A DISSERTATION FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Taxonomic Study of Korean *Chrysanthemum* based on Morphological, Molecular, and Chemotaxonomical Characteristics

한국산 국화속 식물의 형태적 · 유전적 · 화학적 분류 연구

BY

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FEBRUARY, 2015

MAJOR IN FLORICULTURE AND LANDSCAPE PLANTS

DEPARTMENT OF HORTICULTURAL SCIENCE & BIOTECHNOLOGY

THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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UNDER THE GUIDANCE OF DR. KIM KI SUN SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL OF SEOUL NATIONAL UNIVERSITY

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ABSTRACT

Korean *Chrysanthemum* is Korean native plant known as Korean wild chrysanthemum, which is very useful genetic resource for landscaping or medicinal plant. Taxonomic relationships among Korean *Chrysanthemum* were investigated using principal component analysis (PCA) and cluster analysis (CA) which were conducted by grouping the taxa based on the morphological, molecular, and chemotaxonomic data. Korean *Chrysanthemum* was collected from natural habitats in different regions of Korean Peninsula from 2006 to 2009. Plants were grown in the greenhouse at the Highland Agricultural Research Institute of National Institute of Crop Science, Rural Development Administration (Pyeongchang, Gangwon, Korea), from 2006 to 2014.

The aim of this study was to obtain clear morphological classification within Korean *Chrysanthemum* using PCA and cluster analysis by comparing 35 horticultural qualitative and quantitative morphological characteristics. As a

result, the shape, length, and *L/W* ratio in leaf were the most remarkable morphological characteristics for the classification, which varied significant differences among 15 *Chrysanthemum* taxa. Group I included *C. zawadskii* subspecies, which had great potential as ground cover material with dense branches (average 18.5 cm in length) and simultaneous flowering with longer flowering period of white or pink flowers. *C. zawadskii* ssp. *acutilobum* var. *alpinum* in Group I also showed potential as useful breeding species for the regulation of various flowering periods due to its spring flowering time while the other taxa bloomed in autumn. Group II had only one species, *C. lineare*, which had unique pale yellow and cone-shaped seed. The seed length of *C. lineare* was 3.2 mm and it was longer than the other groups. *C. lineare* had exceptionally longer leaves with no petiole in both basal and cauline leaves. Group III included three *C. indicum* subspecies, *C. boreale*, and *C. makinoi*. Taxa in Group III were mostly bushy plants with lignified stems at flowering time. The diameter of flower head ranged from 15.1 to 27.3 mm, relatively smaller than the other groups (Groups I and II). Group III seemed to be desirable species as edible medicinal plants for flower tea or cosmetics because of their small flower heads (15.1-27.3 mm in diameter) and more numbers of flowers (80-223 ea) with white or yellow petals. Phylogenetic studies were conducted to evaluate interspecific relationships among Korean *Chrysanthemum* taxa using internal transcribed spacer (ITS 1, ITS 2) region sequences of nuclear ribosomal DNA (nrDNA) and chromosome complement.

The taxa had well-developed series of polyploids (4x, 6x, 8x) from diploids (2n = 2x = 18) with basic chromosome number (n=9). The divergence rate was very low within *C. zawadskii* subspecies ranging from 0.0 to 1.5%. *C. lineare* was basally branched within *Chrysanthemum*. Our results suggested that ITS region sequences were not effective for morphological classification of *Chrysanthemum*, such as *C. zawadskii* and *C. indicum* subspecies. Final study was conducted to reveal taxonomic relationships between the taxa through chemical classification by analyzing flavonoids and volatile flavor compounds. As a result, five flavonoids were identified from *Chrysanthemum* using HPLC/MS: luteolin 7-*O*-rutinoside, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin, and acacetin-7-*O*-rutinoside. In particular, *C. zawadskii* ssp. *acutilobum*, ssp. *yezoense*, ssp. *latilobum*, and *C. indicum* var. *acuta*, which showed effective anti-inflammatory activities, and had four flavonoids (luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, and acacetin-7-*O*-rutinoside), indicating that these four taxa would be used as functional materials. In addition, in 15 taxa from GC/MS analysis, 45 volatile flavor compounds were detected. Camphor, borneol, phytol, phytol, α-pinene, camphene, 1,8-cineole, and germacrene-D were main volatile compounds in *Chrysanthemum*, with differences of significant content among the taxa. Camphor, which is known for its antimicrobial properties, was the most abundant volatile compound in *C. zawadskii* ssp. *latilobum* and var. *leiophyllum*. In particular, *C. indicum* subspecies and *C. boreale* contained α-thujone, which

has outstanding anti-bacterial, anti-cancer, anti-inflammatory, and anti-diabetic efficacies. Especially, *C. indicum* var. *albescens* could be used as perfumes since it showed twenty one times more camphene content than *C. indicum*. In addition, *C. indicum* var. *acuta* contained fairly high content of 1,8-cineole. Therefore, the present study will provide the basic materials needed for species selection and cultivation of *Chrysanthemum* species, which are useful in food, cosmetic, and medicin industry.

Keywords: *Chrysanthemum*, classification, flavonoids, flavor, polyploidy

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GENERAL INTRODUCTION

The genus *Chrysanthemum* (tribe Anthemideae Cass., Asteraceae) is one of the world's most important and popular ornamental and medicinal plant (Kim et al., 2014a). It is distributed in temperate zones of eastern Asia and in central China, Japan, Korea, and Siberia (Iwatsuki et. al., 1997; Trehane, 1995). Korean *Chrysanthemum* includes 18 taxa and is characterized by its obvoid, generally mucilaginous cypselae without pappus, and involucral bracts with dark brown margins (Bremer and Humphries 1993; Lee, 2006; Zhao et. al., 2009). It blooms mainly between September to November and has white, yellow or pink colored flower. The structure of the flower head consists of ray florets on the outer side and disc florets inside. Ray floret is the only pistil and unisexual. Disc floret consists of hundreds of pistils and stamens which are bisexual. Since its flower is protandrous, it does not self-fertilize but almost always cross-fertilizes. Its fruit is usually achene (Oh et al., 1994).

Particularly, *C. zawadskii* of Korean *Chrysanthemum* is the major and most popular native plant in Korea, which occupies 8.1% (3.9 million pots) of Korean native plant production and generates about 38% (USD 6.3 million) of the total Korean native plant sales (KWFA, 2004; KWPA, 2010).

Korean *Chrysanthemum* is being used for natural medicine, traditional food, and as an ornamental plant since the ancient times (Park and Kwon, 1997). It

also has potential for ground covers, cut flower, pot-plant, bedding plant and valuable hereditary resource in the research of genetics and breeding of chrysanthemum due to their origin of cultivated chrysanthemum (Kim et al., 2014a; Lee and Kim, 2000; Oh et al., 1994). Since Korean *Chrysanthemum* contains a lot of flavonoids or terpene compounds (Kim et al., 2014b, 2014c), it has been used as an ingredient in cosmetics, food, and folk medicine for a long time (Matsuda et al., 2002; Kim et al., 1998; Ko and Jeon, 2003). Recently, antioxidant activation and anti-inflammatory effects of the flavor compounds in Korean *Chrysanthemum* have been reported (Bae and Lee, 2008; Sung et al., 2007; Yoon and Cho, 2007). Food processing studies with its health benefits also have been conducted, such as cookie powder (Bae et al., 2009) and rice cake powder (Park and Shin, 1998). *Chrysanthemum* has also been proposed as potential ingredient for herbal cosmetics involving the effects of tyrosinase inhibitory activity associated with anti-oxidant activation, whitening, and moisturizing effects (Bae et al., 2009; Sung et al., 2007; Yoon and Cho, 2007).

Several taxonomic studies are reported the morphological variations among Korean *Chrysanthemum* based on leaf characteristics (Kim and Tobe, 2009; Oh et al., 1994), flowers (Kim et al., 2011), roots (Oh et al., 1994), and genetic characteristics based on Random Amplified Polymorphic DNA (RAPD) (Lee and Kim, 2000), somatic chromosome number and karyotype analysis (Kim, 2003) and chemical composition of petals (Park and Kwon, 1997). Korean *Chrysanthemum* also received a lot of attention, recognizing the importance of

its resource utilization for medicinal and ornamental value. However, only few studies on morphological characteristics, genetic characteristics, and chemotaxonomy have been conducted.

According to the development of numerical taxonomy, phylogenetic taxonomy by morphological characteristics, genetic analysis, and biochemical characteristics have been conducted by multivariate analysis such as Principal Component Analysis (PCA) or cluster analysis, investigating through qualitative and quantitative trait analysis on leaves, fruit, and flower morphology, and taxonomic study based on morphological characteristics. By using these analyses, the correlated variants were found looking for ways to define the characteristics among the species (Kim et al., 2014a, 2014c).

The purpose of this study was to investigate and analyze the classification traits of 15 Korean *Chrysanthemum* taxa based on morphological, molecular, and chemotaxonomical characteristics.

LITERATURE REVIEW

Morphological classification

The taxonomic system of Korean *Chrysanthemum* is as follows. Four species, *C. sibiricum*, *C. indicum*, *C. sinense*, and *C. coronarium*, were reported by a Russian botanist Palibin (1898). while Nakai (1911) reported six species and three varieties. However, Lee (1996) recognized two varieties such as *C. indicum* var. *acutum* with thin leaves and *C. indicum* for. *albescense* with white flower.

Phenotypic characteristics are important for the classification of plant forms at the intergeneric, interspecific, and even intraspecific levels both in the field repositories (Zhao et al., 2009). Morphological characteristics used for diagnostic key to the species have been almost identical (Kim et al., 2014a). Classification of Korean *Chrysanthemum* was based on the head size, color of ray flowers, number and arrangement of heads, and leaf morphology. These morphological characteristics, however, greatly vary within species, and their respective features in species were often overlapped with those in other species (Kim, 2003). Pollen size, spine length and number of spine rows between colpi are important characteristics for distinguishing genera in systematic studies (Clark et al., 1980). Seed morphology such as size, color, or shape is also of systematic significance at specific levels (Brochmann, 2008; Kim et al., 2014a).

Cytological and molecular study

The cytological system of Korean *Chrysanthemum* was reported by Lee (1975). Unlike the species in the Mediterranean that is mainly annual diploids, Korean *Chrysanthemum* is perennial and mainly polyploid (Dowrick, 1952; Kim, 2003). The *C. zawadskii* was basically diploid; most of subspecies were tetraploid, hexaploid and octaploid according to polyploid phenomena and unique divergence depending on habitat (Kim, 2003). It would have been doubled from diploid to octaploid through natural crossing (Nakata et al., 1987). The *C. zawadskii* subsp. *lucidum* was confirmed to be tetraploid (Kim, 1999; Lee, 1975; Oh, 1991) while *C. zawadskii* subsp. *latilobum* was reported as tetraploid or hexaploid (Kim, 1999; Kim, 2003). On the other hand, *C. zawadskii* subsp. *acutilobum* and var. *tenuisectum* and var. *alpinum* were reported hexaploid (Kim, 1999; Lee, 1975) and *C. zawadskii* subsp. *coreanum* was reported 10 diploid (Kim, 1999). Moreover, *C. boreale* was diploid, which was consistent with Kim (1999) and Dowick (1952). It was reported that *C. indicum* was diploid (2n = 18) and tetraploid (2n = 36) and that *C. indicum* for. *albescens* was diploid (2n = 18) or hexaploid (2n = 54), thus *C. indicum* subspecies were diverged into diploid, tetraploid and hexaploid (Kim, 1999).

The chloroplast sequence of diploid *C. boreale* was closer diploid *C. indicum* than diploid *C. zawadskii* (Kim, 1999). The cultivated chrysanthemum was genetically closer *C. boreale* and *C. indicum* than *C. zawadskii* (Lee and Kim, 2000). Besides, *C. zawadskii* subspecies was diverged based on diploid *C.*

zawadskii, from which, some species showed unique divergence patterns according to polarization and habitat. Kim (1999) reported that 2n = 2x = 18 (diploid) in *C. indicum*, *C. zawadskii* was the most primitive and *C. zawadskii* was diverged into 10 diploid *C. zawadskii* ssp. *coreanum*. In the case of chromosome number, there were many variations within the same species according to spontaneous land (Kim, 1999). Hence, diversified genetic analysis and sufficient cytogenetic studies are urgent in order to discuss the phylogeny of the genus *Chrysanthemum*.

Internal transcribed spacer (ITS) approach has been widely used for the phylogenetic analysis (Baldwin et. al., 1995). It is a non-coding region that exists among 18S, 5.8S, and 26S ribosomal DNA (rDNA) and is referred to as ribosomal internal transcribed spacer region. Typically, compared to protein sequence in the course of evolution or rRNA coding gene, variations are severe in terms of sequence and divergence percentage is faster, hence it is used in classifying not only subspecies with close phylogenetic relationships but also in genus or species (Baldwin et al., 1995).

Chemotaxonomy with flavonoids and volatile flavor compounds

Korean *Chrysanthemum* is perennial Asteraceae plant that is have been used as natural medicine or food as well as ornamental plant since the ancient times (Choi, 1992). Since Korean *Chrysanthemum* contains a lot of flavonoid and terpene compounds, it has been used as ingredient in cosmetic (Kim et al., 1995; Matsuda et al., 2002; Shin et al., 1995; Yu and Xie, 1987), food (Ko and Jeon, 2003), and folk medicine (Kim et al., 1998; Nam and Yang, 1995). Recently, antioxidant activity and anti-inflammatory effects of the flavonoid and flavor compounds in Korean *Chrysanthemum* have been reported (Bae and Lee, 2008; Sung et al., 2007; Yoon and Cho, 2007), and food processing studies with its healthful benefits have been conducted (Bae et al., 2009; Park and Shin, 1998). *Chrysanthemum* has also been proposed as potential ingredient for herbal cosmetics involving the effects of tyrosinase inhibitory activity associated with antioxidant activation, whitening, and moisturizing effects (Bae et al., 2009; Sung et al., 2007; Yoon and Cho, 2007).

Flavonoids such as acacetin, apigenin, luteolin, kaempferol, quercetin, and myricetin are the important bioactive molecules in herbal plants (Ha et al., 2006; Matsuda et al., 2002; Shin et al., 1995; Uehara et al., 2012). The flavonoids of *Chrysanthemum* have also been reported from a few species. Eriodictyol 7-*O*-glucuronide was isolated from the flowers of *C. indicum* together with luteolin and its 7-*O*-glucoside (Matsuda et al., 2002). Acacetin and its 7-*O*-galactoside and 7-*O*-rutinoside, apigenin and its 7-*O*-glucoside and 7-*O*-glucuronide,

luteolin and its 7-*O*-glucoside and 7-*O*-glucuronide, kaempferol, quercetin and its 3-*O*-glucoside, 3-*O*-galactoside, 3-*O*-rutinoside and 3,7-di-*O*-glucoside, and myricetin have been reported from the flowers of *C. indicum* (Matsuda et al., 2002; Shin et al., 2004; Wu et al., 2010; Yu and Xie, 1987). From the flowers of *C. arcticum*, apigenin 7-*O*-glucuronide, patuletin 7-*O*-glucoside, quercetin and its 7-*O*-glucoside were isolated (Harborne et al., 1970). Acacetin and its 7-*O*-galactoside, apigenin and luteolin were reported from the whole plants of *C. boreale* (Shin et al., 1995).

Some volatile flavors have been isolated from a few species of *Chrysanthemum*. Previous research reported that the main compounds of volatile flavors derived from *C. indicum* and *C. boreale* were 1,8-cineole, germacrene-D, camphor, α-pinene, and camphene, whereas those from *C. zawadskii* ssp. *latilobum* were mainly terpenoids such as camphene and ocimene (Hong, 2002; Jiang et al., 2005; Kim, 1997; Wang et al., 2008; Wang and Yang, 2006). The *C. indicum* also contained several healthy volatile flavor compounds and various vitamins, which improve blood flow in long-term use, refresh the body, help digestion, alleviate fever and headaches, lower blood pressure, and offer efficacy such as inhibitory effects against tuberculosis and various viruses (Sung et al., 2007). This species has also been used as medicinal wine for patients with hypertension (Choi, 1992) and natural flavoring ingredient in traditional food or tea (Bae et al., 2009; Yoon and Cho, 2007).

Byun et al. (2006) reported that antioxidant compounds such as flavonoids,

terpenoids, and phenolic compounds were the main functional compounds of Korean *Chrysanthemum*. It was also studied for flavonoids and volatile flavor compounds as pharmacological, and cosmetics ingredients, and health food (Kim et al., 2014b). However, flavonoids and volatile flavor compounds were hardly reported from the leaves. Especially, there are few relative to Korean *Chrysanthemum* has slight information on the classification of species based on volatile compounds.

Recently, plants were studied based on numerical taxonomic classification using multivariate data from the biochemical characteristics (Kim et al., 2014c; Sung, 2000). In particular, on the basis of the chemical components like the volatile compounds in plant, multivariate analysis such as PCA and culser analysis have been performed (Chung, 1999; Yun et al., 2002). Cluster analysis is a kind of multivariate analysis that divides data with close similarities into groups (clusters) that are meaningful and useful. As such, this technique for grouping into clusters of similar traits based on the diverse characteristics of certain entities or subjects, cluster analysis can be utilized in situations where there are no clear or known classification criteria (Sung, 2000).

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CHAPTER I

Taxonomic Relationship of Korean *Chrysanthemum* based on Morphological Characteristics

ABSTRACT

In the analysis of genetic relationship among Korean *Chrysanthemum*, the morphological characteristics of 15 *Chrysanthemum* taxa were investigated. Principal Component Analysis (PCA) and cluster analysis were conducted for the grouping based on the morphological data. Fifteen *Chrysanthemum* taxa were classified into three groups through PCA and cluster analysis based on the general plant growth and flowering characteristics. Groups I and II included non-bushy type and large sized flower plants, while Group III included bushy type and small sized flower plants. Group I had nine *C. zawadskii* subspecies: *acutilobum*, *acutilobum* var. *tenuisectum*, *acutilobum* var. *alpinum*, *lucidum*, *coreanum*, *naktongense*, *yezoense*, *latilobum*, and *latilobum* var. *leiophyllum*. Group I was found to be desirable species as garden plants because of white or pink flowers with relatively large size flower head (diameter of 43.5-67.6 mm), good plant height (19.3-64.6 cm), and long flowering period (24-39 days). The *C. lineare* was the only species included in Group II with unique cone head

shaped seeds and no petiole. Group III included five *C. indicum* species and related species: *C. indicum*, *C. indicum* var. *albescens*, *C. indicum* var. *acuta*, *C. boreale*, and *C. makinoi*. Group III had great potential as edible medicinal resources; e.g., flower tea, which was abundant in number and had small sized flowers with white or yellow petals. This study provided a clear diagnostic key for the species-specific classification based on leaf, branch type, plant height, flower size, and flowering types.

Keywords: *Chrysanthemum*, Classification, Cluster, Morphology, Phylogenetic

INTRODUCTION

The tribe Anthemideae of Asteraceae has 12 subtribes, 108 genera and 1,741 species (Bremer and Humphries, 1993). The subtribe Artemisiinae has a total of 18 genera and 634 species and is characterized by disciform or discoid, commonly paniculate capitula, smooth or short spined pollen, obovoid, thin-walled, and ribless cypselae without pappus, and involucral bracts with dark brown margins (Fukai, 2003; Zhao et. al., 2009). The genus *Chrysanthemum* named according to the International Code of Nomenclature consists of 41 species and is distributed in temperate zones in eastern Asia and Siberia (Iwatsuki et. al., 1997). Although most Korean *Chrysanthemum* is distributed throughout Korean Peninsula (Kim, 1999; Lee, 2006), some subspecies are found only in specific regions in Korea. *Chrysanthemum zawadskii* is the major and most popular native plant in Korea, accounting for 8.1% (3.9 million pots) of Korean native plant production (KWPA, 2010) and 38% (US$ 6.3 million) of the total Korean native plant sales (KWFA, 2004).

The diagnostic key for identifying the morphological characteristics within *Chrysanthemum* taxa was almost identical among previous studies, including the size of flower head, color of ray flowers, number and arrangement of head flowers, and leaf morphology, such as leaf shape, leaf thickness, leaf glossiness, and leaf color (Kim, 2003; Kim and Tobe, 2009; Lee, 2006; Oh et al., 1994; Zhao et al., 2009). There are several existing taxonomic studies identifying the

morphological variations among Korean *Chrysanthemum* based on the leaf characteristics (Kim and Tobe, 2009; Oh et al., 1994), flowers (Kim et al., 2011), roots (Oh et al., 1994), and genetic characteristics based on Random Amplified Polymorphic DNA (RAPD) (Lee and Kim, 2000), somatic chromosome and karyotype analysis (Kim, 2003) and chemical composition of petal (Park and Kwon, 1997).

Recently, multivariate analysis for numerical taxonomy has been developed for identification within the species via the morphological and taxonomic characteristics of the species. The method uses not only quantitative but also qualitative data, thereby providing better identification within species (Kim and Lee, 1995; Kim et al., 1999; Sung, 2000).

Korean *Chrysanthemum* has gained a lot of attention as an important medicinal and ornamental plant, and its market is expected to increase significantly. To establish the proper classification and usage of the species, better identification, understanding of the species, clear classification and grouping will be important helping hand.

This study was aimed to obtain clear classification within Korean *Chrysanthemum* by using PCA and cluster analysis to categorize the 15 taxa into groups by morphological characteristics.

MATERIALS AND METHODS

Plant materials and growing conditions

Lee (2006) reported that Korean *Chrysanthemum* included 18 taxa characterized by morphological criteria in New Flora of Korea. In accordance with the flower head diameter, flower size, leaf margin, seed shape, and others, Korean *Chrysanthemum* has been classified into 8 species, 9 subspecies and 1 variety, and listed 18 taxa: *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*, *C. zawadskii* ssp. *acutilobum* var. *alpinum*, *C. zawadskii* ssp. *lucidum*, *C. zawadskii* ssp. *coreanum*, *C. zawadskii* ssp. *naktongense*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*, *C. indicum*, *C. indicum* var. *albescens*, *C. boreale*, *C. morifolium*, *C. makinoi*, *C. coronarium*, *C. intermedium*, *C. pallasianum* and *C. lineare*. Among the 18 taxa, 15 taxa were investigated in the study: wherein four taxa, namely, *C. coronarium*, *C. morifolium*, *C. intermedium*, and *C. pallasianum* were excluded and one variety, *C. indicum* var. *acuta*, was added in accordance with Lee (1996).

Fifteen taxa of five Korean *Chrysanthemum* species with five subspecies and five varieties were collected from the natural habitats in different regions of Korean Peninsula from April to October during 2006-2009 (Table I-1 and Fig. I-1). The classification and nomenclature of species in *Chrysanthemum* were mainly in accordance with Lee (1996, 2006) and the International Code of

Botanical Nomenclature (Trehane, 1995). Collected plants were transplanted into square plastic pots (20 cm × 20 cm × 16 cm) filled with a mixture (1:1, v:v) of commercial horticultural substrate (SsukSsukyi: 60% peat moss, 30% perlite, Nongwoo Co., Suwon, Korea) and sandy loam (Masato, Hwaboonworld, Chilgok, Korea). Voucher specimens were deposited in the Korea Medicinal Resources Herbarium (KMRH) at Rural Development Administration (RDA), Korea. These species have also been deposited in the herbarium at the Highland Agricultural Research Institiute (HARI, 37°40'N, 128°43'E, altitude 772 m). Individual plants were then grown in the greenhouse at HARI. Foliar spray of nutrient solution (6-10-5 New Hyponex, Hyponex Co., Osaka, Japan) was applied bi-weekly throughout the growing period from June to September, 2010, 2011, and 2012. When flowering occurred, the plants were sampled and preserved at the herbaria of RDA. Each of five individuals were randomly selected and investigated for morphological characteristics for three years from 2010 to 2012.

Table I-1. List of Korean *Chrysanthemum* used in this study.

Scientific name	Korean name	KMRH voucher[z]	Cites (Natural habitats)
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa	Ganeunip-gujeolcho	MPS003031	Mt. Yumyeong, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *tenuisectum* (Kitagawa) Y. Lee	Pocheonganeunip-gujeolcho	MPS003032	Pocheon, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *alpinum* (Nak.) Y. Lee	Bawi-gujeolcho	MPS003033	Mt. Baekdu, Hambuk, Korea
C. zawadskii Herbich ssp. *lucidum* (NAK.) Y .Lee	Ulleung-gughwa	MPS003034	Ulleung, Gyeongbuk, Korea
C. zawadskii Herbich ssp. *coreanum* (NAK.) Y. Lee	Hanla-gujeolcho	MPS003035	Mt. Halla, Jeju, Korea
C. zawadskii Herbich ssp. *naktongense* (NAK.) Y. Lee	Nakdong-gujeolcho	MPS003037	Gimhae, Gyeongnam, Korea
C. zawadskii Herbich ssp. *yezoense* (Maekawa.) Y. Lee	Nam-gujeolcho	MPS003039	Goheung, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa	Neolbeunip-gujeolcho	MPS003041	Wando, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa var. *leiophyllum* (Nak.) Y. Lee	Seoheungneolbeunip-gujeolcho	MPS003043	Gangneung, Gangwon, Korea
C. indicum Linné	Gamguk	MPS003044	Anmyeondo, Chungnam, Korea
C. indicum Linné var. *albescens* Makino	Huin-gamguk	MPS003046	Jeongseon, Gangwon, Korea
C. indicum var. *acuta* (Uyeki) Kitam.	Ganeunip-gamguk	MPS003047	Byeonsanbando, Jeonnam, Korea
C. boreale (Mak.) Makino	Sanguk	MPS003049	Pyeongchang, Gangwon, Korea
C. lineare Matsumura	Kikeun-sanguk	MPS003050	Mt. Chilbo, Gyeonggi, Korea
C. makinoi Matsumura et Nakai	Makino-gughwa	MPS003051	Daegu, Korea

[z]KMRH, Korea Medicinal Resource Herbarium at Rural Development Administration in Korea.

