citrus and Date Crop Germplasm Committee, 2004; FAOSTAT, 2010). Since the year of 1753 that the genus *Citrus* was established by Carole Linneaus, the taxonomy of *Citrus* and closely related genera, and the number of species belonging to the genus *Citrus* have become the focus of argument. Until now, there are two principal systems of *Citrus* taxonomy: Swingle & Reece (1967) system & Tanaka (1977) system. Swingle & Reece's system recognized three groups of the subtribe Citrinae (Citreae, Aurantioideae, Rutaceae), i.e., the "primitive citrus fruit trees", the "near-citrus fruit trees", and the "true citrus fruit trees" groups. In the "true citrus fruit trees" group, there were six subgenera including *Fortunella* Swingle, *Microcitrus* Swingle, *Eremocitrus* Swingle, *Clymenia* Swingle, *Poncirus* Raf., and *Citrus* L. The genus *Citrus* L. has 16 species distributed in two subgenera, *Citrus* (consisting of 10 species) and *Papeda* (consisting of 6 species). Tanaka (1977) accepted the genus *Citrus* in a broad term and included a total of 159 species and 14 variant species under two subgenus citrus, *Archicitrus* Tanaka and *Metacitrus* Tanaka. Both systems seemed different, however, their divergence of views only focused whether they accepted most of hybrids, cultivars, bud sports, and variant species as true botanical species. Tanaka (1977) considered *Citrus* hybrids, cultivars, bud sports, and variant species as absolute botanical species, but not Swingle & Reece (1967) did not accept them as good taxonomic species.

To understand *Citrus* taxonomy and examine their phylogenetic relationships, many scientists have indicated their own attitudes based on various analysis data, i.e., isozymes (Fang *et al.*, 1993; Herrero *et al.*, 1996), morphological and biochemical data (Scora, 1975; Barrett & Rhodes, 1976; Potvin *et al.*, 1983; Zhou, 1992), Microsatellites (Susheel *et al.*, 2010; Amar *et al.*, 2011; Biswas *et al.*, 2011), and DNA markers (Nicolosi *et al.*, 2000; Abkenar *et al.,* 2004; Pang *et al.,* 2007). According to the chemical classification and morphological analysis, Scora (1975) and Barrett & Rhodes (1976) recognized that the subgenus *Citrus* in the "true citrus fruit trees" group only included three true botanical species, *C. grandis*, *C. medica*, and *C. reticulata* and other species were all derived from hybrids, cultivars, or variant species, that usually consists of some commercially important fruits, such as *C. limon* (lemon), *C. paradisi* (grapefruit), *C. sinensis* (sweet orange), *C. aurantium* (sour orange), and *C. aurantifolia* (lime). Zhou (1992) analyzed morphological characters of 24 *Citrus* species populations, and recognized five groups, *C. hystrix*, *Citrophorum*, *Cephalocitrus*, *Acrumen*, and *Microacrumen*. However, Mabberley (1998) suggested that the genera *Fortunella*, *Microcitrus*, and *Eremocitrus* should be reabsorbed back

into the genus *Citrus*. Seen from these, the systematic of *Citrus* is still an argument focus for current comprehension. The reason for the complication of *Citrus* taxonomy and phylogeny is considered as the apomixis, wide cross-compatibility, high frequency of bud mutation, and long history of cultivation (Moore, 2001). Thus, the wide controversy concerning the *Citrus* taxonomy and the phylogenetic relationships, especially among the genera of the "true citrus fruit trees" group still exist (Pang *et al.*, 2003). In the view of our authors, six subgenera discrimination system by Swingle & Reece (1967) is supported, and the *Citrus* is considered to be composed of six tribes including *Citrophorum*, *Cephalocitrus*, *Aurantium*, *Sinocitrus*, *Papeda*, and *Papedocitrus*.