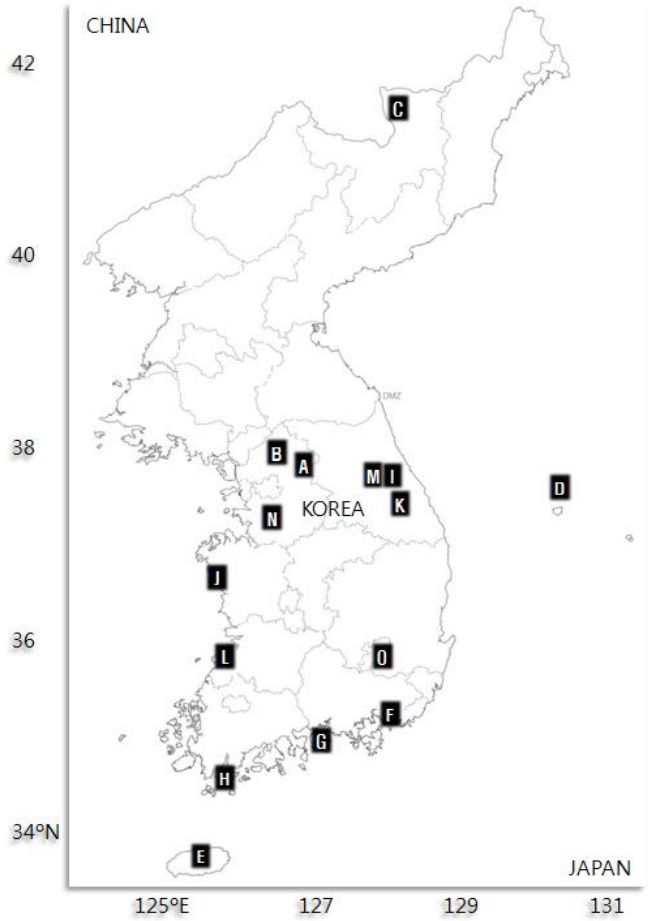

Fig. I-1. Collection sites of Korean *Chrysanthemum* from 15 different regions in Korea. Sites are indicated with black dots. A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

Meteorological data

Meteorological data was collected based on 30 year average value from Korea Meteorological Administration from 1981 to 2010 (KMA, 2012). The climatological data of local areas were obtained from the national weather service website. Annual meteorological conditions, such as average, maximum and minimum temperatures, precipitations, wind speed, humidity, and daylight durations were investigated (Table I-2).

Analysis of morphological characteristics

As shown in Fig. I-2, the general morphological characteristics were investigated to determine the average plant growth and flowering based on the Chrysanthemum Test Guidelines criteria set by the International Union for the Protection of New Varieties of Plants (UPOV, 2008) and the Korea Seed and Variety Service (Song et al., 2000).

Plant height was measured from the tallest point of the canopy to the base of the plant. Winter suckers were photographed and the color was determined using the Royal Horticultural Society (RHS) chart with the visual appearance. Glossiness of winter suckers was visually rated as either strong, weak, or absent. Basal leaf morphology was measured during the growing period and cauline leaf morphology was measured during the flowering time. Leaf length was measured from the lamina tip to the intersection of lamina and petiole along the lamina mid-rib.

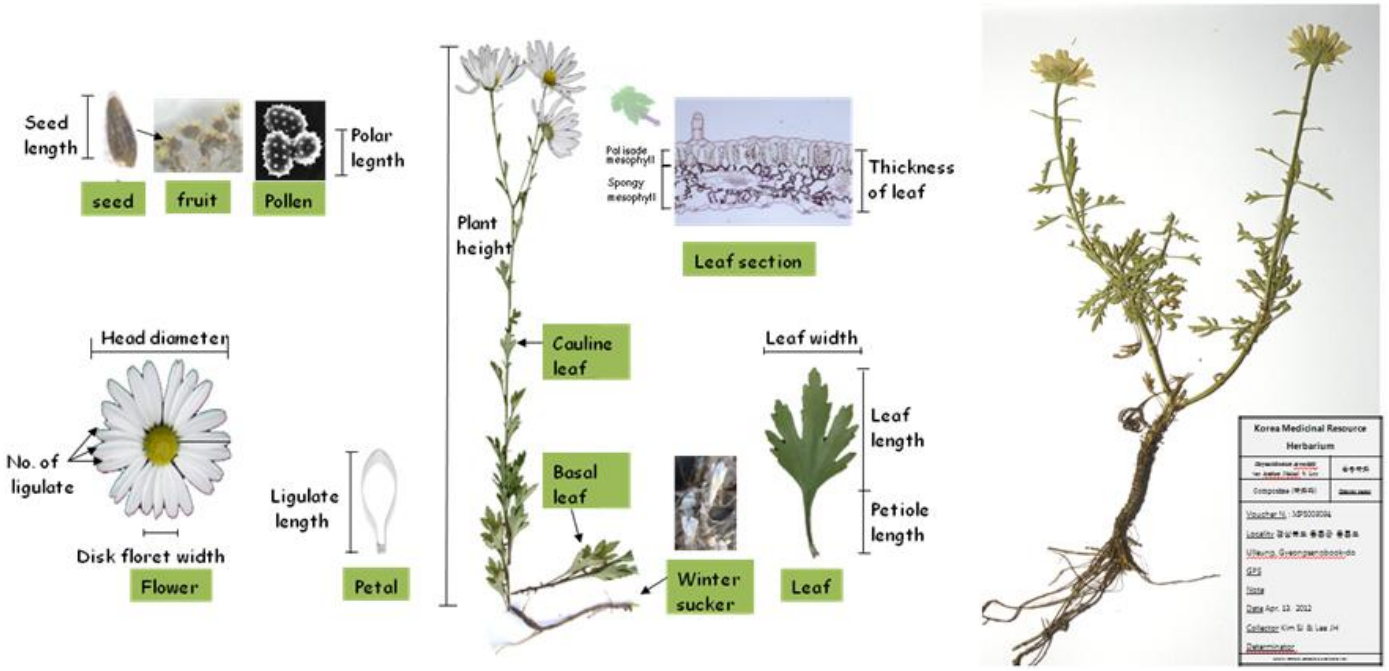

Fig. I-2. Basic survey standard (left) and herbarium voucher (right) of Korean *Chrysanthemum*.

Leaf width was measured from the widest lamina lobes. Petiole length was measured from the stem margin to the end of leaf lamina. Leaf area was measured using leaf area meter (LI-3100, Li-Cor Inc., Lincoln, NE, USA), and the average leaf area was calculated from 10 leaves.

The number of flowers was counted during the flowering time of each species. Flower head diameter was measured from the widest point of the flower. Disk floret width was measured from the widest point of yellow floret. The length of ligulate petal was measured, after taking out the petals from the flower. Colors of the flower were determined using Royal Horticultural Society (RHS) color chart. Flowering time was determined at 40% flowering stage during the flowering period under natural environmental condition and the flowering period was recorded.

Measurement of leaf anatomy

To measure leaf thickness and cell density, leaf tissue sections were fixed with modified Kamovsky fixative (Oh and Kim, 2010) containing 2.5% glutaraldehyde with air bubbles removed at 4˚C for 90 min, and washed with 0.1 M sodium phosphate buffer (pH 7.2) five times, with each wash lasting 15 min. The tissue sections were post-fixed with 1% osmium tetroxide at 4˚C for 90 min and washed with the same phosphate buffer five times. The post-fixed tissue sections were dehydrated in grade ethanol series of 40, 60, 80, 90, and 95% and three times in 100%, with each dehydration lasting 5 min. Tissue sections

were then treated in grade mixed solution series of propylene oxide:epon (2:1; v:v), propylene oxide:epon (1:1; v:v), and propylene oxide:epon (0:1; v:v) with each treatment lasting 3 h. After the tissue sections were treated with 100% epon and 1.5% epon in 2,4,6-Tris (dimethylaminomethyl) phenol solution for 15 min and inserted in silicon mold, these were kept for polymerization in an oven at 60˚C for 4 days. The polymerized epon block was cut up into leaf samples with 1,500 nm thickness using microtome (Ultracut R, Leica Microsystems GmbH, Wetzlar, Germany), followed by staining with 0.5% periodic acid staining solution, and was washed with distilled water two times with each wash lasting 10 min.

The stained section was treated with Schiff's reagent for 15 min and 1% sodium bisulfate solution for 5 min and then washed with distilled water for 30 min. The stained section was dried on 60˚C hot plate for 5 h and dropped into histomount and then covered with cover glass. The stained sections were photographed using 100X optical microscope (Axioskop 2, Carl Zeiss, Jena, Germany). Leaf thickness was measured using leaf cross-sections and determined by observing the cell density with NIS-D version 3.22 software (Nikon NIS-D, Nikon Co., Tokyo, Japan).

Pollen morphology

Pollen morphology was examined through natural mode using scanning electron microscopy (SEM, Hitachi N-2460, Hitachi Co., Tokyo, Japan) and the

size of pollen was calculated by the Leica Application Suite version 2.8.1 software (Leica Microsystems GmbH, Wetzlar, Germany).

Seed morphology

Seeds were collected from mature achene for each species at 2nd month after the flowering time. Seeds were photographed and documented using optical microscope (Nikon SMZ 800, Nikon Co., Tokyo, Japan). Seed size was calculated through the Nikon Application Suite version 2.8.1 software (Nikon Microsystems, Nikon Co.).

Data analysis

To evaluate the morphological diversity structure and establish relationships among species, several statistical procedures were conducted. Analysis of variance (ANOVA) of 35 qualitative and quantitative morphological characteristics (Yun et al., 2002) of the 15 *Chrysanthemum* taxa was done using SAS version 9.2 software (SAS Institute Inc., Cary, NC, USA) of 2010-2012 data. Levels of significance were calculated using ANOVA test at $p < 0.01$, 0.05, and 0.001.

Two multivariate analyses, PCA and cluster analysis were used for identification within species via the morphological and taxonomic characteristics (Zar, 1984). Quantitative and qualitative data were used to provide better identification within taxa. In particular, cluster analysis was to

aggregate any population, species or varieties that had very similar characteristics into few species groups.

The PCA was conducted to detect the differences among 15 taxa by considering 35 morphological characteristics simultaneously using *proc factor* with SAS software. The first three principle components were extracted through PCA and figured out with two-dimensional plot using SAS software. Cluster analysis was performed using morphological characteristics of the species based on quantitative and qualitative variations.

Six hierarchical methods was used for clustering: Ward's minimum variance method (Ward, 1963), weighted pair-group method using centroids (WPGMC), the single linkage method, complete linkage method, the average linkage method (also called UPGMA-the unweighted pair-group method using arithmetic averages), and maximum likelihood estimate algorithm. In each cluster, the mean morphological characteristics and their standard deviation were calculated for each horticultural qualitative and quantitative trait. The semi-R^2 value showing the amount of the variability in the data set captured by the clusters was plotted and assessed. The Cubic Clustering Criterion (CCC), Calinski and Harabasz index (the pseudo-*F*) and statistics based on the Je(2)/Je(1) rule (pseudo-T^2) were also used to determine the number of clusters that best described the data set. The number of groups in cluster analysis was decided based on the cluster history. Dendrogram of 15 *Chrysanthemum* taxa was drawn using PROC TREE of the SAS version 9.2 software.

RESULTS

Meteorological environment condition

Averages of 30 years meteorological data of 15 natural habitats of Korean *Chrysanthemum* were obtained from the Korea Meteorological Administration (Table I-2). The altitude of 15 natural habitats ranged from 26 m to 1386 m, where *C. zawadskii* ssp. *acutilobum* var. *alpinum* was collected at the highest altitude in Mt. Baekdu while *C. zawadskii* ssp. *latilobum* var. *leiophyllum* was collected at the lowest altitude in Gangneung. Average temperature was the highest in Mt. Halla at 15.8˚C and lowest in Mt. Baekdu at 0.5˚C. Accumulated precipitation was the lowest in Mt. Baekdu with 915 mm and highest in Pyeongchang with 1898 mm. Wind speed was the strongest in Pyeongchang and weakest in Mt. Yumyeong. Relative humidity and daylight duration were the highest in Byeonsanbando.

For *C. zawadskii* subspecies, in the case of *C. zawadskii* ssp. *acutilobum*, ssp. *acutilobum* var. *tenuisectum*, ssp. *acutilobum* var. *alpinum*, and ssp. *lucidum* from low temperature regions where the average temperature of collection sites was 0.5-12.5℃, the leaf margin was deep-shaped. However, in the collection sites of *C. zawadskii* subspecies with 13.1-14.1˚C, the leaf margin was shallow-shaped (Table I-3; Fig I-4). The result consisted of collected samples directly from the field and compiled climatic data from nearby weather stations. Leaf

habit could also affect the leaf climate relationships. Adams et al. (2008) presented the results of systematic spatially distributed analysis by showing the relationship between the leaf margins and temperature demonstrating the strong relationship between the percentage leaf margin and temperature on regional scale. This result indicated promising tool for analyzing the relationship between leaf margin and climate (Greenwood et al., 2004).

High elevation plants with very short growing season can photosynthesize very efficiently in low-temperature conditions and also adapt to the high intensity light (Körner and Larcher, 1988). This could be attributed to the plants effective protection mechanism against oxidative damages (Polle, 1997; Streb and Feierabend, 1999). In addition, flower bud development in *Chrysanthemum* is affected by temperature conditions (Nothnagl et al., 2004). This caused early termination of the vegetative growth and relatively earlier start of the reproduction growth of *C. indicum* (Kim et al., 2007).

In this study, *C. zawadskii* subspecies, in general, bloomed in September and October whereas *C. zawadskii* ssp. *acutilobum* var. *alpinum* adjusted to the environment of high elevation of 1386 m which resulted in the early blooming in April. This could be due to average temperature of 0.5˚C in the high mountain which is considered very low regions (Table I-2). These studies proved that enhanced stress tolerance in chrysanthemum is possible by crossing commercial cultivars with wild relative species (Sun et al., 2010).

Table I-2. Meteorological data of natural habitats of 15 Korean *Chrysanthemum* taxa.

Region	Lat.(N)	Long.(E)	Altitude (m)	Average temp. (˚C)	Maximum temp. (˚C)	Minimum temp.(˚C)	Accumulated precipitation (mm)	Wind speed (m/s)	Humidity (%)	Daylight Hours (hr)
Mt. Yumyeong, Gyeonggi, Korea	37° 34'	127° 29'	860	11.6	17.7	6.3	1,438	1.2	70.5	2,265
Pocheon, Gyeonggi, Korea	38° 08'	127° 18'	154	10.2	16.2	4.7	1,391	1.8	70.4	2,050
Mt. Baekdu, Hambuk, Korea	41° 98'	128° 02'	1,386	0.5	6.7	-5.7	915	1.6	74.6	No data
Ulleung, Gyeongbuk, Korea	37° 28'	130° 53'	222	12.5	15.8	9.8	1,384	3.7	74.3	1,856
Mt. Halla, Jeju, Korea	33° 21'	126° 31'	1,320	15.8	18.9	12.9	1,498	3.5	69.6	1,854
Gimhae, Gyeongnam, Korea	35° 02'	128° 53'	37	13.3	19.4	11.1	1,229	2.2	63.8	2,145
Goheung, Jeonnam, Korea	34° 37'	127° 16'	53	13.6	19.3	8.4	1,454	1.5	69.7	2,370
Wando, Jeonnam, Korea	34° 23'	126° 42'	35	14.1	18.1	10.8	1,533	3.6	72.5	2,067
Gangneung, Gangwon, Korea	37° 45'	128° 53'	26	13.1	17.5	9.2	1,464	2.6	61.4	2,106
Anmyeondo, Chungnam, Korea	36° 30'	126° 24'	28	11.9	17.3	7.2	1,286	2.4	74.1	2,179
Jeongseon, Gangwon, Korea	37° 27'	128° 33'	712	8.8	14.2	3.8	1,324	1.7	67.0	2,145
Byeonsanbando, Jeonnam, Korea	34° 41'	126° 43'	12	12.7	18.0	8.0	1,250	1.6	76.0	2,473
Pyeongchang, Gangwon, Korea	37° 47'	128° 32'	772	6.6	11.5	2.0	1,898	4.3	73.3	2,195
Mt. Chilbo, Gyeonggi, Korea	37° 16'	126° 59'	238	12.0	17.2	7.5	1,312	1.7	69.6	2,163
Daegu, Korea	35° 53'	128° 37'	64	14.1	19.5	9.5	1,064	2.8	63.5	2,230
Average			395	11.5	16.5	7.0	1,384	2.4	70.0	2,150

*Values represent the averages for 30 years (1981-2010) from the Korea Meteorological Administration.

Growth characteristics

A total of 35 morphological characteristics based on the Chrysanthemum Test Guidelines criteria set (UPOV, 2008) and the Korea Seed and Variety Service (Song et al., 2000) were evaluated and found to be slightly different among taxa (Table I-3). The plant heights of 15 *Chrysanthemum* taxa were divided into three types: short (10-40 cm), medium (40-70 cm) and tall (70-100 cm). The growth habitats of 15 taxa were grouped into bushy type or non-bushy type, where five taxa were bushy type and 10 taxa were non-bushy type (Table I-3). There were nine taxa with weakly glossy winter suckers and five taxa with non-glossy winter suckers (Table I-3 and Fig. I-3).

Table I-3. Mean values of 35 qualitative and quantitative traits in 15 Korean *Chrysanthemum* taxa used for multivariate analysis.

Species[z]	Plant		Winter sucker		Basal leaf								Cauline leaf							
	Plant height (cm)	Plant type[y]	Glossi-ness[x]	Tricoh-ome density[w]	Leaf length (cm)	Leaf width (cm)	L/W ratio	Petiole length (cm)	Leaf thickness (mm)	Leaf area (cm^2)	Leaf shape	Leaf margin	Leaf length (cm)	Leaf width (cm)	L/W ratio	Petiole length (cm)	Leaf thickness (mm)	Leaf area (cm^2)	Leaf shape	Leaf margin
A	64.6	NB	W	M	4.5	4.6	1.0	3.5	1.2	10.3	Ovate	Deep	2.5	3.4	0.7	3.3	0.9	1.1	Ovate	Deep
B	39.2	NB	W	S	4.8	5.2	0.9	4.6	1.4	3.9	Ovate	Deep	0.9	3.8	0.2	3.8	0.9	0.5	Ovate	Deep
C	18.5	NB	W	C	2.6	3.3	0.8	2.6	1.2	2.4	Heart	Deep	1.2	3.3	0.4	2.5	1.1	1.7	Oblong	Deep
D	41.6	NB	S	A	4.6	5.6	0.8	4.3	1.7	12.8	Ovate	Deep	1.6	4.4	0.4	3.6	1.1	3.6	Ovate	Deep
E	19.3	NB	W	M	3.2	4.1	0.8	3.4	1.0	4.7	Ovate	Deep	0.6	2.3	0.3	2.5	0.7	0.8	Ovate	Deep
F	49.5	NB	W	M	4.3	4.9	0.9	3.3	1.4	14.7	Ovate	Medium	2.0	4.1	0.5	2.8	1.1	2.4	Spatulate	Shallow
G	45.6	NB	S	M	4.1	4.1	1.0	3.7	1.3	12.7	Ovate	Shallow	0.5	3.3	0.1	4.3	0.9	5.6	Spatulate	Shallow
H	35.5	NB	W	M	4.1	4.6	0.9	4.5	1.3	11.2	Heart	Shallow	1.2	3.7	0.3	4.7	1.0	5.5	Spatulate	Shallow
I	60.1	NB	W	S	4.6	4.3	1.1	2.5	1.3	13.3	Heart	Shallow	0.9	3.9	0.2	3.1	0.8	2.7	Spatulate	Shallow
J	76.6	BU	A	M	4.0	3.9	1.0	1.6	1.0	12.9	Ovate	Medium	4.2	4.4	1.0	1.7	1.1	8.8	Ovate	Medium
K	65.2	BU	A	M	4.2	3.9	1.1	1.6	1.2	9.9	Ovate	Medium	3.1	3.9	0.8	1.9	0.9	6.0	Ovate	Medium
L	43.2	BU	A	M	4.1	4.1	1.0	1.6	1.2	7.3	Ovate	Medium	2.7	3.2	0.9	1.1	0.7	5.7	Ovate	Medium
M	89.9	BU	A	M	4.9	4.7	1.0	1.6	1.3	15.2	Oblong	Medium	5.2	5.5	0.9	2.0	1.0	11.0	Ovate	Medium
N	93.3	NB	W	S	9.2	2.2	4.2	-[v]	0.9	0.7	Linear	Deep	8.6	2.3	3.7	-	0.5	4.1	Lanceolate	Deep
O	58.3	BU	W	S	5.2	3.6	1.4	1.4	1.0	7.0	Ovate	Medium	2.2	2.6	0.9	1.4	1.0	7.4	Ovate	Medium

[z]A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. Indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi.*

[y]NB, Non bushy; BU, bushy.

[x]Glossiness, Glossiness of leaf surface express A (Absent or very weak), W (Weak), or S (Stong).

[w]Hair, The trichome density of winter sucker express A (Absent or very scare), S (Scare), M (Middle) or C (compact).

[v]Petiole, The absence of petiole denote dash (-).

[u]RHS: Royal Horticultural Society.

[t]Pollen shape: Oblate (P/E ratio)=0.5-0.75, Suboblate=0.75-0.88, Spheroidal=0.88-1.14, Subprolate=1.14-1.33, Prolate=1.33-2.00.

(Continued)

Species	Flower							Pollen			Seed				
	No. of flower	Head diameter	Disk floret width	Ligulate length	Flower color	Flowering time	Flowering period	Polar length	Equatorial diameter	Pollen shape[t]	Seed length	Seed width	L/W ratio	Seed shape	Seed color
	(ea)	(mm)	(mm)	(mm)	(RHS)[u]	(date)	(Days)	(um)	(um)		(mm)	(mm)			
A	48	52.2	12.7	21.1	WHITE	Sep. 22	33	40.9	32.5	Subprolate	2.1	0.9	2.3	Lanceolate-ovate	Brown
B	35	52.6	9.6	22.6	WHITE	Sep. 18	34	38.5	32.3	Subprolate	2.2	0.9	2.4	Lanceolate-ovate	Brown
C	4	44.5	12.5	17.3	W155B	Apr. 23	35	38.9	29.1	Prolate	2.6	0.9	2.9	Lanceolate-oblong	Brown
D	29	58.5	14.0	22.8	WHITE	Sep. 22	39	39.0	28.0	Prolate	2.6	0.9	2.9	Lanceolate-oblong	Brown
E	15	45.7	9.4	20.1	WHITE	Oct. 15	24	48.5	35.6	Prolate	2.0	0.9	2.2	Lanceolate-ovate	Brown
F	26	58.1	12.4	24.7	WHITE	Oct. 1	33	36.3	33.9	Spheroidal	2.2	0.9	2.4	Lanceolate-ovate	Brown
G	23	59.4	12.4	23.8	WHITE	Oct. 14	38	43.5	35.4	Subprolate	2.7	1.0	2.7	Lanceolate-oblong	Brown
H	14	67.6	14.3	26.7	WHITE	Oct. 11	32	45.2	34.9	Prolate	2.5	0.9	2.8	Lanceolate-oblong	Brown
I	54	43.5	8.7	17.8	P75C	Oct. 6	24	41.6	38.9	Spheroidal	2.1	0.9	2.3	Lanceolate-oblong	Dark brown
J	106	27.3	9.8	9.4	Y3B	Oct. 8	39	40.4	29.7	Prolate	1.7	0.8	2.1	Lanceolate-ovate	Dark brown
K	146	25.4	7.8	8.9	WHITE	Oct. 13	34	28.3	27.2	Spheroidal	1.5	0.7	2.1	Lanceolate-ovate	Brown
L	80	22.9	6.5	8.2	Y6B	Oct. 8	28	39.1	31.3	Subprolate	1.8	0.8	2.3	Lanceolate-ovate	Dark brown
M	223	15.1	5.4	5.8	Y7A	Oct. 12	29	35.3	26.3	Prolate	1.3	0.6	2.2	Lanceolate-ovate	Dark brown
N	19	42.4	14.9	15.1	WHITE	Sep. 25	25	40.4	32.0	Subprolate	3.2	1.2	2.7	Cone	Pale yellow
O	12	26.0	8.6	13.1	WHITE	Nov. 26	34	36.8	26.4	Prolate	1.6	0.7	2.3	Lanceolate-ovate	Dark brown

Table I-4. List of 35 qualitative and quantitative traits and their range of values in 15 Korean *Chrysanthemum* taxa.

Plant part	Trait	Unit	Mean±SD	F value[z]	Range and parameter code[y]
Plant	Plant height	cm	53.3±22.39	6.82*	18.5-93.3
	Plant type	degree	1.3±0.49	2.36***	Non bushy (1), Bushy (2)
Winter sucker	Glossiness	degree	2.7±1.28	8.60**	Absent or very weak (1), Weak (3), Strong (5)
	Tricohome density	degree	4.5±1.77	0.33^{NS}	Absent or very scare (1), Scare (3), Middle (5), Compact (9)
Basal leaf	Leaf length	cm	4.6±1.44	26.19***	2.6-9.2
	Leaf width	cm	4.2±0.82	6.99**	2.2-5.6
	L/W ratio	ratio	1.2±0.85	281.50***	0.8-4.2
	Petiole length	cm	2.7±1.36	26.95***	0.0-4.6
	Leaf thickness	mm	1.2±0.20	3.47^{NS}	0.9-1.7
	Leaf area	LAI	9.3±4.65	2.20^{NS}	0.7-15.2
	Leaf shape	degree	2.1±1.83	7.54**	Ovate (1), Heart (3), Oblong (5), Linear (7)
	Leaf margin	degree	5.4±1.55	0.67^{NS}	Shallow (3), Medium (5), Deep (7)
Cauline leaf	Leaf length	cm	2.5±2.16	35.75***	0.5-8.6
	Leaf width	cm	3.6±0.85	1.67^{NS}	2.3-5.5
	L/W ratio	ratio	0.8±0.87	218.87***	0.1-3.7
	Petiole length	cm	2.6±1.27	19.50***	0.0-4.7
	Leaf thickness	mm	0.9±0.18	4.15*	0.5-1.1
	Leaf area	LAI	4.5±3.08	10.53**	0.5-11.0
	Leaf shape	degree	2.7±2.49	10.30**	Ovate (1), Oblong (3), Spatulate (5), Lanceolate (9)
	Leaf margin	degree	5.3±1.67	0.57^{NS}	Shallow (3), Medium (5), Deep (7)

	No. of flower/plant	ea	55.6±60.73	5.66*	4-223
	Head diameter	mm	42.7±15.94	28.46***	15.1-67.6
	Disk floret width	mm	10.6±2.94	10.00**	5.4-14.9
Flower	Ligulate length	mm	17.2±6.74	30.21***	5.8-26.7
	Flower color	RHS	1.7±1.23	0.73^{NS}	White (1), Yellow (3), Pink (5)
	Flowering time	date	269±46.3	0.92^{NS}	Apr. 23-Nov. 26
	Flowering period	day	32.1±5.06	1.06^{NS}	24-39
	Polar length	um	39.5±4.63	2.76^{NS}	28.3-48.5
Pollen	Equatorial diameter	um	31.6±3.77	4.75^{NS}	26.3-38.9
	Shape	degree	5.9±3.20	0.39^{NS}	Suboblate (1), Spheroidal (3), Subprolate (5), Prolate (9)
	Seed length	mm	2.1±0.52	25.66***	1.3-3.2
	Seed width	mm	0.9±0.14	38.46***	0.6-1.2
Seed	L/W ratio	ratio	2.4±0.28	4.11*	2.1-2.9
	Seed shape	degree	1.8±1.26	9.12**	Lanceolate ovate (1), Lanceolate oblong (3), Cone (5)
	Seed color	degree	1.9±1.28	14.37***	Brown (1), Dark brown (3), Pale yellow (5)

[z]The variables in 15 taxa expressed [NS]non-significant, [*] Significant at $p < 0.05$, [**] Significant at $p < 0.01$, or [***] Significant at $p < 0.001$.
[y]The Chrysanthemum Test Guidelines Criteria by the Seed and Variety Service.

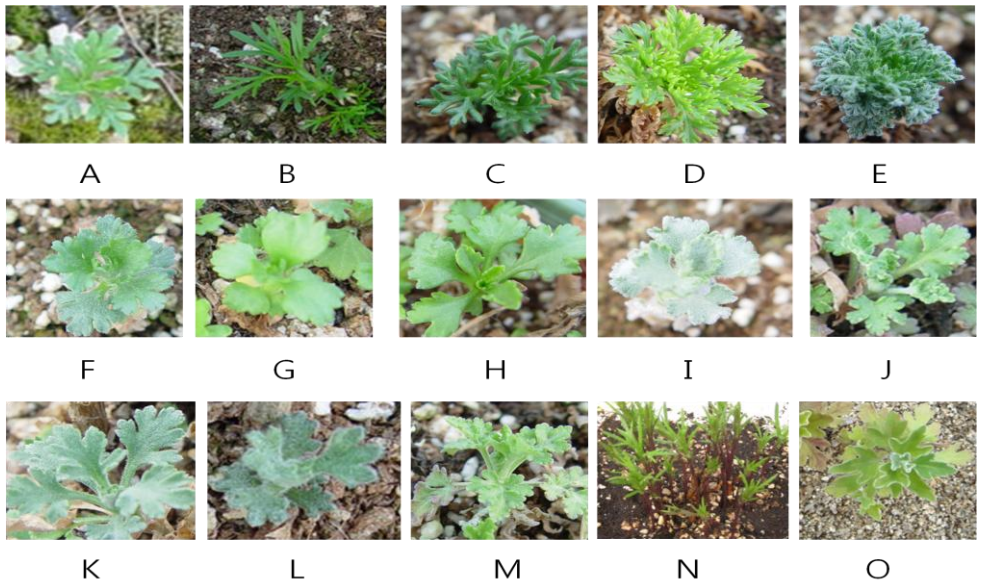

Fig. I-3. Images of winter suckers in 15 Korean *Chrysanthemum* taxa. A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

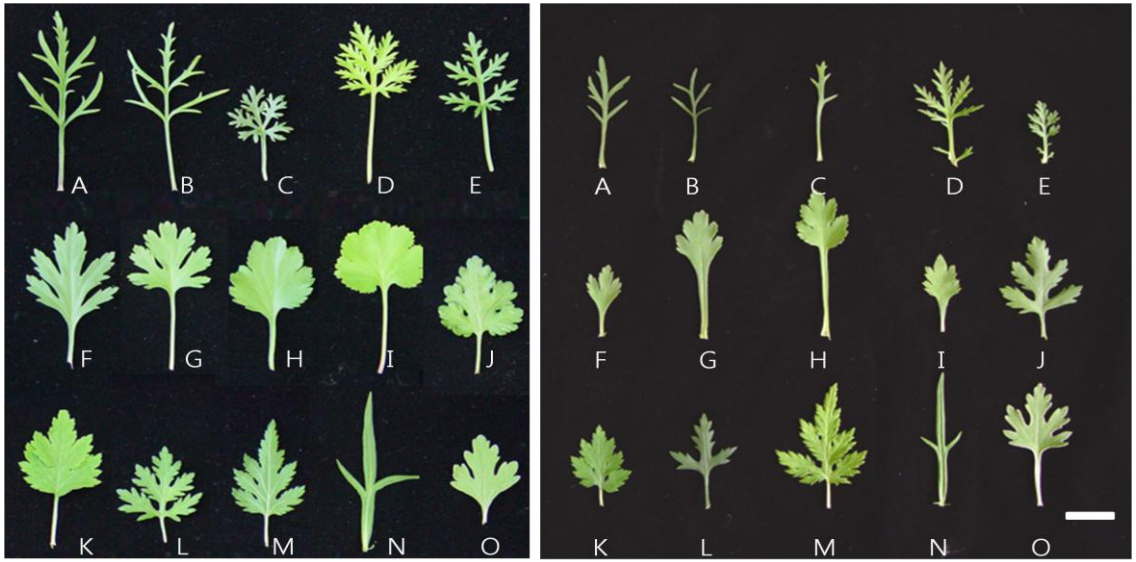

Fig. I-4. Images of radical leaf (left) and cauline leaf (right) of Korean *Chrysanthemum* (scale bar = 2 cm). A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

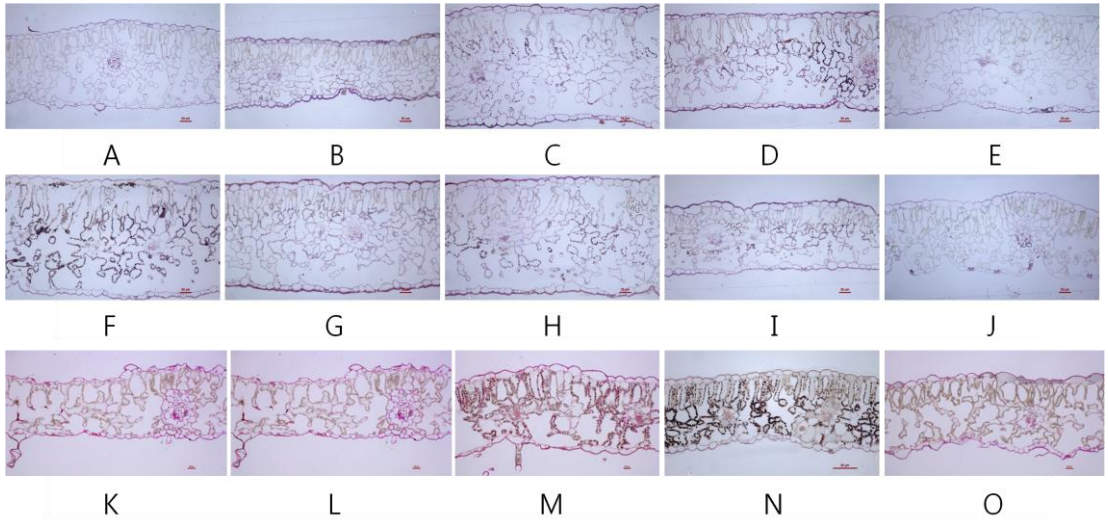

Fig. I-5. Leaf cross-sections of 15 Korean *Chrysanthemum* taxa observed under light microscope (scale bar = 20 μm). A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi.*

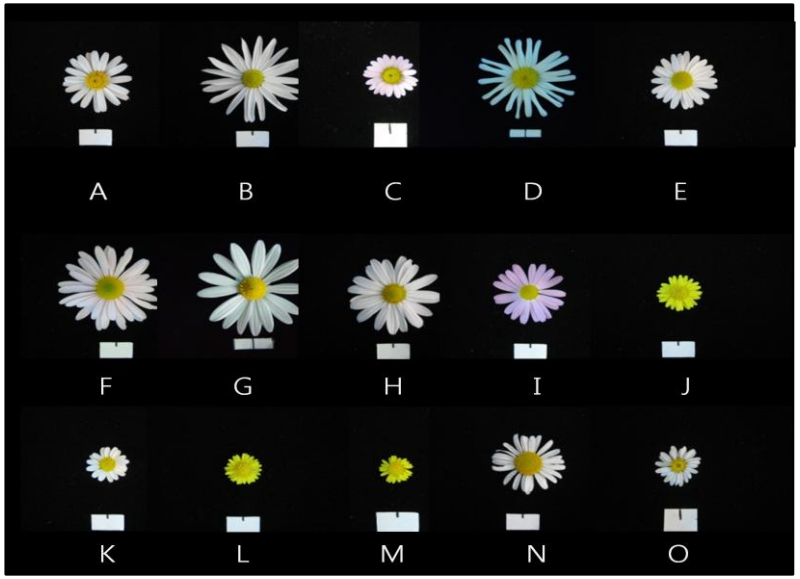

Fig. I-6. Images of flower in 15 Korean *Chrysanthemum* taxa (scale bar = 2 cm). A; *C. zawadskii* ssp. *acutilobum*, B; *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*, C; *C. zawadskii* ssp. *acutilobum* var. *alpinum*, D; *C. zawadskii* ssp. *lucidum*, E; *C. zawadskii* ssp. *coreanum*, F; *C. zawadskii* ssp. *naktongense*, G; *C. zawadskii* ssp. *yezoense*, H; *C. zawadskii* ssp. *latilobum*, I; *C. zawadskii* ssp. *latilobum* var. *leiophyllum*, J; *C. indicum*, K; *C. indicum* var. *albescens*, L; *C. indicum* var. *acuta*, M; *C. boreale*, N; *C. lineare*, O; *C. makinoi.*

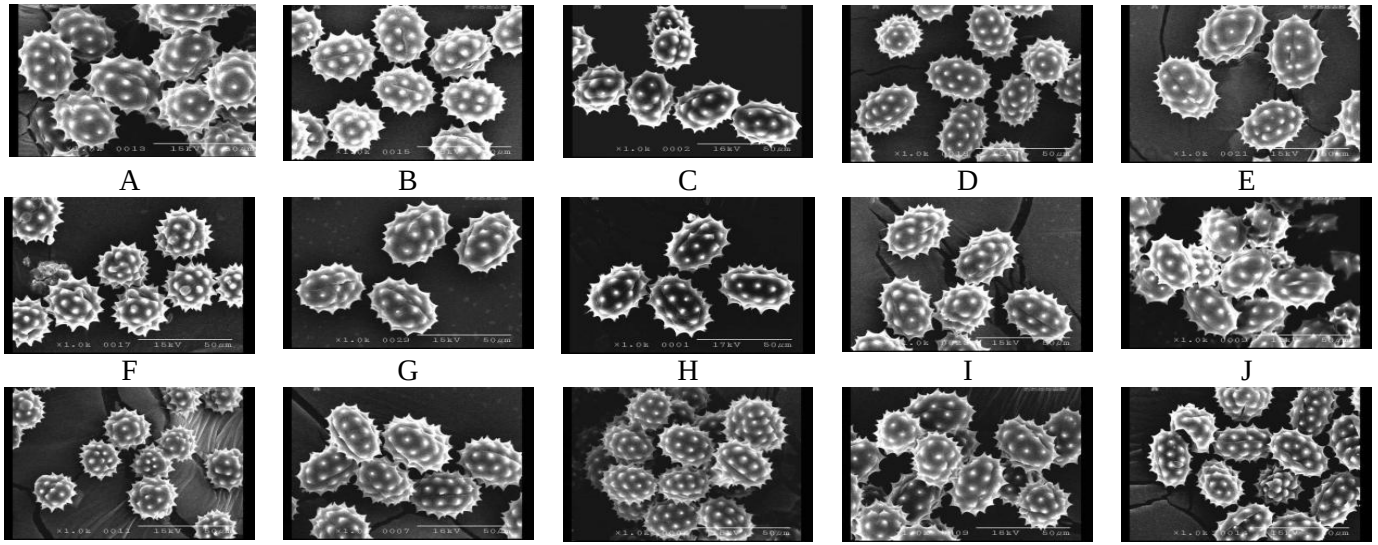

Fig. I-7. Scanning electron microscope (SEM) obserbation of pollen grains of Korean *Chrysanthemum* (x 1,000). A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

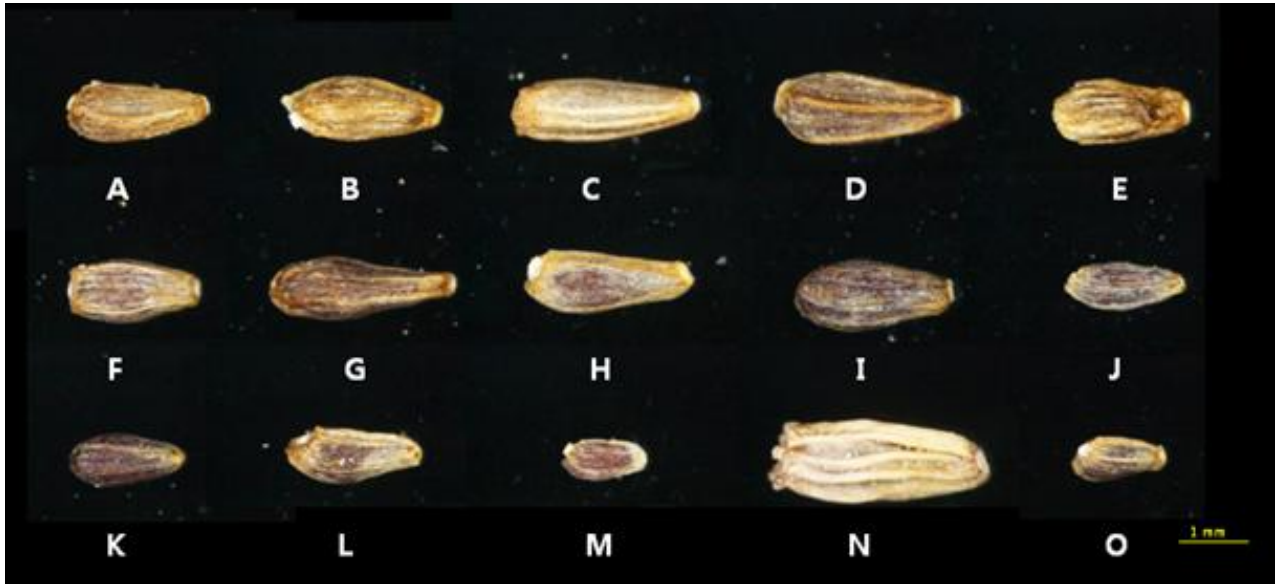

Fig. I-8. Seed shape of 15 Korean *Chrysanthemum* taxa (scale bar = 1 mm). A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

The *C. zawadskii* ssp. *lucidum* and ssp. *yezoense* has strong glossy winter suckers. The trichome densities of the winter suckers were classified into four types. Among 15 taxa, one taxa showed the absence or very scarce trichome on winter suckers, four taxa showed scarce trichome density; nine taxa showed medium trichome density, and only one taxa had high trichome density.

Chrysanthemum generally has basal and cauline leaves (Lee, 2006). Figure I-4 shows leaves of 15 *Chrysanthemum* taxa. The basal leaf lengths of 15 *Chrysanthemum* taxa ranged from 2.6 to 9.2 cm, leaf width from 2.2 to 5.2 cm and leaf L/W ratio ranged from 0.8 to 4.2 (Table I-3). The petiole lengths of basal leaves were from 0 (petiole-absent) to 4.6 cm. Thickness of the basal leaves ranged from 0.9 to 1.7 mm. Microscopic images of the cross section of the basal leaves showed differences in leaf anatomy among 15 *Chrysanthemum* taxa. *C. boreale* had thinner basal leaves with more compact palisade and spongy parenchyma than other taxa (Fig. I-5).

Leaf areas of *C. boreale* and *C. lineare* were 15.2 and 0.7 cm^2, respectively. Although basal leaves were mostly ovate shaped, these were classified into four types: ovate-, heart-, oblong-, and linear-shaped leaves (Table I-3 and Fig. I-4). Basal leaf margins were divided into three types: shallow-, medium- and deep-types (Table I-3).

The leaf length of cauline leaves ranged from 0.5 to 8.6 cm. Cauline leaf width ranged from 2.3 to 5.5 cm and leaf L/W ratio ranged from 0.2 to 3.7. The petiole length of cauline leaves ranged from 0 (petiole-absent) to 4.7 cm. The

thickness of cauline leaves ranged from 0.7 to 1.1 mm. However, the *C. boreale* had the largest cauline leaf area at 11.0 cm^2 and *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* had the smallest leaf area at 0.5 cm^2. Cauline leaf shapes were classified into four types: ovate-, oblong-, spatulate-, and lanceolate-shaped. Cauline leaf margins were divided into three types: shallow, medium and deep-like basal leaves.

Moreover, the *C. boreale* had the higher number of flowers (223 ea) whereas *C. zawadskii* var. *alpinum* had the least (4 ea) (Table I-3). *C. boreale* had the shortest flower head diameter at 15.1 mm and *C. zawadsii ssp. latilobum* had the widest diameter at 67.6 mm. *C. boreale* had the shortest disk floret width (5.4 mm) while *C. lineare* had the longest (14.9 mm). *C. zawadskii* ssp. *latilobum* had the longest ligulate length at 26.7 mm whereas *C. boreale* had the shortest ligulate length at 5.8 mm. Flower colors were white, pink or yellow (Fig. I-6). Flowers bloomed mainly from September to November but only *C. zawadskii* ssp. *acutilobum* var. *alpinum* bloomed in April.

The pollens of *Chrysanthemum* were observed under SEM. *C. zawadskii* ssp. *coreanum* showed the longest pollen polar length (PL) at 48.5 μm and *C. indicum* var. *albescens* had the shortest PL at 28.3 μm (Table I-3 and Fig. I-7). *C. zawadskii* ssp. *coreanum* showed the longest equatorial diameter (ED) at 35.6 μm, while *C. boreale* showed the shortest ED at 26.3 μm. Depending on the pollen shape, 15 taxa were categorized into three types: spheroidal-, subprolate-, and prolate-shaped.

Table I-5. Eigenvalues and contributions of the first 10 principal components using 35 morphological characteristics in 15 Korean *Chrysanthemum* taxa.

Principal component	Eigenvalue	Difference	Contribution (%)	Cumulative contribution (%)
PC 1	10.69	1.31	30.54	30.54
PC 2	9.38	5.63	26.79	57.33
PC 3	3.75	0.49	10.72	68.05
PC 4	3.26	0.89	9.32	77.37
PC 5	2.37	0.69	6.79	84.15
PC 6	1.69	0.36	4.81	88.97
PC 7	1.32	0.55	3.79	92.75
PC 8	0.77	0.15	2.21	94.97
PC 9	0.62	0.22	1.78	96.75
PC 10	0.41	0.13	1.17	97.91
PC 11	0.28	0.03	0.79	98.70
PC 12	0.24	0.13	0.69	99.40

Table I-6. List of three principal components among 35 morphological characteristics in 15 Korean *Chrysanthemum* taxa.

Plant part	Trait	Loading of principal component		
		PC 1	PC 2	PC 3
Plant	Plant height	-0.215896[y]	0.096219	0.217543
	Plant type	-0.267704	-0.106813	-0.049356
Winter sucker	Glossiness	0.251113	0.062145	0.071200
	Tricohome density	-0.001317	-0.043492	-0.219025
Basal leaf	Leaf length	-0.086444	0.257790	0.158311
	Leaf width	0.110560	-0.241245	0.109707
	L/W ratio	-0.080480	0.306432	0.063344
	Petiole length	0.255216	-0.150792	0.026045
	Leaf thickness	0.147031	-0.162461	0.133991
	Leaf area	-0.021218	-0.204270	0.341788
	Leaf shape	-0.085395	0.224670	0.121299
	Leaf margin	0.020124	0.093833	-0.387187
Cauline leaf	Leaf length	-0.198713	0.203443	0.044642
	Leaf width	-0.067687	-0.199350	0.184866
	L/W ratio	-0.136711	0.278683	0.004625
	Petiole length	0.235348	-0.158091	0.137216
	Leaf thickness	0.029511	-0.224254	-0.013148
	Leaf area	-0.219232	-0.061175	0.162787
	Leaf shape	0.074429	0.239976	0.255962
	Leaf margin	0.002071	0.098887	-0.393070
Flower	No. of flower/plant	-0.233085	-0.125537	0.082627
	Head diameter	0.290873	0.037169	0.105816
	Disk floret width	0.215693	0.162713	0.033772
	Ligulates length	0.291785	0.015444	0.089610
	Flower color	-0.138154	-0.063220	0.239575
	Flowering time	-0.096488	-0.038946	0.270201
	Flowering period	0.070464	-0.135453	-0.072263
Pollen	Polar length	0.163180	0.075612	0.056041
	Equatorial diameter	0.166108	0.053654	0.234593
	Shape	-0.036146	-0.015680	-0.091921
Seed	Seed length	0.204846	0.222460	0.044516
	Seed width	0.172317	0.250039	0.062817
	L/W ratio	0.203660	0.119763	-0.000989
	Seed shape	0.101469	0.244506	0.039495
	Seed color	-0.205067	0.192371	0.131745

[y]Underlined loading was the trait that had higher correlation with principal component of column.

The *C. boreale* had the smallest seed (1.3 mm length and 0.6 mm width) and *C. lineare* had the biggest seed (3.2 mm length and 1.2 mm width) (Table I-3 and Fig. I-8). The *L/W* ratio of seed was the highest in *C. zawadskii* ssp. *alpinum* and ssp. *lucidum* at 2.9 and the lowest in *C. indicum* and *C. indicum* var. *albescens* at 2.1. Most seed shapes were lanceolate-ovate or lanceolate-oblong, except *C. lineare* which had unique cone-shaped. Seed color was mostly brown or dark brown except *C. lineare* with pale yellow.

Taxonomic relationship using morphological characteristics through principal component and cluster analysis

A total of 35 qualitative and quantitative morphological characteristics of *Chrysanthemum* were used as identification keys for classification in multivariate analysis (Table I-4). Variance analysis of morphological characteristics showed that 23 variables were significantly different among the taxa, whereas 12 variables were not. Of the 23 significant variables, 12 variables were highly significant at $p < 0.001$ which included plant type, basal and cauline leaf length, basal and cauline *L/W* ratio, basal and cauline petiole length, flower head diameter, ligulate length, seed length, seed width, and seed color (Table I-4).

Through PCA of 35 morphological variables of 15 *Chrysanthemum* taxa, 12 principal components were determined with 99.4% cumulative contribution. Table I-5 shows the eigenvalue, the difference, and contribution of the first 12

principal components from PCA of 35 morphological variables of 15 *Chrysanthemum* taxa. The eigenvalues of each principal component were the total number of hypothetic variables analyzed by PCA, indicating that the first, second, and third principal components represented approximately 11, 9 and 4 variables among the 35 morphological characteristics of *Chrysanthemum*, respectively. These three major principal components covered 24 characteristics and explained 68% of the results.