In the past few decades, many molecular marker techniques have been developed to overcome the limitations of morphological and biochemical markers in plant phylogenetics, such as chloroplast DNA (cpDNA) *rbc*L, *trn*H-*psb*A, *trn*L-*trn*F, and *mat*K, and nrDNA 5S, 16S, 18S, and ITS (Agarwal *et al.*, 2008). The application of these molecular marker techniques is to examine and analyze the genome-wide variability. Among them, the internal transcribed spacer (ITS) region of 18S-28S nuclear ribosomal DNA (nrDNA) is mostly widely used for phylogenetic studies (Baldwin *et al.*, 1995). It allows high nucleotide variability of ITS sequence, easily PCR amplification, and high primer universality (Alvarez & Wendel, 2003; Kress *et al.*, 2005). This region has been used for phylogenetic studies of microbe, plants, and even animals (Martin & Rygiewicz, 2005; Karehed *et al.*, 2008; Dai *et al.*, 2010). In this study, we selected five tribes of the genus *Citrus* to investigate. We evaluated the discrimination capacity and efficiency of ITS marker for genetic diversity and species identification of *Citrus* species, and determined the genetic relationship among the *Citrus* species.

Materials and Methods

Plant materials: Twenty-six *Citrus* plant materials belonging to 22 different *Citrus* species, provided by Prof. Ho-Min Kang, Department of Horticulture, Kangwon National University, Korea, were investigated in the present study. Fresh mature leaves were collected from these *Citrus* species and immediately stored in liquid nitrogen condition. Their specimens and relevant information listed here have been deposited in the National Centre for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/). The NCBI GenBank accession numbers of *Citrus* species investigated in this study is shown in Table 1. Among them, 14 *Citrus* species (No. 11-25 of Table 1) were long cultivated in Jeju Island, including *C. natsudaidai*, *C. obovoidea*, *C. tachibana*, *C. grandis*, *C. leiocarpa*, *C. tangerina*, *C. ichangensis*, *C. nippokoreana*, *C. aurantium*, *C. pseudogulgul*, *C. benikoji*, *C. erythrosa*, *C. sunki*, and *C. platymamma*.

Table 1. List of plant materials investigated in this study and their relevant information of specimen voucher and NCBI accession number.

No.	Species	Tribe	Specimen voucher	GenBank accession No.
1.	*Citrus kinokuni*	*Sinocitrus*	kk-8	JQ990159
2.	*Citrus unshiu*	*Sinocitrus*	kk-13	JQ990160
3.	*Citrus unshiu*	*Sinocitrus*	p-13	JQ990161
4.	*Citrus medica* var. *sarcodactylis*	*Citrophorum*	p-19	JQ990163
5.	*Citrus medica* var. *sarcodactylis*	*Citrophorum*	p-19-1	JQ990164
6.	*Citrus sinensis*	*Aurantium*	kk-22	JQ990165
7.	*Citrus hassaku*	*Sinocitrus*	kk-28	JQ990166
8.	*Citrus grandis*	*Cephalocitrus*	p-29	JQ990169
9.	*Citrus hybrid*	-	p-30	JQ990171
10.	*Citrus* spp.	-	p-53	JQ990174
11.	*Citrus limon*	*Citrophorum*	kk-55	JQ990175
12.	*Citrus natsudaidai*	*Sinocitrus*	kk-57	JQ990176
13.	*Citrus obovoidea*	*Sinocitrus*	kk-66	JQ990177
14.	*Citrus tachibana*	*Sinocitrus*	kk-69	JQ990178
15.	*Citrus grandis*	*Cephalocitrus*	kk-70	JQ990179
16.	*Citrus leiocarpa*	*Sinocitrus*	kk-71	JQ990180
17.	*Citrus tangerina*	*Sinocitrus*	kk-72	JQ990181
18.	*Citrus ichangensis*	*Papedocitrus*	kk-73	JQ990182
19.	*Citrus nippokoreana*	*Sinocitrus*	kk-74	JQ990183
20.	*Citrus aurantium*	*Sinocitrus*	kk-75	JQ990184
21.	*Citrus pseudogulgul*	*Sinocitrus*	kk-76	JQ990185
22.	*Citrus benikoji*	*Sinocitrus*	kk-77	JQ990186
23.	*Citrus erythrosa*	*Sinocitrus*	kk-78	JQ990187
24.	*Citrus sunki*	*Sinocitrus*	kk-79	JQ990188
25.	*Citrus platymamma*	*Sinocitrus*	kk-80	JQ990189
26.	*Citrus unshiu*	*Sinocitrus*	kk-98	JQ990190