The first principal component (PC 1) showed highly correlated eigenvalue with 11 morphological characteristics including plant height, plant type, glossiness of winter sucker, petiole length of basal and cauline leaf, cauline leaf area, flower number, flower head diameter, disk floret width, ligulate length, and seed color (Table I-6). Most of the variables in PC 1 were related to leaf and flower quantitative characteristics of the species. The second principal component (PC 2) was closely related with six leaf characteristics, including basal leaf length and *L/W* ratio, cauline leaf *L/W* ratio and leaf thickness, both basal and cauline leaf shapes, and three seed morphological characteristics such as seed length, seed width, and seed shape. All of the four characteristics in the third principal component (PC 3) were related to qualitative or quantitative characteristics such as basal leaf area, basal leaf margin, cauline leaf margin, and flowering time.

Based on PC 1 and PC 2 from PCA, each species was plotted on scatter plot (Fig. I-9) and 15 *Chrysanthemum* taxa were categorized into three groups. Nine

C. zawadskii subspecies having non-bush type, longer petiole length of basal and cauline leaves, larger head diameter, and longer ligulate length were located at the right part of plots 1 and 2. Most of the species in the group were characterized by high positive values in PC 1 (Fig. I-9; Group I). In the result of PCA, *C. lineare* was not closely related with other two groups due to the remarkably longer basal and cauline leaf length with no petiole, greater seed size, unique cone seed shape, and unique pale yellow seed color. *C. lineare* was located in the upper part of plot 1 and middle part of plot 2 due to high positive values in both PC 2 and PC 3 (Fig. I-9; Group II). *C. indicum*, *C. indicum* var. *albescens*, *C. indicum* var. *acuta*, *C. boreale*, and *C. makinoi* having bush type, shorter petiole length of basal and cauline leaves, smaller flower head diameter, and shorter ligulate length were located at the left part of plots 1 and 2. Difference in the morphology of Group III with smaller flower, greater number of flowers and later flowering time could be related to flower traits of wild species in Korea. The species in the group were characterized by more negative values in PC 1 and PC 2 (Fig. I-9; Group III).

In this study, the morphological variation among *Chrysanthemum* was clearly revealed through PCA. ANOVA showed that the number of flower, head diameter, disk floret width, and ligulate length of flower morphological traits, considered as part of PC 1, were significantly different among the taxa. However, flowering time of the traits for PC 3 was not significantly different among the taxa (Table I-4).

Cluster analysis using Ward's minimum variance method of the standardized first three principal components of the *Chrysanthemum* was categorized into three groups based on *pseudo F*, *pseudo* t^2 (PST^2) and RSQ (R^2) values (Fig. I-10). The dendrogram showed three distinct groups: *C. zawadskii* subspecies (Group I), *C. lineare* (Group II) and *C. indicum* subspecies and related species (Group III). Group I included nine *C. zawadskii* subspecies that had non-bushy plant type and various leaf shapes. These were slightly different between the basal leaf (ovate, heart, oblong, and spatulate) and cauline leaf (ovate, oblong, spatulate, and lanceolate). The flower head diameters of these species were relatively longer than the other groups. Group II had only one species, *C. lineare*, which had unique pale yellow and cone-shaped seed. The seed length of *C. lineare* was 3.2 mm and longer than the other groups. *C. lineare* had exceptionally longer leaves with no petiole in both basal and cauline leaves. Group III included three *C. indicum* species, *C. boreale* and *C. makinoi*. Taxa in Group III were mostly bushy plants and had the same ovate-shaped basal leaf and cauline leaf. Flower head diameter ranged from 15.1 to 27.3 mm, which is relatively smaller than the other groups (Groups I and II).

This was concordant with the PCA result confirming that the 15 taxa could be categorized into three groups. Dendrogram from the cluster analysis showed that the data can be grouped into three clusters and the proportion of the variance was accounted for the clusters.

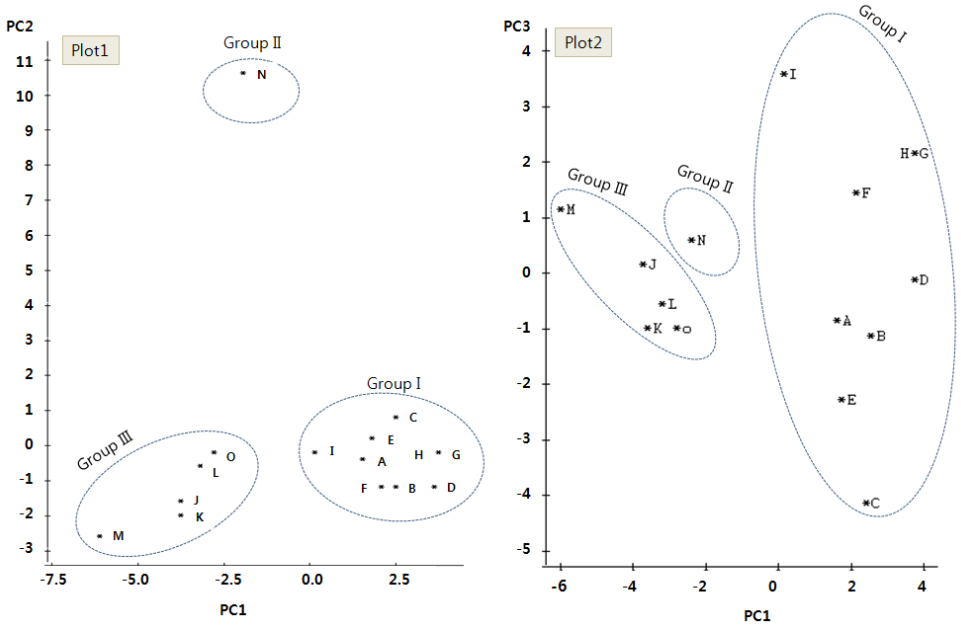

Fig. I-9. Scatter plot of morphological characteristics in Korean *Chrysanthemum* using the principal components. A, *C. zawadskii* ssp. *acutilobum*; B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*; C, *C. zawadskii* ssp. *acutilobum* var. *alpinum*; D, *C. zawadskii* ssp. *lucidum*; E, *C. zawadskii* ssp. *coreanum*; F, *C. zawadskii* ssp. *naktongense*; G, *C. zawadskii* ssp. *yezoense*; H, *C. zawadskii* ssp. *latilobum*; I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*; J, *C. indicum*; K, *C. indicum* var. *albescens*; L, *C. indicum* var. *acuta*; M, *C. boreale*; N, *C. lineare*; O, *C. makinoi*.

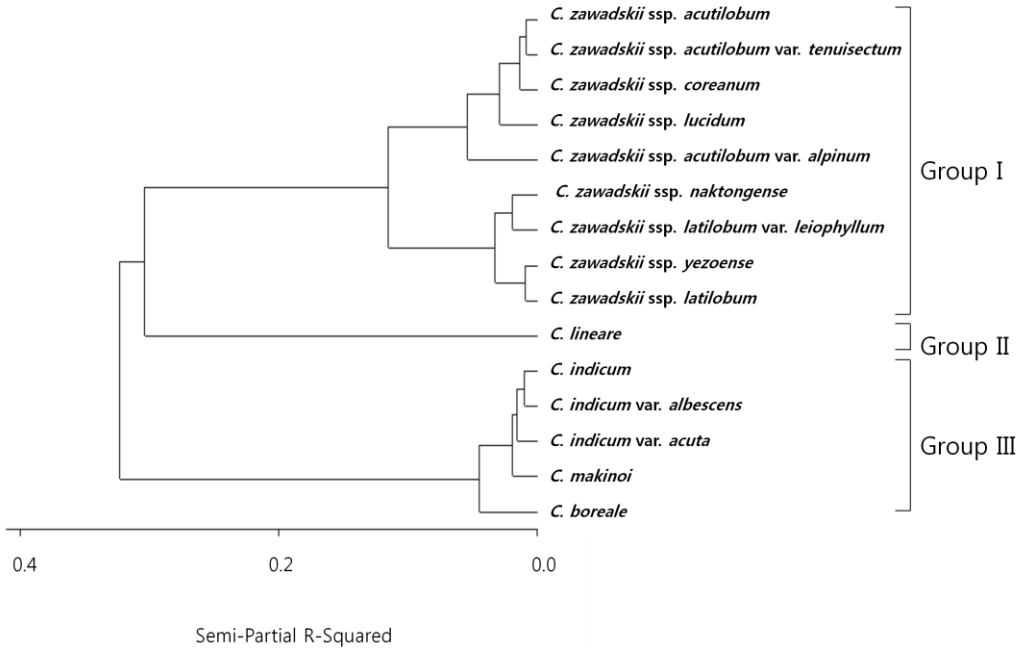

Fig. I-10. Dendrogram of grouping based on morphological characteristics of 15 Korean *Chrysanthemum* taxa by cluster analysis using Ward's minimum variance method.

DISCUSSION

Most of the morphological characteristics showed variation among 15 Korean *Chrysanthemum* taxa. Some morphological characteristics of *C. zawadskii* also seemed to be distinct. Although 12 morphological characteristics were not significantly different among the taxa, 23 morphological characteristics, such as plant type and height, winter sucker (glossiness and trichome density), basal and cauline leaf (leaf length, basal leaf width, basal leaf length/width *L/W* ratio, petiole length, leaf thickness, leaf area, leaf shape, and leaf margin), flower (flower number, head diameter, disk floret, flower color, flowering time, and flowering period), pollen (polar length, equatorial diameter, and pollen shape), and seed (seed length, seed width, seed *L/W* ratio, seed shape, and seed color) were found to be useful key factors for the classification of Korean *Chrysanthemum*. In particular, the basal leaf *L/W* ratio was the most remarkable feature of the study (*F* value of 281.50), which exhibited significant differences among the 23 morphological characteristics (Table I-4).

According to the PCA and cluster analysis using horticultural qualitative and quantitative morphological characteristics, 15 *Chrysanthemum* taxa could be further classified into three groups. In the same environmental condition during the three years of the study, 15 *Chrysanthemum* taxa displayed unique plant shapes in terms of plant height, leaves, particularly basal and cauline leaf

shapes, glossiness of the winter suckers, and flowering habits.

Cluster analysis reconstructed smaller variance as group from large number of correlated variances within the species, and the calculated distances among the groups defined the differences in characteristics among the species (Chung and Ko, 1995; Jang et al., 2006; Yun et al., 2002). This method was used for *Allium cepa* (Kwon et al., 1995), *Carya illinoensis* (Shin et al., 1997), *Chionanthus retusa* (Park et al., 2008), *Diospyros kaki* (Kim and Ko, 1995), *Pragaria ananassa* (Kim et al., 2009), *Prunus cerasifera* (Chung, 1999), and *Zea mays* (Hur et al., 2013) with different morphological traits for each species. These results showed clear classification for identifying the species. Previous studies also indicated that cluster analysis could be used as management tool for large size Asian pear germplasm (Kim, 2004), good germplasm database for breeding of cherries (Shahi-Gharahlar et al., 2010) and environmental and genetic factors for determining the bioactive compound levels of *C. indicum* (Fang et al., 2012).

Findings of this study provided clear diagnostic key for classification showing the species-specific plant form including the unique leaves, branch type, plant height, flower size, and flowering habits. Group I consisted of nine *C. zawadskii* subspecies as shown in their morphological characteristics through PCA and cluster analysis. Most results confirmed the Korean *Chrysanthemum* classification of Lee (2006) except the *C. zawadskii* ssp. *acutilobum* var. *alpinum*. Lee (2006) assumed that *C. zawadskii* ssp. *acutilobum* var. *alpinum*

resembled as *C. zawadskii* ssp. *acutilobum*, and ssp. *acutilobum* var. *alpinum* but had a small plant height, high density trichome in leaf, shorter petiole length in cauline leaf, and larger flower size. This study showed cluster analysis of morphological characteristics confirming two species, *C. zawadskii* ssp. *acutilobum* and ssp. *acutilobum* var. *alpinum* which were not closely related due to differences in the flowering time (Table I-3 and Fig. I-10). Kim and Tobe (2009) reported that *C. zawadskii* sub-species displayed variations in leaf shape and leaf thickness and difference in basal and cauline leaf shapes.

Group I including *C. zawadskii* subspecies had great potential as ground cover material with dense branches and various leaf shapes from shallow, medium and deep margins (Table I-3 and Fig. I-4). *C. zawadskii* ssp. *acutilobum* var. *alpinum* under Group I also showed potential as breeding resource for flowering period since the flowering period of the taxa is spring while the other taxa bloom in autumn. These taxa also had shorter plant height of 18.5 cm than other species with an average height of 53.3 cm (Table I-3).

The *C. lineare* was the only species in Group II and had very unique leaf and seed shapes among taxa. Generally, the plant habitats requiring protection specially due to their extremely small or decreasing population size, the category of rarity is set by the geographical distribution areas of the species, degree of habitat specificity and local population size (KNA, 2012). Rare plants are referred as endangered plants, protected plants, declining species, special plants, legally protected plants, and red list plant species. Regarding the

evaluation categories for the list of rare plants, International Union for Conservation of Nature (IUCN) classifies them into extinct-in-the-wild, critical species, endangered species, vulnerable species, and deficient species. The evaluation criteria for endangered plant species recommended by IUCN are most stable as evaluation criteria for the rare/endangered plant species and adopted as standard programs by most countries.

Group III consisted of five taxa, including *C. indicum* subspecies, *C. makinoi*, and *C. boreale*. Cluster analysis showed that *C. makinoi* was closely related to *C. indicum* in terms of morphological characteristics. Group III seemed to be desirable species as edible medicinal resources for flower tea because of its small flower head diameter (15.1-27.3 mm) and more number of flowers (80-223 ea) with white or yellow petal. However, further studies on the analysis of fragrance of the species could be worth pursuing prior to its use as edible resources.

The study highlighted the combined application of leaf and flower morphological traits of 15 *Chrysanthemum* taxa for genetic diversity. *C. lineare* displayed more different morphological characteristics than the other 14 taxa. In addition, *C. zawadskii* subspecies had more accepted sub-species and varieties in the regions. Further, *C. zawadskii* ssp. *acutilobum* var. *alpinum* was differentiated from *C. zawadskii* subspecies. The study proved that the evolution of *C. zawadskii* sub-species with various morphological leaf shapes occurred from shallow to deep margins of leaf shape.

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CHAPTER II

Phylogenetic Analysis of Korean *Chrysanthemum* based on Cytological and Molecular Characteristics

ABSTRACT

Korean *Chrysanthemum* naturally distributed throughout Korea is regarded as one of the most popular ornamentals and medicinal herbs. Phylogenetic studies were conducted to evaluate interspecific relationships in 21 populations of Korean *Chrysanthemum* including 16 taxa and two outgroups using internal transcribed spacer (ITS 1, ITS 2) region sequences of nuclear ribosomal DNA (nrDNA) and chromosome number. Phylogenetics analysis was conducted using strict consensus methods. The taxa had well-developed series of polyploids (4x, 6x, 8x) from diploids (2n = 2x = 18) with basic chromosome number (n = 9). The length of ITS sequences aligned was 663-664 bp, and the length of ITS 1 and ITS 2 regions were 268-269 bp and 231 bp, respectively. The divergence rate among *Chrysanthemum zawadskii* subspecies was very low within 1.5%. In the phylogenetic tree obtained by strict consensus analysis, the taxa were divided into three major groups. *C. lineare* was basally branched within *Chrysanthemum*. Our results suggest that Korean *Chrysanthemum* is morphologically independent group such as *C. zawadskii*, *C. boreale* and *C.*

indicum species were unresolved with ITS region sequences. These findings contributed to our understanding of the phylogeny, origin, and classification of *Chrysanthemum*. *Chrysanthemum* could have been derived to develop polyploidy for the phylogenetic analysis based on ITS sequence and chromosome data.

Keywords: Asteraceae, Chromosome, nrDNA, Phylogeny, Polyploidy

INTRODUCTION

Korean *Chrysanthemum* was established by Russian botanist (Palibin, 1898). The genus consists of 41 species and is distributed in temperate zones in eastern Asia such as Korea, Japan, China, and Siberia (Iwatsuki et. al., 1997; Oberprieler et. al., 2007). Eighteen *Chrysanthemum* taxa were distributed in Korea including the commercial and hybrid chrysanthemum (Lee, 2006). Cultivars in the genus have been bred to improve the shape, color, size, and number of flowers for more than 20 years, cultivated in 636 ha with 613 new registered cultivars in Korea (MIFAFF, 2012; KSVS, 2012).

Korean *Chrysanthemum* was reported to be diploid, tetraploid, hexaploid, octoploid, and 10 diploids (Dowrick, 1952; Hwang et al., 2013; Inceer and Hayirlioglu-Ayaz, 2010; Kim, 2003; Lee, 2006; Nakata et al., 1987). Polyploidy played an important role in chromosome evolution with the basic chromosome number (Abd El-Twab and Kondo, 2003). Often, there is a need to differentiate between the genomes of closely related species and to identify the ancestors of species (Abd El-Twab and Kondo, 2003).

Molecular tools like nuclear ribosomal DNA of the internal transcribed spacer (ITS), chloroplast DNA (*trnL-trnF*, *atpB-rbcL*, *rpoC1*, *trnH-psbA*), nuclear DNA of inter generic spacer (IGS), external transcribed spacer (ETS), and simple sequence repeat (SSR) technology have been used in plant

classification (Ahn et al., 2010; Felsenstain, 1985; Masuda et al., 2009). Molecular studies based on nuclear markers (nrDNA ITS) have been frequently used to resolve genetic delimitation and circumscription in several plant taxa (Oberprieler et al., 2007; Zhao et al. 2010). These tools have been reported to resolve phylogenetic relationships among Asteraceae, such as *Artemisia* (Lee et al., 2010), *Aster* (Hong et al., 2012), and *Ligularia* (Ahn et al., 2010). The sequence analysis of internal transcribed spacer (ITS) region of nuclear ribosomal DNA (nrDNA) between the small sub-unit (18S) and the large subunit (26S) of nrDNA has been used for analysis of phylogenetic relationships within and among closely related genera and identification of species (Baldwin et al., 1995; Suh et al., 1993; Yapwattanaphun et al., 2004). Ribosomal DNAs (rDNAs) with repeated sequences are organized as families in tandem arrays at the nucleolus organizer regions of chromosomes in all eukaryotes. Copy numbers of rDNA in different species vary from a few hundred to several thousands (Tsai et al., 2004).

Studies on polyploidy of *Chrysanthemum* had contributed to phylogenetic relationships and constitution of breeding cultivars (Lee and Kim, 2000; Zhao et al., 2009). This study was performed to evaluate the performance of chromosome and nrDNA in *Chrysanthemum*, analyze its genetic diversity and establish molecular database for breeding programs for the management and conservation of Korean *Chrysanthemum* germplasm in national level.

MATERIALS AND METHODS

Plant materials and nomenclature

Sixteen taxa (19 populations) of five *Chrysanthemum*, five subspecies, and five varieties were used as plant materials for the analysis of the molecular phylogeny. In addition, two taxa of *Aster glehnii* and *Aster indicus* as out-group were obtained from the GenBank. Total of 18 taxa (21 populations) with voucher information and accession numbers were shown in Table II-1. The classification and nomenclature of *Chrysanthemum* were based on Lee (2006) and the International Code of Botanical Nomenclature (ICBN) (Nicolson, 1999; Trehane, 1995). The taxa were deposited in the herbarium at Pyeongchang in the Highland Agricultural Research Institute, National Institute of Crop Science, Rural Development Administration in Korea (lat. 37°40'N, long. 128°43'E, altitude 772 m).

Table II-1. List of taxa used for the ITS analysis with voucher and GenBank numbers of Korean *Chrysanthemum*.

Scientific name	KMRH[z]	NCBI[y]	Sites (Natural habitats)
C. zawadskii Herbich	GenBank[x]	EF577309	Nanjing, China
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa	MPS003031	KJ183117	Mt. Yumyeong, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *tenuisectum* (Kitagawa) Y. Lee	MPS003032	KJ183118	Pocheon, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *alpinum* (Nak.) Y. Lee	MPS003033	KJ183132	Mt. Baekdu, Hambuk, Korea
C. zawadskii Herbich ssp. *lucidum* (NAK.) Y .Lee	MPS003034	KJ183119	Ulleung, Gyeongbuk, Korea
C. zawadskii Herbich ssp. *coreanum* (NAK.) Y. Lee	MPS003035	KJ183120	Mt. Halla, Jeju, Korea
C. zawadskii Herbich ssp. *naktongense* (NAK.) Y. Lee	MPS003037	KJ183121	Gimhae, Gyeongnam, Korea
C. zawadskii Herbich ssp. *naktongense* (NAK.) Y. Lee	GenBank[w]	AF314595	Beijing, China
C. zawadskii Herbich ssp. *yezoense* (Maekawa.) Y. Lee	GenBank[x]	EF577307	Nanjing, China
C. zawadskii Herbich ssp. *yezoense* (Maekawa.) Y. Lee	MPS003039	KJ183122	Goheung, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa	MPS003041	KJ183123	Wando, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa var. *leiophyllum* (Nak.) Y. Lee	MPS003043	KJ183124	Gangneung, Gangwon, Korea
C. indicum Linné	MPS003044	KJ183125	Anmyeondo, Chungnam, Korea
C. indicum Linné	GenBank	JN315940	Hunan, China
C. indicum Linné var. *albescens* Makino	MPS003046	KJ183126	Jeongseon, Gangwon, Korea
C. indicum var. *acuta* (Uyeki) Kitam.	MPS003047	KJ183127	Byeonsanbando, Jeonbuk, Korea
C. boreale (Mak.) Makino	MPS003049	KJ183128	Pyeongchang, Gangwon, Korea
C. lineare Matsumura	MPS003050	KJ183129	Mt. Chilbo, Gyeonggi, Korea
C. makinoi Matsumura et Nakai	MPS003051	KJ183130	Daegu, Korea
Aster glehnii F. Schmidt (Out group)	GenBank[v]	AY722010	Pyeongchang, Gangwon, Korea
Aster indicus L. (Out group)	GenBank[u]	EF108396	Taichung, Taiwan

[z]Herbarium voucher number indicate Korea Medicinal Resource Herbarium, Rural Development Administration (KMRH) numbers in Korea.

[y]Accession numbers indicate National Center for Biotechnology Information (NCBI) accession numbers in USA.

[x]Zhao et al., 2010 in NCBI, [w]Zhao et al., 2010 in NCBI, [v]Hong et al., 2010 in NCBI, [u]Hsieh et al., 2006 in NCBI.

Chromosome analysis

Root tips (2 mm from the end) of *Chrysanthemum* were taken and washed with distilled water. The root tips were pre-treated with 2 mM 8-hydroxyquinoline solution at 16℃ for 4 h in dark room. It was fixed in aceto-ethanol solution (acetic acid glacial: 95% ethanol = 1:3, v/v) for more than 12 h and stored in 70% ethanol at -20℃. The stored root tips were sufficiently washed with distilled water for 20 min and later subjected to mixture of enzymes in 0.3% pectolyase, 0.3% cellulase, 0.3% cytohelicase, and 150 mM of citrate buffer before being treated for 1-2 h at 37℃. The solution of 60% acetic acid glacial was loaded using pincette and placed on slide applying squash method. The root tip sample was dried in an oven at 50℃ for 1 h before being stored at -20℃. The dried slides were stained with 4',6'-diamidino-2-phenylindole (DAPI) in Vectashield (Vectashield mounting medium, Vecta laboratories Inc., California, USA) and examined under fluorescent microscope (Nikon BX 61, Nikon, Newyork, USA) with 200, 400, and 1000 magnification. Images were captured and then processed with the Genus image analysis workstation software (Genus version 3.8 program, Applied Imaging Corporation, UK). The individual chromosome was measured by software program and determined chromosome number.

DNA extraction, PCR amplification, and DNA sequencing

Genomic DNA was extracted from 200 mg of lyophilized leaves using DNeasy Plant Mini Kit (Qiagen Mini Kit, Qiagen Sciences, Germantown, MD, USA). Quality and quantity of samples were analyzed based on electrophoresis on 1.8% agarose gel containing ethidium bromide (EtBr), and performed ND-1000 spectrophotometer (NanoDrop, Fisher Thermo, Wilmington, DE, USA), respectively. The ITS region including 5.8S gene of the nuclear ribosomal DNA was amplified using the primer pair 5′-TCCTCCGCTTATTGATATGC-3′ and 5′-TCCGTAGGTGAACCTGCGG-3′ (Ahn et al., 2010) in 20 μL reactions containing 4 μg template, 1 μL of each primer (10 pmol), and 14 μL of sterilized water in PCR premix tube. The temperature cycling regime of ITS consisted of initial incubation at 94℃ for 5 min, followed by 35 cycles at 94℃ for 1 min, 50℃ for 1 min and 72℃ for 10 min, and terminated with incubation at 72℃ for 2-10 min. Following the PCR process, the reaction products were purified using the purification kit (MinElute PCR Purification Kit, Qiagen, Duesseldorf, Germany). The purified products were sequenced directly by DNA sequences (ABI 3730, Applied Biosystems, Foster City, MO, USA) using PCR amplification primers. To confirm the sequences, each sample was sequenced three times. The reactions were performed based on the recommendations of the manufacturers. The derived ITS sequences were confirmed by appropriate program Blast (NCBI) to search the DNA sequence databases for high similarity with other *Chrysanthemum* sequences (ncbi.nlm.nih.gov).

Sequence Alignment and Phylogenetic Analysis

Nucleotide sequence was aligned using CLC Main Workbench (CLC Main Workbench ver. 4.0, CLC bio A/S, Aarhus, Denmark). The alignment was manually checked using the program MEGA 6 and the pair wise sequence divergence between taxa in total ITS, ITS 1, and ITS 2 was determined by Kimura-2 method (Kimura, 1980). The G+C contents were counted using Generunner 3.0. The resultant divergence matrix was then computed to generate phylogenetic trees using the strict consensus method (Saitou and Nei, 1987; Swofford, 1998).