- Means indeterminate

Isolation of DNA, PCR amplification and sequencing: DNA extractions were performed by using the modified cetyltrimethylammonium bromide (CTAB) method described by Doyle & Doyle (1987). The ITS1-5.8S-ITS2 region was amplified using universal primers ITS1 (forward primer) and ITS4 (reversed primer, White *et al*., 1990) in 20 µl PCR reaction. The reaction components for effective PCR amplification are 1 µl of template DNA (~1-100 ng), 10 µl 2 × PCR Dye Master Mix (containing 2 × Taq DNA polymerase, 2 × PCR buffer, 2 × dNTP, and moderate loading dye, QIAGEN, Korea), and 0.1 µmol l^{-1} of each primer (including forward primer and reversed primer). PCR amplification was conducted using this set of primers with the following program: 35 cycles of denaturation at 95°C for 1 min, annealing 54-57°C for 1 min, and a final extension step at 72°C for 1 min. The amplification products were checked by electrophoresis through 1.0% agarose gel, and then purified before DNA sequence analysis using a QIAquick PCR Purification Kit (QIAGEN, Korea) or Gel Purification Kit (QIAGEN, Korea) according to the manufacturer's instructions. Purified PCR products were then sequenced at MACROGENE Advancing through Genomics (Korea, http:// dna.macrogen.com/kor/).

Sequence editing and alignment: For editing and assembly of the complementary strands, the software program DNAMAN version 6.0 (Lynnon Biosoft Corporation, USA, www.lynon.com) was used. Analogue of our sequences and nucleotide sequence comparisons were detected with Basic Local Alignment Search Tool (BLAST) network services against National Center for Biotechnology Information (NCBI) GenBank databases (http://www.ncbi.nlm.nih.gov/). The multiple sequence alignment of ITS1-5.8S-ITS2 region was also performed using DNAMAN version 6.0 software, to detect single nucleotide polymorphisms.

Phylogenetic analysis: We assessed intraspecific genetic divergences by using pairwise distance calculations (Meyer & Paulay, 2005). Jaccard coefficients used to represent identity among the ecotypes were calculated by similarity coefficient [Sj = a/(a+u)]. In the total ITS region, ITS1 and ITS2 region, '1' was used for base variation and '0' was used for no variation; 'a' represents the number of the same bases and 'u' represents the number of different bases between the two varieties. The phylogenetic relationships among 26 *Citrus* materials was estimated after the construction of a phylogram based on multiple sequence alignment of various DNA sequences with the DNAMAN version 6.0 software (Lynnon Biosoft Corporation, USA, www.lynon.com). Genetic distance (GD) was obtained with the help of MEGA software and mean GD of the intraspecific distance was calculated by sum of individual GD divide by number of samples.

Results and Discussion

PCR amplification: PCR amplification of nrDNA ITS region of 26 *Citrus* species investigated in this study generated a monomorphic band of ~750 bp in length using ITS universal primer sets, ITS1 and ITS4. The analogue of the PCR products was detected using the BLAST on NCBI server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The identity of our sequencing results is above 93% to 99% compared to existing sequence sources of existent *Citrus* species in GenBank database. This result suggested that the ITS universal primers could be successfully performed for the genus *Citrus* plants; the nrDNA ITS region could be successfully amplified using ITS universal primer sets.

Sequence length: The sequence length of ITS region of 26 *Citrus* species varied narrowly (Table 2). The length of ITS1 ranged from 241 bp (*C. limon*) to 251 bp (*C. medica* var. *sarcodactylis*, *C. obovoidea* and *C. benikoji*), with the most length of 247 bp. The shortness of ITS1 region of *C. limon* mainly resulted in a array deletion of 10 bp (locating in 167 bp to 226 bp of ITS1 region) compared to other ITS1 sequences (Fig. 1). The array deletion in the ITS1 region was not considered to be the specific characteristic of *Citrophorum*, because two *C. medica* var. *sarcodactylis* materials (*Citrophorum*) did not show this array deletion in ITS1 region (Fig. 1).

The 5.8S region evolves relatively slowly compared to ITS1 and ITS2 region, in generally, it is highly conserved. Due to high conservation of this region, it is generally used not for plant phylogenetic studies but as an alignment tool (Cullings & Vogler, 1998). In this study, high conservation and low sequence variation were found in the 5.8S region, mostly with 163 bp in length from 26 *Citrus* sequences, except of *C. obovoidea*, *C. pseudogulgul*, and *C. benikoji* having 162 bp in length (Table 2). The difference of sequence length of 5.8S region was not related with tribe discrimination.

Because of high sequence variation and differentiation of ITS2, this intergenic spacer has been shown to be more valuable in identifying interspecies and intraspecies (Chiou *et al*., 2007). Among 26 *Citrus* species investigated in this study, high variation and differentiation ability was shown in nucleotide substitution, deletion, or addition, but not in sequence length. Nearly all ITS2 sequence included 227 bp in length, while *C. kinokuni*, *C. tachibana*, *C. grandis*, *C. leiocarpa*, *C. tangerina*, and *C. nippokoreana* had one more nucleotide addition, with 228 bp in length (Table 2). The difference of sequence length of ITS2 was also not related with tribe discrimination.

G+C content (%): The G+C content (%) ranged from 59.76% to 71.26% in ITS1 region, with the average G+C content (%) of 68.72% (Table 2). The G+C content (%) largely varied among 26 *Citrus* species in ITS1, induced by not only sequence length but G or/and C content. The 5.8S region showed narrow variation of G+C content (%), ranging from 47.24% to 54.60% (Table 2). Combined with one nucleotide indel in 5.8S, three or four G indels and seven C indels resulted in the decrease of G+C content (%) of *C. obovoidea*, *C. pseudogulgul*, and *C. benikoji* (Table 2). In addition, the sequence length of *C. limon* in 5.8S region was, though, invariable, five G substitutions and seven C substitutions made its G+C content (%) down to the lowest value among all *Citrus* species (47.24%). Likewise ITS1 region, the G+C contents (%) of ITS2 were largely variable, ranging from 63.00% to 71.49% (Table 2). The G+C content variation was mainly induced by G or/and C substitution, while the affect of sequence length was not significant.

Table 2. Sequence length and G+C content (%) of the ITS region from 26 *Citrus* materials investigated in this study.

No.	Sequence length (bp)			G+C content (%)		
	ITS1	5.8S	ITS2	ITS1	5.8S	ITS2
1.	247	163	228	70.04	54.60	71.49
2.	247	163	-	71.26	54.60	68.68
3.	-	163	227	70.59	54.60	69.60
4.	251	163	227	67.73	53.99	69.60
5.	-	163	227	70.59	54.60	69.60
6.	247	163	227	70.85	54.60	66.96
7.	247	163	227	70.85	54.60	69.16
8.	-	163	227	70.17	54.60	69.16
9.	247	163	227	70.04	54.60	67.40
10.	-	163	227	70.46	54.60	68.72
11.	241	163	227	61.41	47.24	63.00
12.	247	163	227	69.64	54.60	68.28
13.	251	162	227	60.56	48.15	63.88
14.	-	163	228	70.59	54.60	71.05
15.	250	163	228	69.20	54.60	71.05
16.	247	163	228	70.04	54.60	71.05
17.	-	163	228	70.29	54.60	71.05
18.	247	163	227	70.85	54.60	70.48
19.	247	163	228	70.04	54.60	70.61
20.	247	163	227	70.85	54.60	69.16
21.	250	162	227	60.80	48.77	63.88
22.	251	162	227	59.76	48.15	63.88
23.	247	163	227	70.85	54.60	70.93
24.	250	163	227	68.40	54.60	68.72
25.	247	163	227	70.85	54.60	70.48
26.	247	163	227	70.04	54.60	68.72