RESULTS

Chromosome numbers

Total of 18 *Chrysanthemum* taxa (21 populations) and *Aster* were analyzed using only ITS anlysis. Fifteen among 18 taxa were checked for chromosome number because 15 taxa were directly collected in natural habitats. However, information in the GenBank (NCBI) was used for 6 taxa. Number of chromosomes in Korean *Chrysanthemum* were observed 2n = 2x = 18 in diploid, 2n = 4x = 36 in tetraploid, 2n = 6x = 54 in hexaploid, and 2n = 8x = 72 in octaploid accessions, respectively (Table II-2). Diploid chromosomes 2n = 2x = 18 were found in 3 taxa of *C. boreale, C. makinoi*, and *C. lineare.* Four taxa of *C. indicum, C. indicum* var. *acuta, C. zawadskii* ssp. *latilobum* var. *leiophyllum,* and *C. zawadskii* ssp. *lucidum* revealed tetraploid chromosomes. Seven taxa of *C. zawadskii* ssp. *acutilobum,* ssp. *acutilobum* var. *tenuisectum,* ssp. *acutilobum* var. *alpinum,* ssp. *naktongense,* ssp. *yezoense,* ssp. *latilobum,* and *C. indicum* var. *albescens* were observed having hexaploid chromosomes (2n = 6x = 54). Single taxa of *C. zawadskii* ssp. *coreanum* was confirmed as octaploid (2n = 8x = 72).

The number of somatic chromosomes in *Chrysanthemum* was investigated to determine the following number of chromosomes: polypolid of 2n = 4x = 36, 6x = 54, and 8x = 72 instead of diploid 2n = 2x = 18, ranging from diploid to octoploid and was then categorized (Fig. II-1).

Table II-2. List of chromosome number and ploidy level in Korean *Chrysanthemum*.

Scientific name	NCBI[z]	Chromosome no.	Ploidy level
C. zawadskii	EF577309	-[y]	-
C. zawadskii ssp. *acutilobum*	KJ183117	54	6x
C. zawadskii ssp. *acutilobum* var. *tenuisectum*	KJ183118	54	6x
C. zawadskii ssp. *acutilobum* var. *alpinum*	KJ183132	54	6x
C. zawadskii ssp. *lucidum*	KJ183119	36	4x
C. zawadskii ssp. *coreanum*	KJ183120	72	8x
C. zawadskii ssp. *naktongense*	KJ183121	54	6x
C. zawadskii ssp. *naktongense*	AF314595	-	-
C. zawadskii ssp. *yezoense*	EF577307	-	-
C. zawadskii ssp. *yezoense*	KJ183122	54	6x
C. zawadskii ssp. *latilobum*	KJ183123	54	6x
C. zawadskii ssp. *latilobum* var. *leiophyllum*	KJ183124	36	4x
C. indicum	KJ183125	36	4x
C. indicum	JN315940	-	-
C. indicum var. *albescens*	KJ183126	54	6x
C. indicum var. *acuta*	KJ183127	36	4x
C. boreale	KJ183128	18	2x
C. lineare	KJ183129	18	2x
C. makinoi	KJ183130	18	2x
Aster glehnii (Out group)	AY722010	-	-
Aster indicus (Out group)	EF108396	-	-

[z]Accession numbers indicate National Center for Biotechnology Information (NCBI) numbers in USA.
[y]Chromosome number not analyzed.

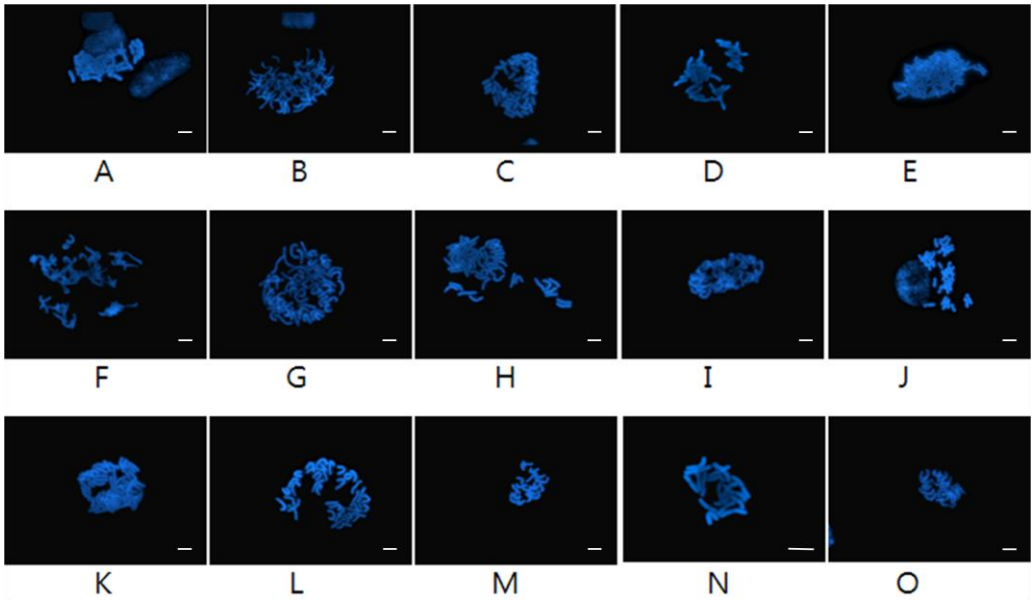

Fig. II-1. Microphotomicrographs of the somatic metaphase chromosome of Korean *Chrysanthemum* with DAPI (scale bar = 5μm). A, *C. zawadskii* ssp. *acutilobum* (2n = 6x = 54); B, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (2n = 6x = 54); C, *C. zawadskii* ssp. *acutilobum* var. *alpinum* (2n = 6x = 54); D, *C. zawadskii* ssp. *lucidum* (2n = 4x = 36); E, *C. zawadskii* ssp. *coreanum* (2n = 8x = 72); F, *C. zawadskii* ssp. *naktongense* (2n=6x=54); G, *C. zawadskii* ssp. *yezoense* (2n = 6x = 54); H, *C. zawadskii* ssp. *latilobum* (2n = 6x = 54); I, *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (2n = 4x = 36); J, *C. indicum* 2n = 4x = 36); K, *C. indicum* var. *albescens* (2n = 6x = 54); L, *C. indicum* var. *acuta* (2n = 4x = 36); M, *C. boreale* (2n = 2x = 18); N, *C. lineare* (2n = 2x = 18); O, *C. makinoi* (2n = 2x = 18).

Phylogeny based on the ITS of nrDNA

From the total DNAs used as templates, the entire ITS region (ITS 1 - 5.8S - ITS 2) was successfully amplified and displayed a single DNA band of 700 bp. To confirm the identity of the derived DNA stretches, BLAST search was performed and mostly significant alignments with available ITS sequences from *Chrysanthemum*, was exhibited.

Derived sequences were analyzed for the variations in length and nucleotide contents (Table II-3). ITS region of *Chrysanthemum* varied in length from 664 bp except for *C. lineare*. ITS regions from *Chrysanthemum* varied from 268-269 bp for the ITS 1 region, 231 bp for the ITS 2 region, and 165 bp for the 5.8S rRNA gene. As shown in these results, the 5.8S rDNA region was highly conserved. The GC contents of the amplified sequences varied either for the entire ITS or the ITS 1 and ITS 2 spacers. In fact, the GC contents of the amplified sequences varied from 51.0-52.5% in the ITS region (ITS 1 + 5.8S + ITS 2), from 47.0-50.7% for ITS 1 and from 52.8-54.1% for ITS 2. The GC contents were nearly similar to those reported for other Asteraceae (Ahn et al., 2010; Hong et al., 2012; Lee et al., 2010). Most of the polymorphic sites were obtained from ITS 1 and ITS 2. These results corresponded with the results of Lau et al. (2001) on the ITS region among 17 *Chrysanthemum* taxa. Interspecific variations in ITS 1 and ITS2 regions among *C. zawadskii* subspecies were very low.

Table II-3. Length and G+C contents of ITS sequences of Korean *Chrysanthemum*.

Scientific name	NCBI	Length (bp)				G+C(%)			
	accession no.[z]	ITS	ITS1	ITS2	5.8S	ITS	ITS1	ITS2	5.8S
C. zawadskii	EF577309	664	269	231	165	52.1	49.8	53.2	54.3
C. zawadskii ssp. *acutilobum*	KJ183117	664	269	231	165	52.1	49.8	53.2	54.3
C. zawadskii ssp. *acutilobum* var. *tenuisectum*	KJ183118	664	269	231	165	52.1	50.0	53.2	54.3
C. zawadskii ssp. *acutilobum* var. *alpinum*	KJ183132	664	269	231	165	52.1	50.2	52.8	54.3
C. zawadskii ssp. *lucidum*	KJ183119	664	269	231	165	52.0	49.4	53.2	54.3
C. zawadskii ssp. *coreanum*	KJ183120	664	269	231	165	52.1	49.8	53.2	54.3
C. zawadskii ssp. *naktongense*	KJ183121	664	269	231	165	52.3	50.2	53.2	54.3
C. zawadskii ssp. *naktongense*	AF314595	664	269	231	165	52.1	49.8	53.2	54.3
C. zawadskii ssp. *yezoense*	EF577307	664	269	231	165	52.5	50.7	53.2	54.3
C. zawadskii ssp. *yezoense*	KJ183122	664	269	231	165	52.1	49.8	53.2	54.3
C. zawadskii ssp. *latilobum*	KJ183123	664	269	231	165	52.3	50.2	53.2	54.3
C. zawadskii ssp. *latilobum* var. *leiophyllum*	KJ183124	664	269	231	165	52.1	49.8	53.2	54.3
C. indicum	KJ183125	664	269	231	165	52.4	50.6	53.2	54.3
C. indicum	JN315940	664	269	231	165	52.3	50.2	53.2	54.3
C. indicum var. *albescens*	KJ183126	664	269	231	165	52.3	50.2	53.2	54.3
C. indicum var. *acuta*	KJ183127	664	269	231	165	52.4	50.2	54.1	54.3
C. boreale	KJ183128	664	269	231	165	52.4	50.2	53.7	54.3
C. lineare	KJ183129	663	268	231	165	51.0	47.0	53.2	54.3
C. makinoi	KJ183130	664	269	231	165	51.7	49.1	52.8	54.3
Aster glehnii (Out group)	AY722010	655	264	224	167	51.3	53.3	53.1	53.3
Aster indicus (Out group)	EF108396	654	264	223	167	51.5	49.2	52.9	52.7

[z]Accession numbers indicate National Center for Biotechnology Information (NCBI) numbers in USA.

Genetic divergence matrix based on entire ITS sequences of 18 taxa (21 populations) was estimated according to the formula of Kimura-2 (Table II-4). Genetic divergence on ITS sequences varied from 0.0 to 26.6%. The ITS sequences in *C. lineare* and *C. indicum* var. *acuta* were found to be the most divergent with 4.4%, while *C. zawadskii* subspecies presented great similarities within same subspecies and varieties. Ten taxa (12 populations) within *C. zawadskii* subspecies were characterized of having great similarities in the ITS sequence within 1.5% divergence rate even though *C. zawadskii* ssp. *lucidum* and var. *alpinum* were most divergent within *C. zawadskii* subspecies.

All the taxa showed intermediate level of similarities. The derived strict consensus phylogenetic tree was illustrated in Fig. II-2. The 16 taxa (19 populations) in *Chrysanthemum*, which was clearly grouped into three main clusters: *C. zawadskii,* ssp. *coreanum,* ssp. *naktongense,* var. *tenuisectum,* ssp. *acutilobum,* ssp. *yezoense,* var. *leiophyllum,* ssp. *lucidum*, ssp. *latilobum, C. makinoi,* and *C. boreale* were grouped with *C. indicum,* var. *albescens,* and var. *acuta* was in cluster I with 92% supported by an interior branch test; and *C. zawadskii* ssp. *acutilobum* var. *alpinum* was grouped in cluster II with 91% supported by an interior branch test. Although alignments of *C. zawadskii* subspecies had great similarities among the subspecies sequences, ITS 1 sequence of var. *alpinum* had the highest divergence within same subspecies. In addition, *C. lineare* was grouped in cluster III by an interior branch test and was supported with bootstrap values of 91%.

Table II-4. Genetic divergence matrix among *Chrysanthemum* based on ITS sequence data using the formula of Kimura-2 parameter method.

No.	Scientific name (NCBI accession no.[z])	Divergence (%)																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*C. zawadskii* (EF577309)	-																				
2	*C. zawadskii* ssp. *acutilobum* (KJ183117)	0.0	-																			
3	*C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (KJ183118)	0.0	0.0	-																		
4	*C. zawadskii* ssp. *acutilobum* var. *alpinum* (KJ183132)	1.1	1.1	1.1	-																	
5	*C. zawadskii* ssp. *lucidum* (KJ183119)	0.5	0.5	0.5	1.5	-																
6	*C. zawadskii* ssp. *coreanum*(KJ183120)	0.0	0.0	0.0	1.1	0.5	-															
7	*C. zawadskii* ssp. *naktongense* (KJ183121)	0.2	0.2	0.2	1.2	0.6	0.2	-														
8	*C. zawadskii* ssp. *naktongense* (AF314595)	0.0	0.0	0.0	1.1	0.5	0.0	0.2	-													
9	*C. zawadskii* ssp. *yezoense* (EF577307)	0.3	0.3	0.3	1.4	0.8	0.3	0.5	0.3	-												
10	*C. zawadskii* ssp. *yezoense* (KJ183122)	0.0	0.0	0.0	1.1	0.5	0.0	0.2	0.0	0.3	-											
11	*C. zawadskii* ssp. *latilobum* (KJ183123)	0.3	0.3	0.3	1.4	0.5	0.3	0.5	0.3	0.6	0.3	-										
12	*C. zawadskii* ssp. *latilobum* var. *leiophyllum* (KJ183124)	0.0	0.0	0.0	1.1	0.5	0.0	0.2	0.0	0.3	0.0	0.3	-									
13	*C. indicum* (KJ183125)	0.3	0.3	0.3	1.1	0.8	0.3	0.5	0.3	0.6	0.3	0.6	0.3	-								
14	*C. indicum* (JN315940)	0.2	0.2	0.2	1.2	0.6	0.2	0.3	0.2	0.5	0.2	0.5	0.5	0.5	-							
15	*C. indicum* var. *albescens* (KJ183126)	0.2	0.2	0.2	0.9	0.6	0.2	0.3	0.2	0.5	0.2	0.5	0.2	0.2	0.3	-						
16	*C. indicum* var. *acuta* (KJ183127)	0.9	0.9	0.9	1.5	1.4	0.9	1.1	0.9	1.2	0.9	1.2	0.9	0.6	1.1	0.8	-					
17	*C. boreale* (KJ183128)	0.3	0.3	0.3	1.2	0.8	0.3	0.5	0.3	0.6	0.3	0.6	0.3	0.6	0.2	0.5	0.9	-				
18	*C. lineare* (KJ183129)	3.6	3.6	3.6	3.6	3.6	3.6	3.7	3.6	3.9	3.6	3.9	3.6	3.9	3.7	3.7	4.4	3.7	-			
19	*C. makinoi* (KJ183130)	0.5	0.5	0.5	1.5	0.9	0.5	0.6	0.5	0.8	0.5	0.8	0.5	0.8	0.6	0.6	1.4	0.8	3.7	-		
20	*Aster glehnii* (Out group)	25.8	25.8	25.8	25.5	26.2	25.8	25.5	25.8	25.6	25.8	26.2	25.8	26.0	25.8	26.0	26.6	25.8	25.1	26.2	-	
21	*Aster indicus* (Out group)	25.9	25.9	25.9	25.2	26.3	25.9	25.6	25.9	25.7	25.9	26.3	25.9	25.9	25.9	25.6	26.5	25.9	25.7	26.3	2.6	-

[z]Accession numbers indicate National Center for Biotechnology Information (NCBI) numbers in USA.

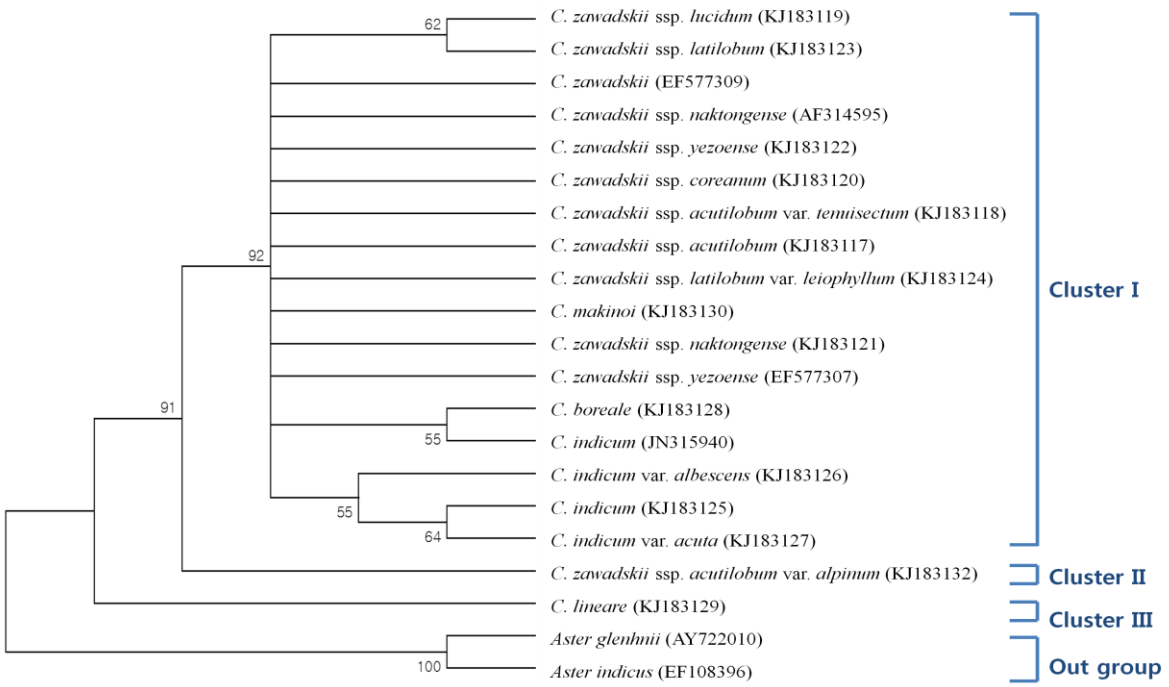

Fig. II-2. The strict consensus tree of *Chrysanthemum* taxa based on nrDNA ITS sequences. Numbers at nodes are bootstrap values. The bootstrap values greater than 50% are above the clades.

In the polymorphisms, the inter-taxa divergence of ITS 1 and ITS 2 was examined using sequences (Table II-5). The divergence rates of ITS 1 and ITS 2 pair wise within *Chrysanthemum* have ranges of 0.0-6.7% and 0.0-4.5%, respectively. Consequently, ITS 1 sequences in *C. lineare* and *C. indicum* var. *acuta,* or *C. lineare* and *C. zawadskii* ssp. *acutilobum* var. *alpinum* were found to be the most divergent while *Chrysanthemum* showed great similarities within same subspecies and varieties. The genetic distance rates based on the ITS 2 spacer ranged from 0.0 to 4.5% (Table II-5). The smallest distance was observed within *C. zawadskii* subspecies. However, *C. lineare* or *C. makinoi, C. inducum var acute,* or *C. zawadskii* ssp. *latilobum* were characterized by the maximum divergence since they exhibited the greatest genetic divergence value of 4.5%.

The phenogram based on ITS 1 sequence analysis illustrated the divergence described above and allowed taxa clustering into two main clusters (Fig. II-3A). The first 15 taxa (18 populations) were composed of cluster I. The *C. lineare* grouped into cluser II which was supported with bootstrap values of 62%. The derived phenogram illustrated divergence within *Chrysanthemum* (Fig. II-3B). The phenogram based on ITS 2 was categorized into three main clusters. The first cluster (I) was composed of 14 taxa (17 populations) that were significantly divergent from *C. zawadskii* ssp. *acutilobum* var. *alpinum* ranged in the second cluster labeled (II). The remaining one taxa of *C. lineare* was grouped in the third cluster (III).

Table II-5. Genetic divergence matrix among *Chrysnathemum* based on ITS 1 (lower diagonal) and ITS 2 (upper diagonal) sequences with Kimura 2-parameter method.

No.	Scientific name (NCBI accession no.[z])	Divergence (%)																				
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
1	*C. zawadskii* (EF577309)	-	0.0	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
2	*C. zawadskii* ssp. *acutilobum* (KJ183117)	0.0	-	0.0	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
3	*C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (KJ183118)	0.0	0.0	-	1.3	0.0	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
4	*C. zawadskii* ssp. *acutilobum* var. *alpinum* (KJ183132)	1.5	1.5	1.5	-	1.3	1.3	1.3	1.3	1.3	1.3	1.8	1.3	1.3	1.3	1.3	1.8	1.3	2.6	1.8	36.8	36.5
5	*C. zawadskii* ssp. *lucidum* (KJ183119)	1.1	1.1	1.1	2.7	-	0.0	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
6	*C. zawadskii* ssp. *coreanum*(KJ183120)	0.0	0.0	0.0	1.5	1.1	-	0.0	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
7	*C. zawadskii* ssp. *naktongense* (KJ183121)	0.4	0.4	0.4	1.9	1.5	0.4	-	0.0	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
8	*C. zawadskii* ssp. *naktongense* (AF314595)	0.0	0.0	0.0	1.5	1.1	0.0	0.4	-	0.0	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
9	*C. zawadskii* ssp. *yezoense* (EF577307)	0.8	0.8	0.8	2.3	1.9	0.8	1.1	0.8	-	0.0	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
10	*C. zawadskii* ssp. *yezoense* (KJ183122)	0.0	0.0	0.0	1.5	1.1	0.0	0.4	0.0	0.8	-	0.4	0.0	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
11	*C. zawadskii* ssp. *latilobum* (KJ183123)	0.4	0.4	0.4	1.9	0.7	0.4	0.7	0.4	1.1	0.4	-	0.4	0.4	0.4	0.4	1.3	0.9	4.5	0.9	39.1	38.7
12	*C. zawadskii* ssp. *latilobum* var. *leiophyllum* (KJ183124)	0.0	0.0	0.0	1.5	1.1	0.0	0.4	0.0	0.8	0.0	0.4	-	0.0	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
13	*C. indicum* (KJ183125)	0.7	0.7	0.7	1.5	1.9	0.7	1.1	0.7	1.5	0.7	1.1	0.7	-	0.0	0.0	0.9	0.4	4.0	0.4	38.3	38.0
14	*C. indicum* (JN315940)	0.4	0.4	0.4	1.9	1.5	0.4	0.7	0.4	1.1	0.4	0.7	0.4	1.1	-	0.0	0.9	0.4	4.0	0.4	38.3	38.0
15	*C. indicum* var. *albescens* (KJ183126)	0.4	0.4	0.4	1.1	1.5	0.4	0.7	0.4	1.1	0.4	0.7	0.4	0.4	0.7	-	0.9	0.4	4.0	0.4	38.3	38.0
16	*C. indicum* var. *acuta* (KJ183127)	1.1	1.1	1.1	1.9	2.3	1.1	1.5	1.1	1.9	1.1	1.5	1.1	0.4	1.5	0.7	-	0.4	4.5	1.3	39.1	38.8
17	*C. boreale* (KJ183128)	0.4	0.4	0.4	1.9	1.5	0.4	0.7	0.4	1.1	0.4	0.7	0.4	1.1	0.0	0.7	1.5	-	4.0	0.9	38.3	38.0
18	*C. lineare* (KJ183129)	5.4	5.4	5.4	6.7	5.4	5.4	5.8	5.4	6.3	5.4	5.8	5.4	6.3	5.8	5.8	6.7	5.8	-	4.5	36.2	37.2
19	*C. makinoi* (KJ183130)	0.7	0.7	0.7	2.3	1.9	0.7	1.1	0.7	1.5	0.7	1.1	0.7	1.5	1.1	1.1	1.9	1.1	5.4	-	39.1	38.8
20	*Aster glehnii* (Out group)	34.2	34.2	34.2	34.2	35.6	34.2	33.6	34.2	33.7	34.2	34.9	34.2	34.9	34.2	34.9	35.5	34.2	34.9	34.9	-	4.6
21	*Aster indicus* (Out group)	34.2	34.2	34.2	32.9	35.6	34.2	33.6	34.2	33.7	34.2	34.9	34.2	34.2	34.2	33.6	34.8	34.2	34.9	34.9	2.3	-

[z]Accession numbers indicate National Center for Biotechnology Information (NCBI) numbers in USA.

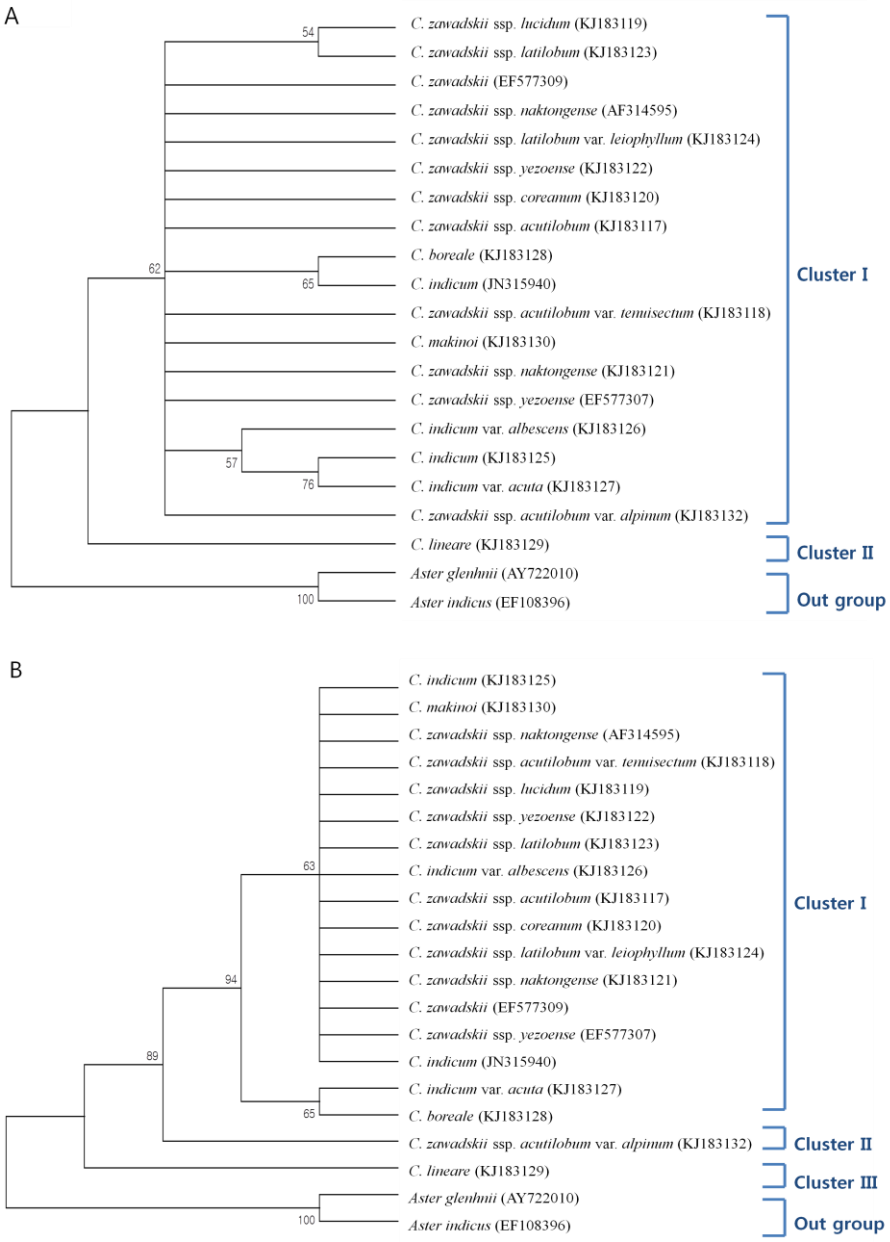

Fig. II-3. The strict consensus tree of *Chrysanthemum* taxa based on ITS 1 (A) and ITS 2 (B) sequences. Numbers at nodes are boot strap values. The bootstrap values greater than 50% are above the clades.

DISCUSSION

Korean *Chrysanthemum* was reported to be diploid, tetraploid, hexaploid, octoploid, and 10 diploids (Dowrick, 1952; Hwang et al., 2013; Kim, 1999; Nakata et al., 1987) with a basic number x = 9 in Asteraceae (Inceer and Hayirlioglu-Ayaz, 2010; Kim, 2003; Lee, 2006). To date, polyploid (2x, 4x, 6x, 8x, 10x) in *C. zawadskii*, polyploid (2x, 4x, 6x) in *C. indicum*, and diploid (2x) in *C. boreale*, *C. lineare*, and *C. makinoi*, respectively, have been reported in various studies (Kim, 2003; Lee, 2006).

The tetraploid and hexaploid individuals were more common than the diploid individulas in *Chrysanthemum* (Kim, 1999). Polyploids were known to have wider geographical ranges, more successful in colonizing new available areas, and have greater tolerance to ecological stress than their diploid counterparts (Inceer and Hayirlioglu-Ayaz, 2010). The polyploid distribution patterns of *Chrysanthemum* showed these general trends. The high frequency of polyploids in the examined plants showed that *Chrysanthemum* continuously developed more in the genetic and ecological evolutionary mechanisms that lead to diversification and speciation (Soltis et al., 2004). The occurrence of polyploidy genotypes was attributed to the prospective of the polyploids species to grow under various habitats and survive better under unfavorable climatic conditions (Abd El-Twab and Kondo, 2006).

Diploid in *C. lineare* and *C. makinoi* especially needed protection due to extremely small or decreasing population size, the category of rarity was set by geographical distribution areas of the species, degree of habitat specificity and local population size (KNA, 2012).

The *C. lineare* was reported to be distributed in Mt. Chilbo, Gyeonggi (Kim et al., 2014). Its mode of formation incorporated genetic diversity from multiple progenitor populations into the polyploid species (Park and Lee, 2004). Thus, genetic diversity in polyploid taxa was found to the higher than expected by models of polyploid formation involving single origin. These duplicated genes and genomes could undergo divergent evolution and evolve new functions. The genetic and genomic attributes of polyploid could have both biochemical and ecological benefits that could contribute to the success of polyploid in nature.

However, ITS is a non-coding region that exists among 18S, 5.8S, and 26S ribosomal DNA (rDNA), and referred to as ribosomal internal transcribed spacer region. The variations in sequence and divergence were used in classifying not only in subspecies with close phylogenetic relationships but also in genus or species (Baldwin et al., 1995). The ITS divergence was used for phylogenetic analysis (Baldwin et al., 1995).

Initially, *C. lineare* was placed in cluster at the base of the phylogenetic tree, while other taxa formed as two clusters. The divergence corresponded to the result in the sequence of *Ligularia* which was ranged 0.95-4.95% within in-group, and it was ranged 0.95-7.76% within out-group (Ahn et al., 2010).

However, the results of the shorter ITS 2 region in *Chrysanthemum* did not correspond to the ITS 2 results in *Kalimeris* which was 1.5% longer than ITS 1 divergence 1.1% (Hong et al., 2012).

The scored size of the ITS 1 and ITS 2 spacers correlated to the results of ITS length of other genus in Asteraceae (Ahn et al., 2010; Lee et al., 2010). In paticular, the results of the longer ITS 1 region in *Chrysanthemum* corresponded with the results of *Ligularia* (Ahn et al., 2010), *Aster* (Hong et al., 2012), and *Artemisia* (Lee et al., 2010). The 5.8S coding region exhibited relatively homogenous size in the studied taxa at 165 bp. This result supported the hypothesis of invariant length, mostly 163 to 166 bp, of this sub-unit in the angiosperm species (Baldwin et al., 1995).

Kim (1999) insisted that hexaploids in *C. zawadskii* subspecies have mostly the same sequence in the intergenic region between *psbA* and *trnH*. According to the analysis on sequence of the chlorophyll DNA, *C. zawadskii* ssp. *lucidum* (tetraploid), *C. zawadskii* ssp. *naktongense* (hexaploid) and *C. zawadskii* ssp. *coreanum* (10 diploid) indicated unique replacement compared to other *C. zawadskii*. However, *Chrysanthemum zawadskii* subspecies could not be easily identified based on the ITS sequence.

In conclusion, the study herein illustrated the use of ITS sequence variation to generate new molecular markers, which was not suitable in the assessment of the genetic diversity within *Chrysanthemum zawadskii* subspecies. Moreover, the *C. indicum* (tetraploid) and *C. indicum* var. *acuta* (tetraploid) were showed

similar sequence indicating close relationship within same sister group. But the *C. indicum* (tetraploid) and *C. indicum* var. *albescens* (hexaploid) formed an independent clade within *C. indicum* subspecies. Thus, genetic relationships among *Chrysanthemum* could not be easily determined based on comparisons of ITS sequences. The study proved that *C. zawadskii* ssp. *acutilobum* var. *alpinum* could be classified as *Chrysanthemum zawadskii* subspecies. *C. lineare* (diploid) formed an independent species from *Chrysanthemum*. Hence, diversified genetic analysis and sufficient cytogenetic studies are demonded to determine the phylogeny of the genus *Chrysanthemum*. Generally, chromosome analysis and ITS sequence analysis of *Chrysanthemum* used in this study provided new information on the phylogenetic relationship among taxa. The outcome of this study will be base for the identification and breeding of Korean *Chrysanthemum* in the future.

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CHAPTER III

Taxonomic Relationships and Anti-inflammatory Effects of Korean *Chrysanthemum* based on Flavonoids

ABSTRACT

This study was conducted to compare flavonoids and anti-inflammatory effect of Korean *Chrysanthemum*. Leaf samples were identified with five flavonoids: luteolin 7-*O*-rutinoside, luteolin-7-*O*-glucoside, apigenin-7-*O*-glucoside, apigenin, and acacetin-7-*O*-rutinoside using HPLC/MS. Flavonoids were categorized into three groups based on cluster analysis. Group I included mostly luteolin-7-*O*-glucoside and acacetin-7-*O*-rutinoside. Group II identified with higher apigenin than other groups. Group III detected with higher luteolin 7-*O*-rutinoside than the other two groups. Leaf extracts from four taxa were dose-dependently suppressed LPS-stimulated NO production and significantly ($p < 0.05$) inhibited production of LPS-induced PGE_2, compared with controls. These extracts reduced the LPS-induced expression of inducible NO synthase and cyclooxygenase-2 and inhibited LPS-induced tumor necrosis factor-α and interleukin-6 production showing anti-inflammatory effects.

Keywords: Anti-Inflammatory, Apigenin, *Chrysanthemum*, Lipopolysaccharide

INTRODUCTION

Flavonoids are naturally occurring low molecular weight and polyphenolic compounds categorized into flavonols, flavones, flavanones, isoflavones, catechins, anthocyanidins and chalcones according to chemical structure (Janssen et al., 1998). The flavonoids distributed in fruits, vegetables, and plants possess potent biological effects including antioxidant, antiplatelet, and anti-inflammatory properties (Park et al., 2008). They raised considerable research interest due to their potential beneficial effects on human health. They are important bioactive compounds found in herbal plants and have been reported with antioxidant and inflammatory activities (Chen et al, 2001; Ha et al., 2006).

The inflammatory reaction is a protective response in tissue against external physical and chemical stimuli, or bacterial infection. It is a mechanism for recovering or regenerating damaged tissues (Willoughby, 1975). The main cells involved in the inflammatory response are macrophages that are activated by multiple stimuli, or cytokines secreted by immune cells, which generate inflammatory cytokines, nitric oxide (NO), prostaglandin E_2 (PGE_2), histamine, serotonine, bradykinin, hydroxyeicosatetraenoic acid (HETE), and leukotriene. These in-turn create inflammation and cause pain, swelling, and increase the body temperature (Lee et al., 2004).

The anti-inflammatory and anti-oxidative effects of Korean *Chrysanthemum*

have not been extensively investigated, but flavonoids have been reported from a few taxa (Han et al., 2009; Hyun et al., 2011; Lee et al., 2008). In addition, minor researches have been reported on Korean *Chrysanthemum* such as the classification of taxa based on flavonoid. However, Korean *Chrysanthemum* is used in traditional Korean medicine for treating pneumonia, bronchitis, common cold, pharyngitis, bladder-related disorders, and hypertension (Yu et al., 2008).

This study showed the analysis of flavonoids and categorized the taxa by cluster analysis methods, as well as by comparing inflammatory responses in LPS-stimulated RAW264.7 macrophage cells of 15 Korean *Chrysanthemum* taxa.

MATERIALS AND METHODS

Plant Material

Fifteen Korean *Chrysanthemum* taxa were grown in the greenhouse at Highland Agriculture Research Institute in Pyeongchang, Gangwon, Korea since 2010. The taxa and collection sites are shown in Table III-1.

Extraction of samples

Fresh leaves were stored in -80℃deep freezer (Revoco Ultima II, Revoco Inc., Asheville, NC, USA) for two days. Samples were freeze dried with freeze-dryer (EYELA FDU-2100, Eyela Corp. Tokyo, Japan) which used trap-cooling temperature at -80℃ with vacuum condition for five days. Freeze-dried samples were blended within 5 min using mini blender (Mini multi deluxe BL126DK, Tefal Co., Kaleo, France). Two grams of freeze-dried powder samples were taken into 250 mL round bottom flask and added with 100 mL of 80% ethanol was added. Extracted solution was heated at 60℃ in a water bath and refluxed for 6 hr with soxhlet extractor (J-BS30, Jisico Co., Seoul, Korea).The extracted solution was filtered using RephiQuik syringe filter (RJN3245NH 0.45um, RephiLe Bioscience Ltd, Shenzhen, China) using vacuum pump (Woo et al., 2010). Sample extracts were concentrated using a rotary evaporator (N-1000, Eyela Co., Tokyo, Japan) and were dissolved in 2 mL of 80% ethanol.

Table III-1. List of Korean *Chrysanthemum* used for flavonoid analysis and anti-inflammatory effects.

Scientific name	Collecting site
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa	Mt. Yumyeong, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *tenuisectum* (Kitagawa) Y. Lee	Pocheon, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *alpinum* (Nak.) Y. Lee	Mt. Baekdu, Hambuk, Korea
C. zawadskii Herbich ssp. *lucidum* (NAK.) Y .Lee	Ulleung, Gyeongbuk, Korea
C. zawadskii Herbich ssp. *coreanum* (NAK.) Y. Lee	Mt. Halla, Jeju, Korea
C. zawadskii Herbich ssp. *naktongense* (NAK.) Y. Lee	Gimhae, Gyeongnam, Korea
C. zawadskii Herbich ssp. *yezoense* (Maekawa.) Y. Lee	Goheung, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa	Wando, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa var. *leiophyllum* (Nak.) Y. Lee	Gangneung, Gangwon, Korea
C. indicum Linné	Anmyeondo, Chungnam, Korea
C. indicum Linné var. *albescens* Makino	Jeongseon, Gangwon, Korea
C. indicum var. *acuta* (Uyeki) Kitam.	Byeonsanbando, Jeonbuk, Korea
C. boreale (Mak.) Makino	Pyeongchang, Gangwon, Korea
C. lineare Matsumura	Mt. Chilbo, Gyeonggi, Korea
C. makinoi Matsumura et Nakai	Daegu, Korea

HPLC/MS flavonoid analysis

Flavonoid compounds were characterized using the Acquity™ ultra performance liquid chromatography (UPLC) system coupled on-line with triplequadrupole (TQ) mass spectrometer detector (ACQUITY TQD, Waters, Milford, MA, USA). Data acquisition and processing were performed using MassLynx control software. The column (ZORBAX Eclipse Plus C_{18}, 3.0 mm × 50 mm, 1.8 μm, Agilent Technologies, Palo Alto, CA, USA) was used with LC solvent at flow rate of 0.4 mL/min. The mobile phase was composed of 0.1% formic acid in water (eluent A) and 0.1% formic acid in acetonitrile (eluent B). Gradient conditions were 0-1 min, 5% B; 5 min, 10% B; 10 min, 20% B; 20 min, 30% B; 25 min, 30% B; 28 min, 65% B; holding at 100% B for 3 min, then holding for 5 min before returning to the initial conditions. Mass spectra were obtained between *m/z* = 100 and 1,000 in negative ionisation mode (ESI^-) at scan rate of 1.5 s/cycle and monitored using photodiode array (PDA) detector. The mass spectrometric conditions were capillary voltage of 2.8 kV, cone voltage of 60 V, desolvation gas flow of 50 L/h (N_2), source temperature of 80°C, desolvation temperature of 200°C, and cone gas flow of 50 L/h (N_2). Collision induced fragmentation experiments were performed in ion trap using helium as the collision gas.

Cell cultures and sample treatment

The RAW 264.7 murine macrophage cells were obtained from the Korea Cell Line Bank. Cells were grown at 37°C in Dulbecco's Modified Eagle's Minimum Essential Medium (DMEM, Life Technologies Inc., Brand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich Corp., St. Louis, MO, USA), penicillin (100 U/mL, Life Technologies Inc.) and streptomycin sulphate (100 μg/mL, Life Technologies Inc.) in humidified atmosphere of 5% CO_2 (Sci 165D, Astec, Tokyo, Japan). Cells were incubated with test compounds at different concentrations (50, 100, or 200 μg/mL), or with the positive controls L-NIL or NS-398, then induced using LPS (1 μg/mL) for 24 h. Different concentrations (50, 100, or 200 μg/mL) of test compounds dissolved in DMSO and LPS were added.

MTT Assay

The MTT assays (Woo et al., 2010) were used to evaluate the cytotoxicity of leaf extracts expressed as IC_{80} values (defined as the concentration that results in 80% decrease in the number of cells compared with that of the control cultures in the absence of an inhibitor). The effective concentration range of non-toxicity was set within the effective concentration range of each sample. Low (50 μg/mL), medium (100 μg/mL), and high (200 μg/mL) concentrations were used.

The MTT assays were conducted prior to the analysis of anti-inflammatory effects of *Chrysanthemum* extracts to determine these inhibitory effects which were not caused by nonspecific cytotoxicity in RAW264.7 cells. Concentration level of more than 80% was the optimum capacity. With IC_{80} concentration of 200 μg/mL, all samples exhibited low toxicity values. As such, by setting concentrations not to exceed the IC_{80} value, inhibition effects against the inflammatory markers NO and PGE_2 were evaluated.

Cells were incubated with an MTT solution for 4 h at 37℃ under 5% CO_2, then 100 μl of DMSO was added to extract MTT formazan and the absorbance of each well was read at 540 nm using an automatic microplate reader (Molecular Devices Inc., Sunnyvale, CA, USA).

Measurement of the nitrite concentration in culture media

The RAW 264.7 macrophages were plated at 2.5 x 10^5 cells/mL in 24-well plates, then incubated (Sci 165D, Astec, Fukuoka, Japan) with and without LPS (1 μg/mL) in the absence and presence of different concentrations (50, 100, or 200 μg/mL) of test compounds for 24 h. The nitrite accumulation in the culture medium was measured as an indication of nitric oxide (NO) production based on the Griess reaction (Rim et al., 2012). Briefly, 100 μl of cell culture medium was mixed with 100 μl of Griess reagent [equal volumes of 1% sulphanilamide (Sigma-Aldrich Corp.) in 5% phosphoric acid (Sigma-Aldrich Corp.) and 0.1% naphtylethylenediamine-HCl (Sigma-Aldrich Corp.)], incubated at room

temperature for 10 min, then the absorbance at 540 nm was measured using microplate reader (VERSAmax, Molecular Devices, CA, USA). Fresh culture medium (DMEM, Life Technologies Inc.) was used as blank in all experiments. The amount of nitrite in samples was measured using serial dilution standard curve of sodium nitrite (Sigma-Aldrich Corp.).

Evaluation of prostaglandin E_2 (PGE_2) generation

The PGE_2 levels in cell culture media were quantified using Enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems, Inc., Minneapolis, MN, USA) according to manufacturer's instructions.

Western blot analysis

Cells treated with leaf extracts of *Chrysanthemum* and control group were collected by centrifugation (Centrifuge 5424R, Eppendorf, Hamburg, Germany) (10,000*g* for 10 min) and washed once with phosphate-buffered saline (PBS). Washed cell pellets were resuspended in the protein extraction solution (PRO-PREP, Intron Biotechnology, Seoul, Korea) and then incubated for 15 min at 4℃. Cells were then centrifuged (Centrifuge 5424R, Eppendorf, Hamburg, Germany) (10,000*g* for 30 min) and the supernatant was used for determination of protein concentration using protein assay reagent (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to manufacturer's instructions. A total of 40 μg of protein was electroblotted (Trans-Blots SD Semi-Dry Electrophoretic

Transfer Cell, Bio-Rad Laboratories, CA, USA) onto PVDF membrane following separation using 10% SDS-polyacrylamide gel electrophoresis (PowerPac Universal Power Supply, Bio-Rad Laboratories, CA, USA). Immunoblots were incubated (BF-350, Biofree, Seoul, Korea) for 1 h with blocking solution (5% skim milk) at room temperature, then incubated (BF-350, Biofree) again overnight with 1:1,000 dilution of iNOS, COX-2, and β-actin antibodies (monoclonal antibodies, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 4℃. Blots were washed three times with Tween 20/Tris-buffered saline (T/TBS), and then incubated (BF-350, Biofree) with 1:2,000 dilution of horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 2 h at room temperature. Blots were again washed three times with T/TBS, and then developed using enhanced chemiluminescence reagent (GE healthcare, NJ, USA).

Determination of TNT-α and IL-6 production

The RAW 264.7 macrophages were treated with leaf extracts of four taxa of *Chrysanthemum* for 1 h, then induced using LPS (1 μg/mL) for 24 h. Levels of TNF-α and IL-6 in culture media were quantified using kits (R&D Systems, Minneapolis, MN, USA).

Statistical analysis

Cell culture experiments were performed at least three times. All values were expressed as mean ± standard deviation (SD). Statistical analysis was performed using SAS version 9.2 software (SAS Institute, Inc., Cary, NC, USA). Analysis of variance (ANOVA) with Duncan's test was used to determine significant differences between means at $p < 0.05$, 0.01, 0.001. Cluster analysis was carried out using 15 taxa in order to categorize taxa into groups and produce dendrogram to represent relationships among taxa with Ward's minimum-variance clustering method.

RESULTS

Flavonoid composition

Flavonoids were identified as luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, apigenin, and acacetin-7-*O*-rutinoside (Table III-2, III-3; Fig. III-1). The retention times of luteolin 7-*O*-rutinoside and 7-*O*-glucosede were 3.78 and 10.15 min, respectively. The retention times of apigenin-7-*O*-glucoside, apigenin, and acacetin-7-*O*-rutinoside were 11.40, 13.69, and 14.68 min, respectively. Moreover, the contents of luteolin-7-*O*-rutinoside were higher in *C. zawadskii* ssp. *naktongense*, *C. zawadskii* ssp. *latilobum*, *C. indicum*, and *C. boreale* than other taxa. The contents of apigenin-7-*O*-glucoside were the highest in *C. indicum*. The contents of acacetin-7-*O*-rutinoside were higher in *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *naktongense*, *C. zawadskii* ssp. *yezoense* and *C. boreale* than other taxa.

Several flavonoids have been isolated from some *Chrysanthemum* species in China (Wu et al., 2010) and Japan (Uehara et al., 2012). *Chrysanthemum zawadskii* var. *latilobum* has luteolin, quercetin, and acacetin which are reported to have anti-cancer activities (Kwon et al., 2006). Acacetin showed significant cytotoxic activity against HCT116 and UO-31 cells (Jang et al., 1997).

Table III-2. Peak assignments, retention times, mass spectral data, and elemental compositions of flavonoids observed in Korean *Chrysanthemum* using HPLC/MS analysis.

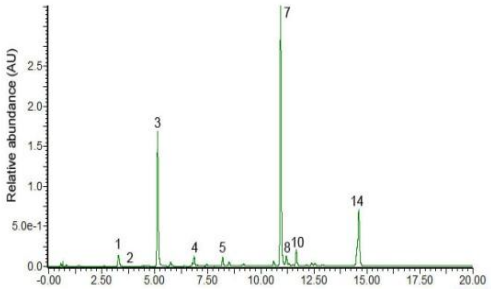
Peak no.	t_R (min) at 254nm	Molecular ion $[M-H]^-$ (*m/z*)	Fragment ions in ESI/MS (*m/z*)	Identification
1	3.36	353 $[M-H]^-$	191 $[(M-H)$-caffeoyl$]^-$	4-*O*-caffeoylguinic acid
2	3.78	593 $[M-H]^-$	285 $[(M-H)$-glucose-rhamnose$]^-$	luteolin-7-*O*-rutinoside
3	5.19	353 $[M-H]^-$	191 $[(M-H)$-caffeoyl$]^-$	3-*O*-caffeoylquinic acid
4	6.91	337 $[M-H]^-$	191 $[(M-H)$-coumaroyl$]^-$	4-*p*-coumaroylquinic acid
5	8.28	337 $[M-H]^-$	191 $[(M-H)$-coumaroyl$]^-$	3-*p*-coumaroylquinic acid
6	10.15	447 $[M-H]^-$	285 $[(M-H)$-glucose$]^-$	luteolin-7-*O*-glucosede
7	10.98	515 $[M-H]^-$	353 $[(M-H)$-caffeoyl$]^-$ 191 $[(M-H)$-2(caffeoyl)$]^-$	4,5-*di*-caffeoylquinic acid
8	11.20	515 $[M-H]^-$	353 $[(M-H)$-caffeoyl$]^-$ 191 $[(M-H)$-2(caffeoyl)$]^-$	3,5-*di*-caffeoylquinic acid
9	11.40	431 $[M-H]^-$	269 $[(M-H)$-glucose$]^-$	apigenin-7-*O*-glucoside
10	11.73	515 $[M-H]^-$	353 $[(M-H)$-caffeoyl$]^-$ 191 $[(M-H)$-2(caffeoyl)$]^-$	3,4-*di*-caffeoylquinic acid
11	11.90	489 $[M-H]^-$	-	unknown
12	12.63	675 $[M-H]^-$	-	unknown
13	13.69	269 $[M-H]^-$	-	apigenin
14	14.68	591 $[M-H]^-$	283 $[(M-H)$-glucose-rhamnose$]^-$	acacetin-7-*O*-rutinoside

Table III-3. Flavonoids distribution of Korean *Chrysanthemum* using HPLC/MS analysis.