- Means uncompleted sequence of our sequencing result

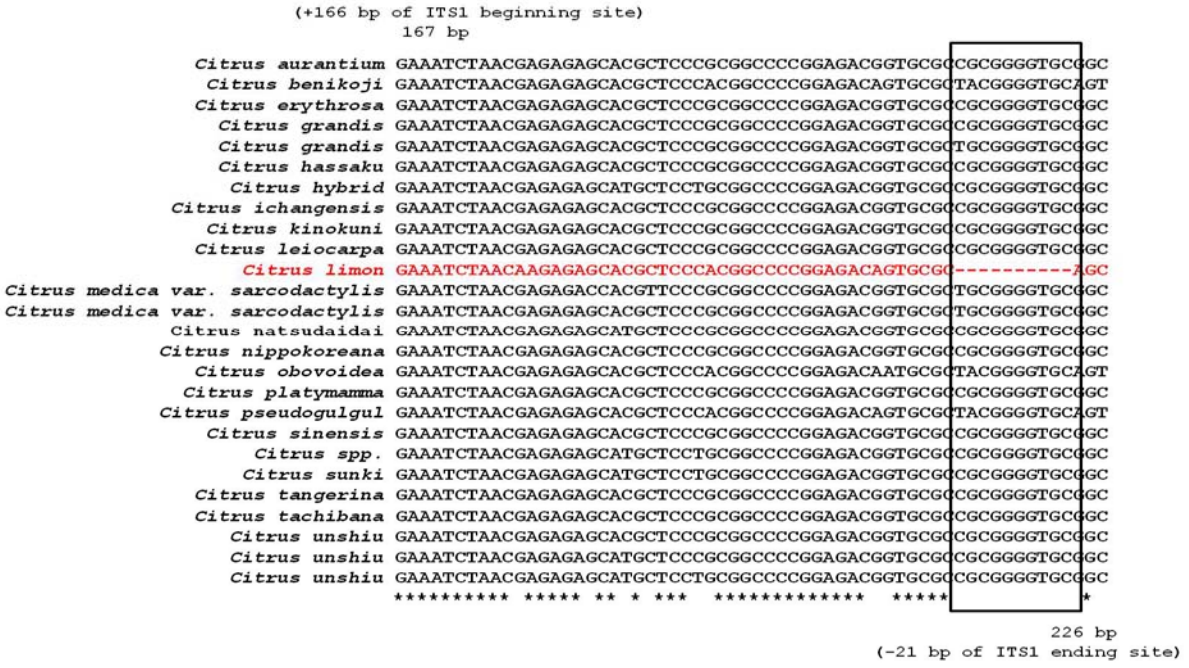

Fig. 1. Sequence substitution, deletion, or addition in the part of ITS1 region among 26 *Citrus* species.

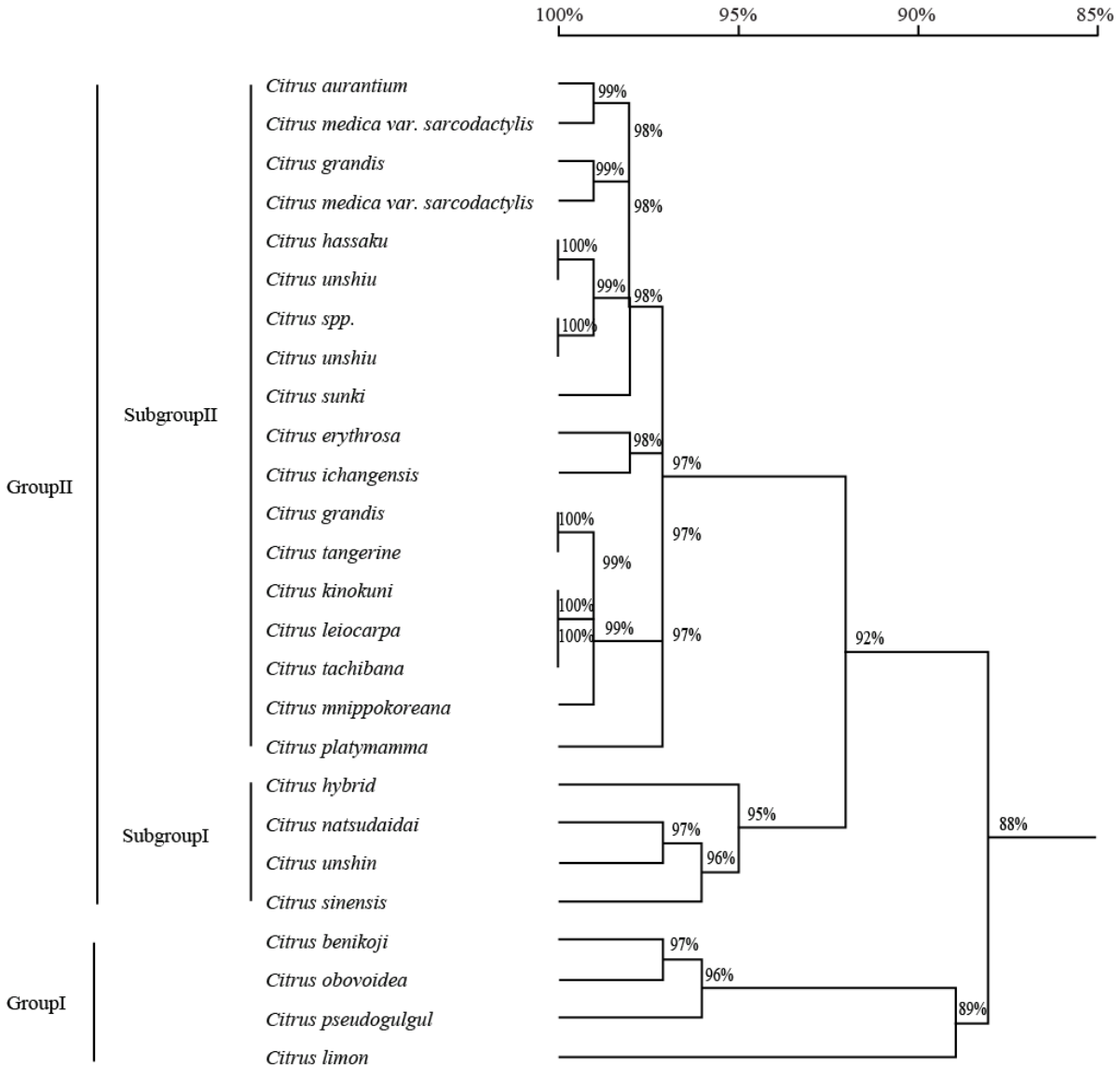

Fig. 2. Phylogenetic tree constructed by the ITS region of 26 *Citrus* species and their grouping.

Phylogenetic relationship among *Citrus* species: A phylogenetic tree was constructed based on the ITS1-5.8S-ITS2 region sequence (Fig. 2). Two groups were recognized: *C. benikoji*, *C. obovoidea*, *C. pseudogulgul*, and *C. limon* formed one group (Group I), while other *Citrus* species formed one (Group II). Both groups shared 88% of identity with each other. In Group I, except *C. limon* belonging to tribe *Citrophorum*, other three species belonged to subgenus *Sinocitrus*. However, this grouping did not relate directly to tribe differentiation, since most species belonging to *Sinocitrus* were grouped to Group II (Fig. 2). In Group II, there were two subgroups showing 92% of identity with each other: *C. hybrid*, *C. natsudaidai*, *C. unshiu* (1 of 3), and *C. sinensis* formed one subgroup (Subgroup I), while other species formed another one (Subgroup II). In Subgroup I, except *C. sinensis* belonging to *Aurantium*, other three species belonged to *Sinocitrus*. Here, interestingly, *C. unshiu* had three plant materials investigated in all, however, one was located in Subgroup I, while two were located in Subgroup II, sharing relatively high similarity rate with *C. hassaku* and *C.* spp., respectively, and forming one monophyletic group (Fig. 2). It was suggested that sequence variation occurred within *Citrus* intraspecies based on the ITS sequence. This situation was also found for *C. medica* var. *sarcodactylis*: both sequences from *C. medica var. sarcodatylis* showed high similarity rate (99%) with *C. aurantium* and *C. grandis*, respectively, forming two respective monophyletic groups (Fig. 2). However, these both *C. medica* var. *sarcodactylis* sequences were not monophyletic. In addition, *C. kinokuni*, *C. leiocarpa*, and *C. tachibana* belonging to *Sinocitrus* shared relatively high similarity rate with each other, and *C. grandis* belonging to *Cephalocitrus* shared high similarity rate with *C. tangerina* belonging to *Sinocitrus*. Combined with *C. nippokoreana*, C. *kinokuni*, *C. leiocarpa*, *C. tachibana*,