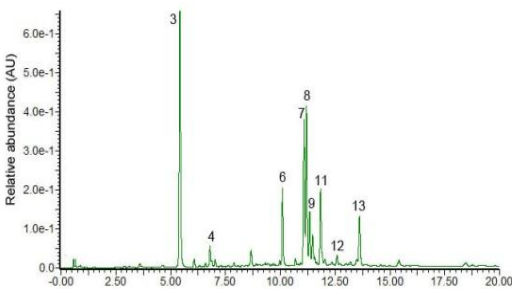
Scientific name	Luteolin-7-O-rutinoside	Luteolin-7-*O*-glucoside	Apigenin-7-*O*-glucoside	Apigenin	Acacetin-7-O-rutinoside
C. zawadskii ssp. *acutilobum*	+[z]	+	+	-	++
C. zawadskii ssp. *acutilobum* var. *tenuisectum*	-	t	+	-	+
C. zawadskii ssp. *acutilobum* var. *alpinum*	-	+	+	-	+
C. zawadskii ssp. *lucidum*	-	t	t	-	+
C. zawadskii ssp. *coreanum*	-	+	t	-	-
C. zawadskii ssp. *naktongense*	++	t	t	t	++
C. zawadskii ssp. *yezoense*	+	t	t	-	++
C. zawadskii ssp. *latilobum*	++	t	t	-	+
C. zawadskii ssp. *latilobum* var. *leiophyllum*	-	t	t	-	+
C. indicum	++	-	++	-	++
C. indicum var. *albescens*	+	t	+	-	+
C. indicum var. *acuta*	t	+	+	+	-
C. boreale	++	-	+	-	++
C. lineare	t	+	+	-	t
C. makinoi	-	t	t	-	-

[z] ++: ≥30% of the total flavonoids, +: from3 to30%, t: <3%, -: not decteced.

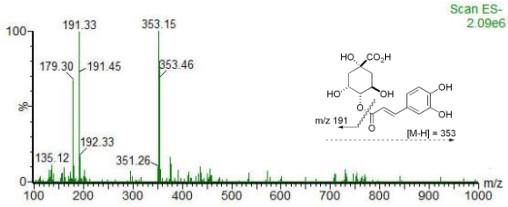
Peak (1,2,3,4,5,7,8,10, and 14)



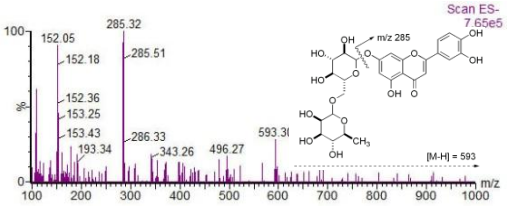
Peak (3,4,5,6,7,8,9,11,12, and 13)



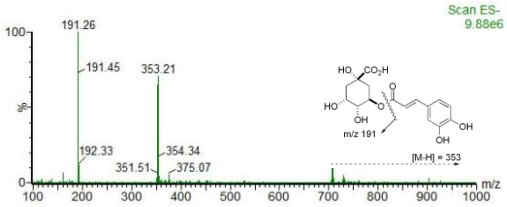
Compound 1 (4-*O*-caffeoylguinic acid)



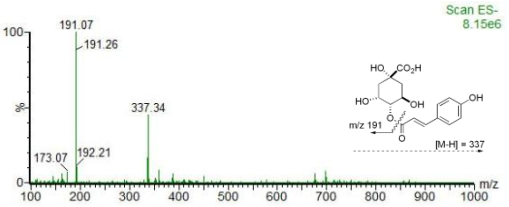
Compound 2 (luteolin-7-*O*-rutinoside)



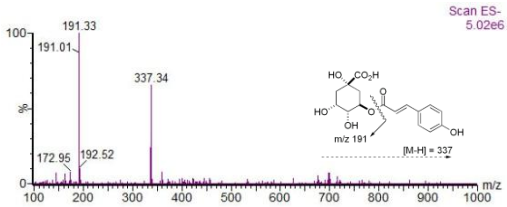
Compound 3 (3-*O*-caffeoylquinic acid)



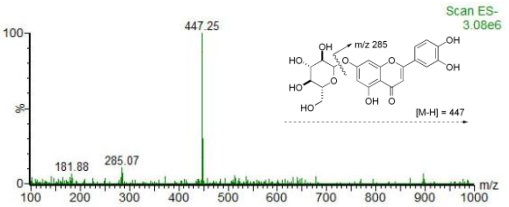
Compound 4 (4-*p*-coumaroylquinic acid)



Compound 5 (3-*p*-coumaroylquinic acid)

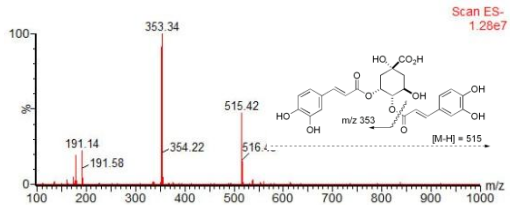


Compound 6 (luteolin-7-*O*-glucosede)

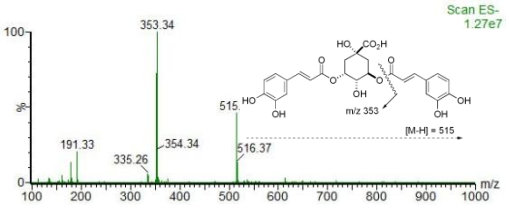


(Continued)

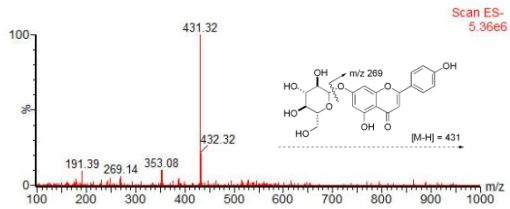
Compound 7 (4,5-*di*-caffeoylquinic acid)



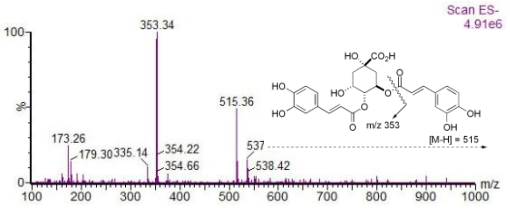
Compound 8 (3,5-*di*-caffeoylquinic acid)



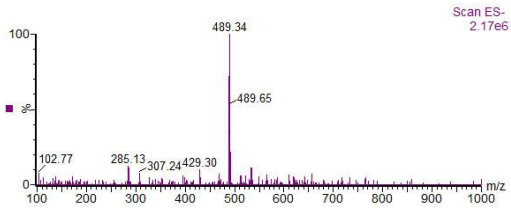
Compound 9 (apigenin-7-*O*-glucoside)



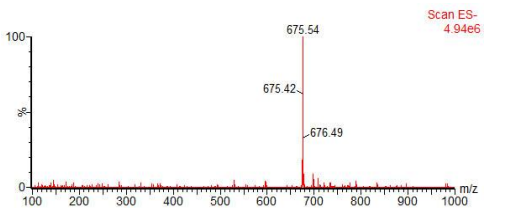
Compound 10 (3,4-*di*-caffeoylquinic acid)



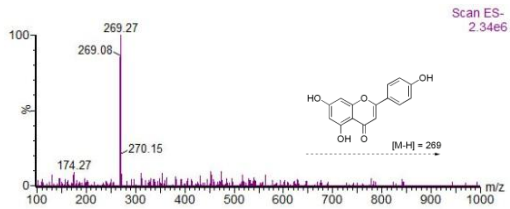
Compound 11 (unknown)



Compound 12 (unknown)



Compound 13 (apigenin)



Compound 14 (acacetin-7-*O*-rutinoside)

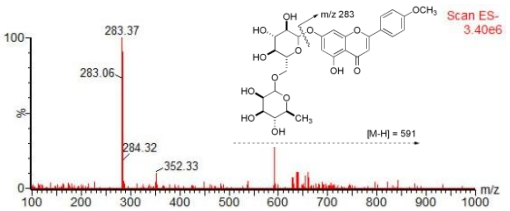


Fig. III-1. HPLC/MS spectrum of identified flavonoids in the leaf extracts of Korean *Chrysanthemum*.

Effect of leaf extracts on LPS-induced NO production and PGE_2 generation

To investigate whether leaf extracts from *Chrysanthemum* have anti-inflammatory activities, LPS-induced NO production was determined in the presence of leaf extracts in LPS-induced RAW 264.7 macrophages (Table III-4). The IC_{50} value inhibitory effects against LPS-induced NO production in four taxa of *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, and *C. indicum* var. *acuta* were 62.7, 64.3, 63.1, and 69.5 μg/mL, respectively. The *C. zawadskii* ssp. *acutilobum* extract showed the most powerful inhibitory effects. L-NIL (IC_{50} = 18.2 μM) and NS398 (IC_{50} = 0.3 μM) were used as positive control inhibitors. In addition, the inhibitory effects of *Chrysanthemum* were not due to non-specific cytotoxicity because the extracts had no effect on cell viability, which was measured using MTT assays at concentrations up to 200 μg/mL. Nine taxa of *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *acutilobum* var. *tenuisectum*, *C. zawadskii* ssp. *coreanum*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, *C. zawadskii* ssp. *latilobum* var. *leiophyllum*, *C. indicum* var. *albescens*, *C. indicum* var. *acuta*, and *C. makinoi* inhibited over 90% of the LPS-induced PGE_2 production (Table III-4). Extracts of *C. zawadskii* var. *latilobum* with high NO inhibitory activities were reported to be effective for inhibition of iNOS protein expression while showing similarity to inhibition of NO generation by *Chrysanthemum* extracts and inhibition of iNOS protein expression (Han et al., 2009).

Table III-4. Effect of Korean *Chrysanthemum* on NO and PGE_2 production in RAW 264.7 cells.

Scientific name	No production IC_{50} (μg/mL)	PGE_2 production inhibition[z] (%)
C. zawadskii ssp. *acutilobum*	62.7±1.3	99.2±3.6
C. zawadskii ssp. *acutilobum* var. *tenuisectum*	137.0±2.3	98.9±4.1
C. zawadskii ssp. *acutilobum* var. *alpinum*	110.4±2.2	69.4±2.9
C. zawadskii ssp. *lucidum*	154.1±2.4	69.9±2.2
C. zawadskii ssp. *coreanum*	107.4±1.9	97.8±4.0
C. zawadskii ssp. *naktongense*	129.1±2.4	87.7±3.7
C. zawadskii ssp. *yezoense*	64.3±1.1	99.2±4.1
C. zawadskii ssp. *latilobum*	63.1±1.5	98.1±4.0
C. zawadskii ssp. *latilobum* var. *leiophyllum*	198.0±3.3	94.4±3.9
C. indicum	> 200	82.7±3.5
C. indicum var. *albescens*	80.6±1.7	98.0±3.7
C. indicum var. *acuta*	69.5±1.6	98.2±3.3
C. boreale	> 200	66.4±2.2
C. lineare	145.5±2.9	64.9±2.1
C. makinoi	76.9±1.4	98.3±3.3
p value	0.0007***[y]	<.0001***

[z]Percentage of PGE_2 production inhibition at 50 μg/mL.

[y] *** Significant at $p < 0.001$ within the same column.

Effects of leaf extracts on LPS-induced iNOS and COX-2 protein expression

Based on the inhibitory effects of extracts against LPS-induced NO and PGE_2 production, *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, and *C. indicum* var. *acuta* were determined that NO and PGE_2 inhibition by *Chrysanthemum* were related to reduction in the protein expression of iNOS and COX-2. LPS-induced iNOS and COX-2 protein levels were significantly ($p < 0.05$) inhibited by ethanol extracts of these species in concentration-dependent manner, compared with control (Fig. III-2).

Inhibitory effects against LPS-induced NO generation and iNOS protein expression were observed. In addition, depending on band density ratios of the house keeping protein α-actin, expressions of iNOS proteins were dose-dependently inhibited. In the non-treated control group (RAW 264.7 cells), iNOS expression was not observed, whereas in the LPS-treated group, iNOS expression was significantly ($p < 0.05$) increased, compared to the control group. Leaf extracts at concentration of 50 μg/mL revealed that iNOS expression was significantly ($p < 0.05$) inhibited, compared to the control group. Thus, iNOS protein expression and anti-inflammatory activation occurred via inhibition of generation of the major inflammatory mediators NO and PGE_2.

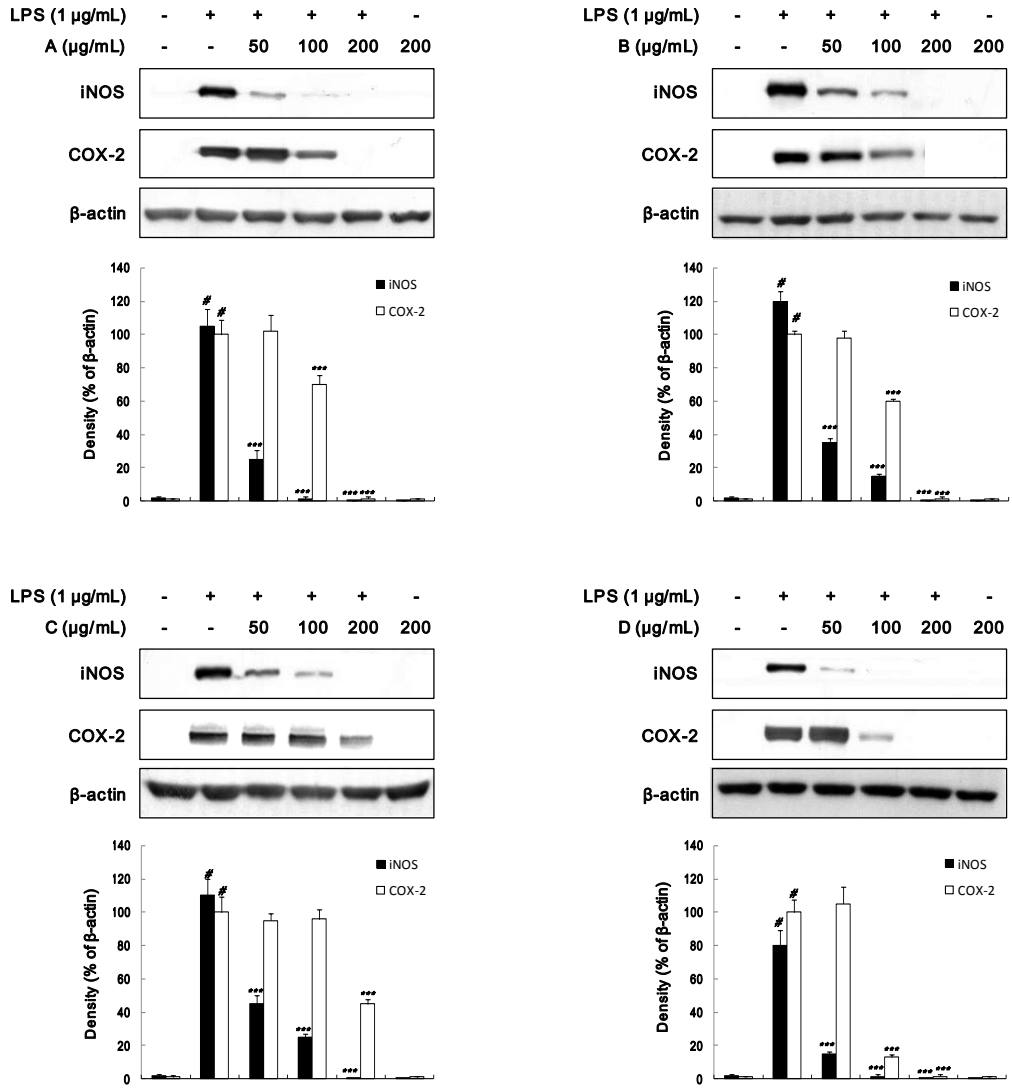
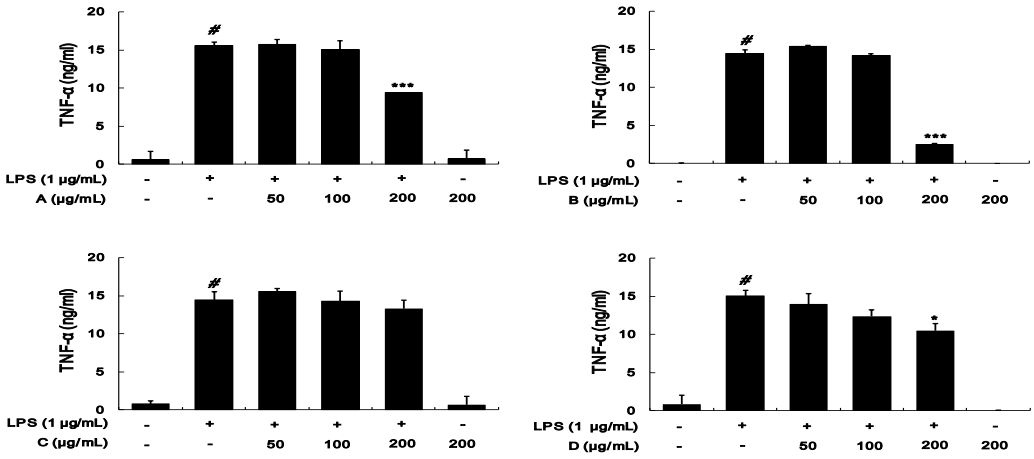


Fig. III-2. Effects of the leaf extracts of *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *yezoense* (B), *C. zawadskii* ssp. *latilobum* (C), *C. indicum* var. *acuta* (D) on LPS-induced expression of iNOS and COX-2 in RAW 264.7 macrophages. $^{\#}p < 0.05$ *vs.* the control group; *** $p < 0.001$ *vs.* the LPS-stimulated group.

Effects of leaf extracts on LPS-induced TNF-α and IL-6 production

The TNF-α and IL-6 production in LPS-induced macrophages treated with four *Chrysanthemum* extracts were examined. Treatment with extracts reduced LPS-induced production of TNF-α and IL-6 (Fig. III-3). The anti-inflammatory effects of *Chrysanthemum* were supported by the effects on pro-inflammatory cytokines (TNF-α and IL-6) in LPS-induced macrophages affecting the effects on regulation of immune reactions, hematopoiesis, inflammation and in some cases, shock and death (Christiaens et al., 2008). *Chrysanthemum* leaf extracts inhibited the expression of iNOS and COX-2 and production of pro-inflammatory cytokines.

(TNF)



(IL)

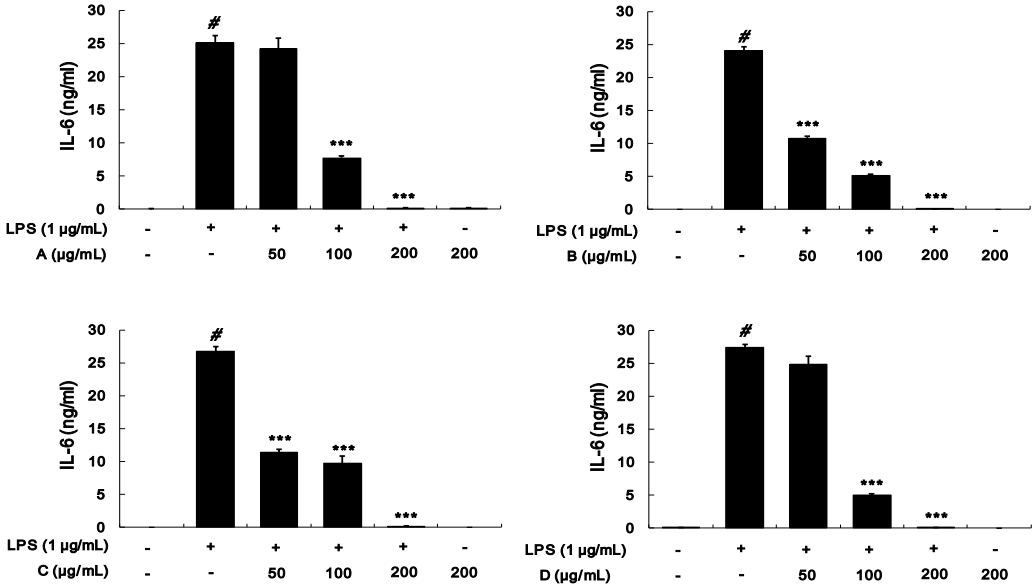


Fig. III-3. Effect of the leaf extracts of *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *yezoense* (B), *C. zawadskii* ssp. *latilobum* (C), *C. indicum* var. *acuta* (D) on LPS-induced production of TNF-α, and IL-6 in RAW 264.7 macrophages (TNF and IL). $^{\#}p < 0.05$ *vs.* the control group; *** $p < 0.001$ *vs.* the LPS-stimulated group.

Classification of *Chrysanthemum* based on flavonoids using cluster analysis

Flavonoids were analyzed and identified using HPLC/MS (Fig. III-4). Cluster analysis was performed for five flavonoids and the dendrogram of relationships was deduced 15 *Chrysanthemum* taxa and categorized into three groups based on flavonoids in *Chrysanthemum*.

Group I was identified mostly with luteolin-7-*O*-glucoside and acacetin-7-*O*-rutinoside. It included *C. zawadskii* ssp. *acutilobum, C. zawadskii* ssp. *acutilobum* var. *alpinum, C. zawadskii* ssp. *acutilobum* var. *tenuisectum, C. zawadskii* ssp. *lucidum, C. zawadskii* ssp. *latilobum* var. *leiophyllum, C. makinoi, C. zawadskii* ssp. *coreanum,* and *C. lineare.* Group II was distinguished with other groups because of higher apigenin contents. This group was only from one taxon, *C. indicum* var. *acuta.* Group III was detected with higher luteolin 7-*O*-rutinoside than other two groups. There were six taxa in this group; *C. zawadskii* ssp. *naktongense, C. zawadskii* ssp. *yezoense, C. zawadskii* ssp. *latilobum, C. indicum,* and *C. boreale*.

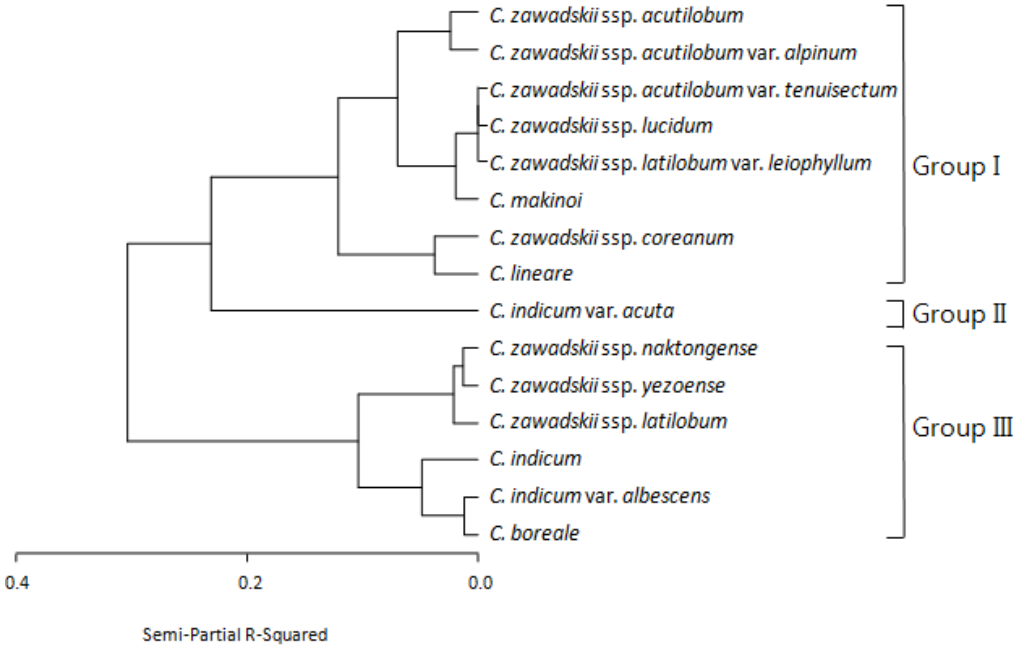


Fig. III-4. Dendrogram of 15 Korean *Chrysanthemum* taxa classified by cluster analysis based on flavonoids.

DISCUSSION

Flavonoids have beneficial biological effects including antioxidant, and anti-inflammation activities (Chen et al., 2001). In this study, flavonoids and anti-inflammatory properties in Korean *Chrysanthemum* were compared.

Chrysanthemum leaf extracts were contained five flavones of luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, apigenin, and acacetin-7-*O*-rutinoside. The flowers of *C. indicum* have been detected with acacetin, apigenin, luteolin, kaempferol, quercetin, and myricetin (Matsuda et al., 2002; Wu et al., 2010).

Bae et al. (1999) reported luteolin compounds in flower of *C. indicum*, which was more than the number of flavonoids, such as luteolin-7-*O*-rutinoside, apigenin-7-*O*-glucoside, and acacetin-7-*O*-rutinoside, detected from the same species used in this study. In addition, whole plants of *C. boreale* have been reported with acacetin, apigenin and luteolin (Shin et al., 1995). Woo (2010) found that extracts of *C. zawadskii* var. *lucidum* exhibited higher concentrations of phenolic compounds and greater antioxidant activities than extracts of *C. boreale* and *C. indicum*. These diverse results were mostly due to extraxts from different parts of the plants or dependent on various taxa. In this study, flavonoid compounds were categorized into three groups of *Chrysanthemum* according to flavonoid distribution.