C. grandis and *C. tangerina* formed one monophyletic group (Fig. 2). The exclusive *Papedocitrus* species, *C. ichangensis* formed one monophyletic group with one *Sinocitrus* species, *C. erythrosa*. Above results suggested that phylogenetic relationships evaluated based on the ITS sequence had no close relations with tribe discrimination of genus *Citrus*; ITS sequence variation did not reflect tribe classification information.

As known, citrus fruit is one of the most famous specialities of Jeju Island. Having favourable climate for *Citrus* cultivation, Jeju Island has early begun to cultivate *Citrus* species. Because of its good taste and high sugar concentration, citrus fruits were cultivated in Jeju Island were even consecrated to King as tribute in North Korean times (1392-1920). To examine whether there is relationship between genetic diversity and geographical habitat, the phylogenetic relationship was also evaluated according to Jeju Island cultivated species and other *Citrus* species. All the fourteen Jeju Island cultivated species included not only *Sinocitrus* species but *Cephalocitrus* and *Papedocitrus* species (Table 1). Thus, based on above result the grouping in phylogenetic tree was not closely related to tribe discrimination, Jeju Island cultivated species was also not grouped according to different tribe adscription. Whereas, most of these cultivated species fell in subgroup II, except of *C. natsudaidai* in subgroup I and *C. obovoidea*, *C. pseudogulgul*, and *C. benikoji* fell in Group I. This result suggested that genetic diversity caused by geographical factors needs a very long time. No obvious genetic diversity was found between species cultivated in Jeju Island and in other origins. It also suggested that geographical factors did not largely affect *Citrus* phylogenetic relationship in this study, based on sequence variation analysis.

To authors' knowledge, Swingle & Reece's six-subgenus differentiation system in "true citrus fruit trees" group is commonly recognized. One sequence report from each subgenus was investigated from GenBank database in NCBI, i.e. one sequence from *Poncirus trifoliate* representing the subgenus *Poncirus*, one from *Fortunella hindsii* representing *Fortunella*, one from *Cylmenia polyandra* representing *Clymenia*, one from *Microcitrus australasica* representing *Microcitrus*, and one from *Eremocitrus glauca* representing *Eremocitrus*. Three *Citrus* species investigated in this study were selected as the representative of species of *Citrus*, including *C. grandis*, *C. kinokuni*, and *C. nippokoreana*. Compared with these representative sequences, clear subgenus differentiation was found in the phylogenetic tree constructed by the ITS sequence (Fig. 3). *Microcitrus*, *Eremocitrus*, and *Clymenia* are considered to be Australian genera, while *Fortunella*, *Poncirus*, and *Citrus* are considered to be Asian genera. Compared with *Microcitrus* and *Eremocitrus*, Clymenia was nearer to Asian genera, forming one monophyletic group with other three Asian genera species (Fig. 3). This result strongly supported the validity of the subgenus discrimination by Swingle and Reece (1967). However, the number of *Citrus* species needs further determination using more samplings and various discrimination methods.

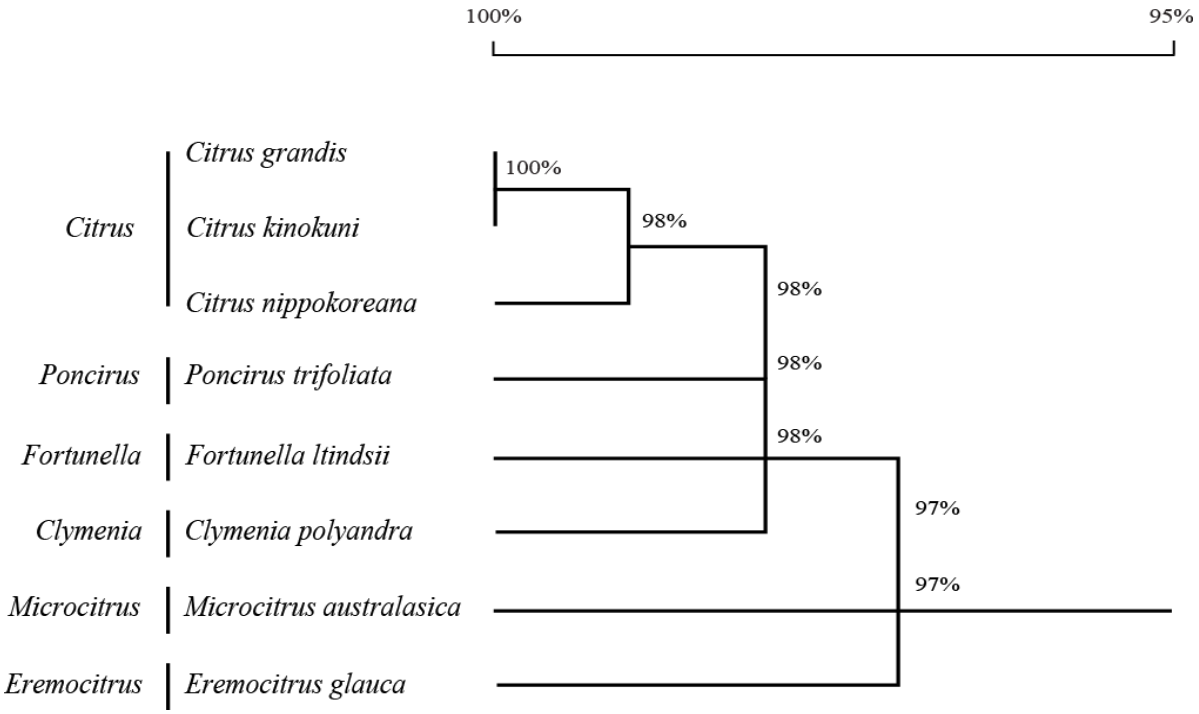

Fig. 3. Phylogenetic tree constructed by the ITS region among three *Citrus* representative species and representative species of other subgenera in the "true citrus fruit tree" group. *Citrus* species used in this analysis were *C. grandis* (GenBank accession number: JQ990169), *C. kinokuni* (GenBank accession number: JQ990159), *C. nippokoreana* (GenBank accession number: JQ990183), *Poncirus trifoliate* (GenBank accession number: FJ434154), *Fortunella hindsii* (GenBank accession number: JN681163), *Cylmenia polyandra* (GenBank accession number: FJ434162), *Microcitrus australasica* (GenBank accession number: AB457061), and *Eremocitrus glauca* (GenBank accession number: FJ434161).

In conclusion, the Swingle & Reece's system of *Citrus* differentiation is strongly validated based on the genetic diversity analysis of ITS sequence in this study. Within the subgenus *Citrus*, six-tribe or subgenera differentiation theory by Swingle & Reece (1967) was, though, accepted by authors, clear tribe discrimination was not found in the phylogenetic tree constructed by ITS sequence. In spite of this, this work provided not only more sequence sources of *Citrus* species but the theoretical, experimental basis of species delimitation in the genus *Citrus*.

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