Luteolin and luteoloside likely played roles in the suppression of protein

expressions of iNOS and COX-2, both were critical, inducible enzymes responsible for the production of nitric oxide and PGE_2 (Hu and Kitts, 2004). Acacetin exerted anti-peroxidant (Chobi et al., 1991), anti-inflammatory (Yin et al., 2008), and anti-cancerous activities by suppressing invasion and migration of human cancer cells (Shen et al., 2010). These results suggested that flavonoids might play role of an electron transport inhibitor in oxidative stress condition and effect antioxidant and anti-inflammation activities

Generation of NO and PGE_2 was inhibited by four taxa of *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, and *C. indicum* var. *acuta* extracts, indicating inhibitory effects against inflammation. Generation of only PGE_2 was inhibited in nine taxa indicating inhibitory effects against inflammation. Variations in inflammatory effects depended on taxa and on leaf extracts used to inhibit generation of the inflammatory mediators NO and PGE_2.

Both NO and PGE_2 are representative inflammatory markers generated by the expression of inducible nitric oxide synthase (iNOS) and cyclooxygenase-2. During inflammation, NO is formed mainly in macrophages by iNOS, the concentration of which is increased by stimulus of inflammatory cytokines or LPS. Prostaglandin (PG) is an unsaturated fatty acid that is generated from the arachidonic acid derived from phospholipids in the cell membrane. PGE_2 in PG, on the other hand, is associated with the inflammatory reaction and contributes to the formation of tumors by inducing angiogenesis (Rim et al., 2012).

Cyclooxygenase (COX) is an enzyme that changes arachidonic acid into PG due to cancer cell-caused inflammation, growth factors, and cytokines (Kim et al., 2011). COX-1 is present in all cells and homeostasis is maintained in normal cells. However, COX-2 is involved in the synthesis of prostaglandins in acute inflammatory response, and COX-2 occurrence is induced by LPS and cytokines (Rim et al., 2012). This was confirmed by COX-2 generation induced using LPS in RAW 264.7 cells and treatment with *Chrysanthemum* extracts at 50 μg/mL, followed by immunoblotting for inhibition of COX-2 generation. However, *Chrysanthemum* extracts in the COX-2 group treated with concentration of 100 μg/mL showed significant ($p < 0.05$) increased in protein expressions due to LPS, compared with control. Moreover, COX-2 expression was significantly ($p < 0.05$) inhibited compared with the control group.

There were four taxa of *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *yezoense*, *C. zawadskii* ssp. *latilobum*, and *C. indicum* var. *acuta* extracts, which were selected based on the inhibitory effects against inflammation. The taxa with effective anti-inflammatory activities were detected mostly with four flavonoids such as luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, and acacetin-7-*O*-rutinoside. The selected taxa can be used as functional materials and as traditional herbs due to these beneficial properties. Finally, this study provided useful information for the effective selection of *Chrysanthemum* for their application in functional herbs and nutraceuticals.

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CHAPTER IV

Taxonomic Relationships of Korean *Chrysanthemum* based on Volatile Flavor Compounds

ABSTRACT

In the analysis of chemotype relationship among Korean *Chrysanthemum*, the volatile flavor compounds in the leaves of 15 taxa were analyzed and identified by gas chromatograph/mass spectrometry. Principal Component Analysis (PCA) and cluster analysis were used for the grouping based on the volatile flavor compounds. Fifteen *Charysanthemum* taxa were categorized into three groups. Groups I and II included higher ketones than Group III. Group I had five *C. zawadskii* subspecies: *acutilobum, acutilobum* var. *tenuisectum*, *acutilobum* var. *alpinum, lucidum,* and *coreanum*. Five *C. zawadskii* subspecies were analyzed with main volatile compounds of D-limonene and *m*-thymol. Group II consisted of four *C. zawadskii* subspecies including *naktongense*, *yezoense, latilobum,* and *latilobum* var. *leiophyllum,* and one species *C. makinoi*. They consisted of main compounds of linalool, *cis*-chrysanthenol, eugenol, and chrysanthenone. Group III included five *C. indicum* species and one species: *C. indicum,* var. *albescens,* var. *acuta, C. boreale,* and *C. lineare*. This study was

able to classify volatile flavor compounds of Korean *Chrysanthemum* attributable to major compounds, such as hydrocarbons (sabinene, cymene, β-selinene), alcohols (1-octen-3-ol, cischrysanthenol, hinesol), ketones (chrysanthenone, camphor), and esters (*cis*-sabiene hydrate, *trans*-chrysanthenyl acetate).

Keywords: Borneol, Camphor, *Chrysanthemum*, Cluster analysis, Principal component analysis

INTRODUCTION

Terpenoids presented in the volatile flavor compounds where two or more isoprenes are combined, and their main ingredients are monoterpene ($C_{10}H_{16}$) and sesquiterpene ($C_{15}H_{24}$) (Kondjoyan and Berdague, 1996). The volatile flavor compounds of Korean *Chrysanthemum* are composed of terpenoids and their oxygen derivatives (Choi et al., 2006). The main volatile flavor compounds were repored to be monoterpenoid compounds such as 1,8-cineole, α-pinene and camphene, and sesquiterpenoid compounds such as β-caryophyllene, germacrene D (Kim et al., 1998; Hong, 2002).

Previous studies on the classification of Korean *Chrysanthemum* were performed via karyotype analysis of somatic chromosome (Kim, 2003), morphological phylogeny (Oh et al., 1994), isozyme genetic studies (Park and Lee, 2004), and genetic characteristics (Lee and Kim, 2000). Whereas, a few studies on the comparison and classification of various Korean *Chrysanthemum* based on volatile flavor compounds have also been conducted (Hong, 2002; Woo et al., 2008). Regarding studies on the flavor compounds of *Chrysanthemum*, some attempts have been made mainly on the comparisons of flavor compounds according to cultivation conditions (Wang et al., 2008), flavor composition depending on extraction methods (Wang and Yang, 2006), and flavor compounds for developing chrysanthemum tea (Choi et al., 2006).

Recently, plants were studied for numerical taxonomic classification using multivariate data based on the morphological, biochemical, and molecular characteristics (Kim et al., 2014; Sung, 2000). In particular, on the basis of the chemical components like the volatile flavor compounds in plant, multivariate analyses such as Principal Component Analysis (PCA) and cluster analysis have been performed (Chung, 1999; Yun et al., 2002). Cluster analysis is a type of multivariate analysis that divides data with close similarities into groups (clusters) that are meaningful and useful. If meaningful groups are the goal, then the clusters should capture the natural structure of the data. Therefore, as a technique for grouping clusters of similar traits based on the diverse characteristics of certain entities or subjects, cluster analysis can be utilized in situations where there are no clear or known classification criteria (Sung, 2000). However, minor research has been performed on the Korean *Chrysanthemum* such as the classification of species based on volatile flavor compounds.

This study was conducted to obtain clear inter-species classification by using multivariate analysis methods such as PCA and cluster analysis, as well as by analyzing and comparing the volatile compounds of 15 Korean *Chrysanthemum* taxa.

MATERIALS AND METHODS

Plant Materials

Fifteen Korean *Chrysanthemum* taxa were used at Highland Agriculture Research Institute of National Institute of Crop Science, Rural Development Administration in Pyeongchang, Korea, since 2010 (Table IV-1). Leaf samples of four individual plants were randomly selected and stored at -70℃ (deep freezer) and freeze-dried using freezing dryer (EDT-12012, Operon Co., Gimpo, Korea) in July 2011.

Extraction of Volatile Flavor Compounds

To extract the volatile flavor compounds, Likens and Nickerson type simultaneous steam distillation and extraction (SDE) apparatus with round flask at each end was used following the method of Schultz et al. (1977). In one of the two round flasks, 2 g freeze-dried samples were added with 500 mL distilled water and heat-refluxed in a 100℃ heating mantle for 2 h. Subsequently, 50 mL n-pentane:diethyl ether mixture (1:1= v:v) was added to the other round flask, and the volatile flavor compounds were extracted by heat-refluxing at 40℃. Anhydrous sodium sulfate was used for dehydration and after concentration using 99.9% nitrogen gas by filtering through filter paper (No. 1, Whatman International, Maidstone, UK), gas chromatograph/mass

spectrophotometer (GC/MS) was used for analysis by dissolving in 0.2 mL diethyl ether.

Analysis of Volatile Flavor Compounds

The extracted volatile compounds were analyzed using GC/MS (7890A, Agilent Co., Nilmington, DE, USA) with a HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness) via split mode (split ratio = 50:1) and identified via gas chromatograph/mass selective detector (GC/MSD) (Table IV-2). In the GC analysis, the inlet and detector temperatures were maintained at 250℃, and the helium carrier gas flow rate was kept at 1.0 mL/min. The oven temperature was kept at 50℃ for 5 min, raised the temperature by 7℃ per min, and maintained at 250℃ for 30 min. In the GC/MSD conditions, the electron ionization energy was 70 eV, the ion source temperature was 250℃ and the mass range was 20-400 a.m.u. Regarding verification of the compounds, tentative identification was made by comparing the mass spectrum obtained using GC/MSD with Wiley 70 database (Wiley 70, Agilent Co., Palto Alto, CA, USA) and mass spectral data from the literature (Kondjoyan and Berdague, 1996). Alkane (Alkane Standard, Aldrich Chemical Co., Milwaukee, WI, USA) of C_5-C_{20} was used as the volatile compound reference material and expressed as percentage (%) of relative peak area.

Statistical Analysis

Based on the results of volatile compound analysis using GC/MSD of the volatile compounds in 15 taxa, ANOVA and multivariate analyses were performed using SAS program (SAS ver. 9.2, SAS institute, Cary, NC, USA). The PCA of multivariate analysis methods was conducted to detect the differences among the 15 taxa with simultaneous consideration of the 45 volatile compounds using proc factor with SAS software. The first three principal components were analyzed through PCA and determined with the two-dimensional plot. Cluster analysis was performed using the volatile compounds of the taxa based on quantitative and qualitative variations, including Ward's minimum variance clustering method displayed dendrogram, the cubic clustering criterion and the *Pseudo* option that displayed *Pseudo F* and *Pseudo* T^2 statistics. The number of groups in cluster analysis was decided based on the cluster history.

Table IV-1. List of taxa used for volatile flavor compounds with natural habitats of Korean *Chrysanthemum*.

Scientific name	Cites (Natural habitats)
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa	Mt. Yumyeong, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *tenuisectum* (Kitagawa) Y. Lee	Pocheon, Gyeonggi, Korea
C. zawadskii Herbich ssp. *acutilobum* (DC.) Kitagawa var. *alpinum* (Nak.) Y. Lee	Mt. Baekdu, Hambuk, Korea
C. zawadskii Herbich ssp. *lucidum* (NAK.) Y .Lee	Ulleung, Gyeongbuk, Korea
C. zawadskii Herbich ssp. *coreanum* (NAK.) Y. Lee	Mt. Halla, Jeju, Korea
C. zawadskii Herbich ssp. *naktongense* (NAK.) Y. Lee	Gimhae, Gyeongnam, Korea
C. zawadskii Herbich ssp. *yezoense* (Maekawa.) Y. Lee	Goheung, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa	Wando, Jeonnam, Korea
C. zawadskii Herbich ssp. *latilobum* (Maxim.) Kitagawa var. *leiophyllum* (Nak.) Y. Lee	Gangneung, Gangwon, Korea
C. indicum Linné	Anmyeondo, Chungnam, Korea
C. indicum Linné var. *albescens* Makino	Jeongseon, Gangwon, Korea
C. indicum var. *acuta* (Uyeki) Kitam.	Byeonsanbando, Jeonnam, Korea
C. boreale (Mak.) Makino	Pyeongchang, Gangwon, Korea
C. lineare Matsumura	Mt. Chilbo, Gyeonggi, Korea
C. makinoi Matsumura et Nakai	Daegu, Korea

[z]KMRH: Korea Medicinal Resource Herbarium at Rural Development Administration in Korea.

Table IV-2. Analytical condition of GC-MS for the analysis of volatile flavor compounds in Korean *Chrysanthemum*.

Instrument	Gas chromatography/mass spectroscopy
Model	Agilent 7890A model (Agilent Technologies 7890A GC System, Wilmington, DE, USA)
Column	HP-5MS (30 m x 0.25 mm, film thickness: 0.25 μm)
Detector	Triple-Axis detector Temperature: 250℃
Carrier gas	He (1 mL/min) Initial temperature: 50℃ Initial time: 5 min
Oven temp.	Rate : 7℃/min Final temperature: 250℃ Final time: 30℃/min
Injection	Port Temperature: 250℃ Injection volume: 1 μl (split ratio 50:1)
Electron voltage	70 eV
Temperature priming	5 min 7℃/min 30min initial 50℃ → 50℃ → 250℃ → 250℃
Data	Wiley 70 database system

RESULTS

Volatile Flavor Compounds in Korean *Chrysanthemum*

Identification of volatile flavor compounds in 15 *Chrysanthemum* taxa was accomplished by comparing the mass spectra of their components with the Wiley database, and their GC retention time with GC/MSD spectral data.

Comparison of the flavor compounds in 15 taxa was shown in Table IV-3. The GC chromatogram showed 45 volatile flavor compounds from 12 to 32 volatile flavors each depending on taxa. To investigate the differences and range in flavor compounds of 15 taxa, the flavor compounds within the six functional categories were analyzed and shown in Table IV-4.The six flavor compounds, including sabinene, *cis*-chrysanthenol, borneol, *m*-thymol, chrysanthenone, and camphor showed the most significant differences ($p < 0.001$) among the taxa (Table IV-4). Sabinene was detected at significant amount only in *C. zawadskii* subspecies and C. makinoi. *cis*-Chrysanthenol was detected only in four taxa of *C. zawadskii* subspecies such as ssp. *naktongense*, *yezoense*, *latilobum*, and *latilobum* var. *leiophyllum*. Significant differences ($p < 0.01$) in γ-terpinene, 1-octen-3-ol, linalool, α-terpineol, hinesol, and *trans*-chrysanthenyl acetate were found among the taxa. In particular, γ-terpinene, 1-octen-3-ol and α-terpineol were detected only in *C. zawadskii* subspecies and *C. makinoi*, while hinesol was detected only in *C. indicum* subspecies and *C. boreale*.

Table IV-3. Components of the flavor compounds in Korean *Chrysanthemum*.

Volatile flavor compounds	Peak area (%) of volatile flavor compounds in *Chrysanthemum* species[z]														
	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O
Alcohols															
1-Octen-3-ol	0.20	0.07	0.30	0.09	0.39	0.58	0.06	0.38	0.25						0.37
1-Decanol														1.84	
Linalool						0.36	0.07	0.17	0.76						0.44
cis-Chrysanthenol						9.24	13.98	14.81	14.13						9.41
Borneol	11.32	12.48	18.52	20.94	18.96	1.10	0.91	0.66	0.62	5.40	18.29	2.86	3.27	1.70	1.82
Epoxylinalol															0.03
α-Terpineol	1.49	0.86	1.08	0.36	1.13	0.86	0.28	0.52	0.66						1.53
Myrtenol	1.05	0.57	1.72	0.32	0.39	1.24	1.34	0.81	0.60	1.68	0.54	2.85	0.51		0.23
Piperitol										0.15	0.14	4.12	12.39		
m-Thymol	2.85	2.98	1.97	4.26	3.39										
Eugenol						1.31	1.80	0.06	0.17						
Hinesol										4.79	2.95	6.73	3.49		
α-Cadinol														2.72	
Phytol	7.36	5.86	10.84	9.92	7.03	12.72	7.37	11.12	9.02	12.21	21.08	4.05	21.62	13.48	11.12
Ketones															
Chrysanthenone						1.15	1.02	0.25	1.99						0.88
Camphor	28.61	25.67	19.61	18.01	10.07	29.77	24.88	30.79	33.22	6.44	8.39	8.91	18.25	13.11	24.88
Pinocarvone	1.07	0.04	0.83	0.02	0.16	0.30	0.29	0.62	0.91	0.99		0.49	0.99		
Thyme camphor	1.22	2.98	1.16	0.26	0.40										
Hydrocarbons															
2,4-Dimethyl-heptene	0.04	0.02	0.10	0.12	0.53	0.03	0.57	0.34	0.32	0.07	0.20		0.04	0.22	0.21
α-Thujene	0.15	0.08	0.14			0.04	0.13	0.03	0.10						
α-Pinene	1.27	0.58	0.25	0.86	0.36	0.44	0.80	1.33	0.59	0.01	1.19	0.45	0.21	0.79	0.99
Camphene	2.04	1.53	1.28	1.23	0.12	0.22	0.10	0.06	0.18	0.17	3.57	0.28	0.98	0.42	0.39
Sabinene	0.38	0.07	0.11	0.06	0.19	0.70	0.65	0.56	0.28						0.23
β-Pinene	0.95	0.17	0.12	0.27	0.10	0.36	1.41	0.07	0.05		0.25	0.08	0.06		0.95
α-Terpinene	0.02	0.26	0.19	1.30	0.99	0.26	0.08	0.25	0.20						
p-Cymene	0.93	1.22	0.09	0.08	1.36	0.99	1.10	1.01	0.26	0.02	0.17	0.13	0.37		1.28
Limonene	0.22	0.12	0.09	0.06	0.97										0.23
γ-Terpinene	0.43	0.61	0.84	0.36	1.21	1.01	0.54	0.53	0.11						0.22
α-Thujone										0.05	0.28	0.08	0.27		
trans-Caryophyllene	0.47	0.84	1.27	1.23	1.15	0.32	0.64	0.60	0.29	2.62	0.21	2.39	0.63		1.17
β-Selinene	0.35	0.19	1.38		0.22	0.60	1.79	1.24	0.96						
Germacrene-D	0.25	0.64	1.16	0.92	1.89	3.71	1.74	0.97	1.11	2.67	2.96	0.18	3.17	1.89	1.01
α-Muurolene										0.29	2.65	3.80	0.15		
δ-Cadinene	0.09	0.66	1.66	8.03	2.56	7.31	5.38	1.88	1.49	1.06	2.59	2.07	0.95		1.39
α-Longipinene				3.80											
Caryophyllene oxide	1.11	3.69	2.23	2.37	1.94	1.51	2.75	1.35	3.87	2.34	3.65	3.90	1.67		
γ-Gurjunene	0.68	1.72	2.84	0.65	1.85	0.66	0.68	0.92	1.40	2.18	1.15	2.09	0.65		
Esters															
1,8-Cineole	1.27	0.73	1.13	0.74	1.06	1.03	0.26	0.91	0.29	0.02	0.55	3.50	0.26	0.20	0.05
trans-Sabinene hydrate	6.97	6.20	1.88	0.36	0.40	2.70	0.49	0.37	0.04						
cis-Sabiene hydrate	1.85	2.21	0.18	0.42	1.48	2.91	0.84	3.37	2.27						
trans-Chrysanthenyl acetate					0.27	0.39	0.36	0.68	0.30						0.14
Bornyl acetate	1.17	0.40	0.58	2.66	1.00									1.98	
Acids															
Pentadecanoic acid														10.88	
Hexadecanoic acid			0.24	0.23			0.63		0.25	4.95	1.45	0.48	0.64		1.50
Aldehyde															
trans-2-Hexenal	0.07	0.10	0.16	0.02	0.23	0.33	0.16	0.80	0.64	0.01	0.37	0.28	0.25		0.13
No. of total compound	**30**	**30**	**31**	**30**	**30**	**31**	**32**	**31**	**32**	**21**	**21**	**21**	**22**	**12**	**25**

[z]For species name, refer to Table 3.

Table IV-4. Flavor compounds and their range in Korean *Chrysanthemum*.

Functional groups	Volatile flavor compound	R.T.	MW	Minimum Value	Maximum value	Mean±SD	*F* value[y]
Alcohols	1-Octen-3-ol	10.13	128	0.06	0.58	0.27±0.17	7.51**
	1-Decanol	12.10	158	1.77	1.77	1.77±0.00	1.00NS
	Linalool	13.41	154	0.07	0.76	0.36±0.27	9.04**
	cis-Chrysanthenol	13.90	152	9.24	14.81	12.31±2.75	100.47***
	Borneol	15.13	154	0.62	20.94	7.92±7.91	14.15***
	Epoxylinalol	15.26	170	0.03	0.03	0.03±0.00	1.00 NS
	α-Terpineol	15.63	154	0.28	1.53	0.88±0.44	10.10**
	Myrtenol	15.79	152	0.23	2.85	0.99±0.73	0.23NS
	Piperitol	17.14	152	0.14	12.39	4.20±5.78	1.98NS
	m-Thymol	17.74	150	1.97	4.26	3.09±0.84	68.62***
	Eugenol	19.25	164	0.06	1.80	0.84±0.86	3.24NS
	Hinesol	24.51	222	2.95	6.73	4.49±1.68	10.49**
	α-Cadinol	24.52	222	2.62	2.62	2.62±0.00	1.00NS
	Phytol	31.36	296	4.05	21.62	10.95±4.96	2.52NS
Ketones	Chrysanthenone	14.12	150	0.25	1.99	1.06±0.63	14.32***
	Camphor	14.61	152	6.44	33.22	20.01±9.03	13.34***
	Pinocarvone	15.05	150	0.02	1.07	0.56±0.39	0.04NS
	Thyme camphor	17.75	150	0.26	2.98	1.20±1.08	6.18*
Hydro-carbones	2,4-Dimethyl-heptene	5.80	126	0.02	0.57	0.20±0.18	1.51NS
	α-Thujene	8.51	136	0.03	0.15	0.10±0.05	2.85NS
	α-Pinene	8.72	136	0.01	1.33	0.67±0.40	0.66NS
	Camphene	9.22	136	0.06	3.57	0.84±0.98	1.90NS
	Sabinene	9.93	136	0.06	0.70	0.32±0.24	14.23***
	β-Pinene	10.01	136	0.05	1.41	0.37±0.44	1.83NS
	α-Terpinene	13.22	136	0.02	1.30	0.39±0.44	3.72NS
	Cymene	11.46	134	0.02	1.36	0.64±0.52	4.62*
	Limonene	11.51	136	0.06	0.97	0.28±0.34	2.34NS
	γ-Terpinene	12.43	136	0.11	1.21	0.59±0.35	7.77**
	α-Thujone	13.93	152	0.05	0.28	0.17±0.12	5.46*
	trans-Caryophyllene	13.90	204	0.21	2.62	0.99±0.74	0.71NS
	β-Selinene	21.63	204	0.19	1.79	0.84±0.60	4.21*
	Germacrene-D	21.73	204	0.18	3.71	1.61±1.09	1.72NS
	α-Muurolene	21.97	204	0.15	3.80	1.72±1.80	3.15NS
	δ-Cadinene	22.44	204	0.09	8.03	2.65±2.46	0.96NS
	α-Longipinene	19.29	204	3.80	3.80	3.80±0.00	1.00NS
	Caryophyllene oxide	23.59	220	1.11	3.90	2.49±1.00	0.14NS
	γ-Gurjunene	23.67	204	0.65	2.84	1.34±0.73	1.28NS
Esters	1,8-Cineole	11.65	154	0.02	3.50	0.80±0.85	0.41NS
	trans-Sabinene hydrate	12.60	154	0.04	6.97	2.16±2.66	3.59NS
	cis-Sabiene hydrate	13.38	154	0.18	3.37	1.72±1.10	4.87*
	trans-Chrysanthenyl acetate	16.68	194	0.14	0.68	0.36±0.18	11.53**
	Bornyl acetate	17.74	196	0.40	2.66	1.28±0.85	3.31NS
Acids	Pentadecanoic acid	28.73	270	0.31	0.31	0.31±0.00	1.00NS
	Hexadecanoic acid	29.32	256	0.23	4.95	1.15±1.50	1.82NS
Aldehydes	*trans*-2-Hexenal	6.25	98	0.01	0.80	0.25±0.23	2.95NS

[yNS], *, **, ***Non-significant or significant at $p < 0.05$, 0.01, or 0.001, respectively within the same row.

Alcohols

Alcohols were the most abundant flavor compounds (19.7-43.0% of peak area) in eight *Chrysanthemum* taxa, which include three subspecies of *C. zawadskii*, and *C. indicum* subspecies, *C. boreale*, and *C. lineare* (Table IV-3). Although a total of 14 alcohols were detected from all taxa, their contents were different depending on the taxa. Chang and Kim (2009) reported that 12 alcohols were detected in the flower of *C. indicum*, including 1,8-cineole, chrysanthenol, isopinocarveol, borneol, terpinen-4-ol, endoborneol, carveol, eugenol, spathulenol, cedrol, vulgarol B, and α-bisabolol. Choi et al. (2006) reported 11 alcohols in the flower of *C. boreale*, which was more than the number of alcohols detected from the same species used in this study. These different results were mostly due to the plant parts where the flavor compounds were extracted.

All *Chrysanthemum* taxa contained two alcohols (phytol and borneol), which consisted of 32-92% alcohols, depending on the species (Fig. IV-1). Phytol was one of the most abundant alcohol compounds in six taxa, with average of 50% of the total alcohols. In particular, the phytol content in *C. lineare* was 67.6% of total alcohols. Phytol was identified as an antioxidant compound in *Melittis melissophyllum* (Maggi et al., 2010) and *Eriobotrya japonica* (Ham et al., 2012), and it was reported to be having high antimicrobial activity against *Staphylococcus aureus* (Inoue et al., 2005). With high phytol contents, these taxa may potentially be a great source for medicinal terpenic compounds.

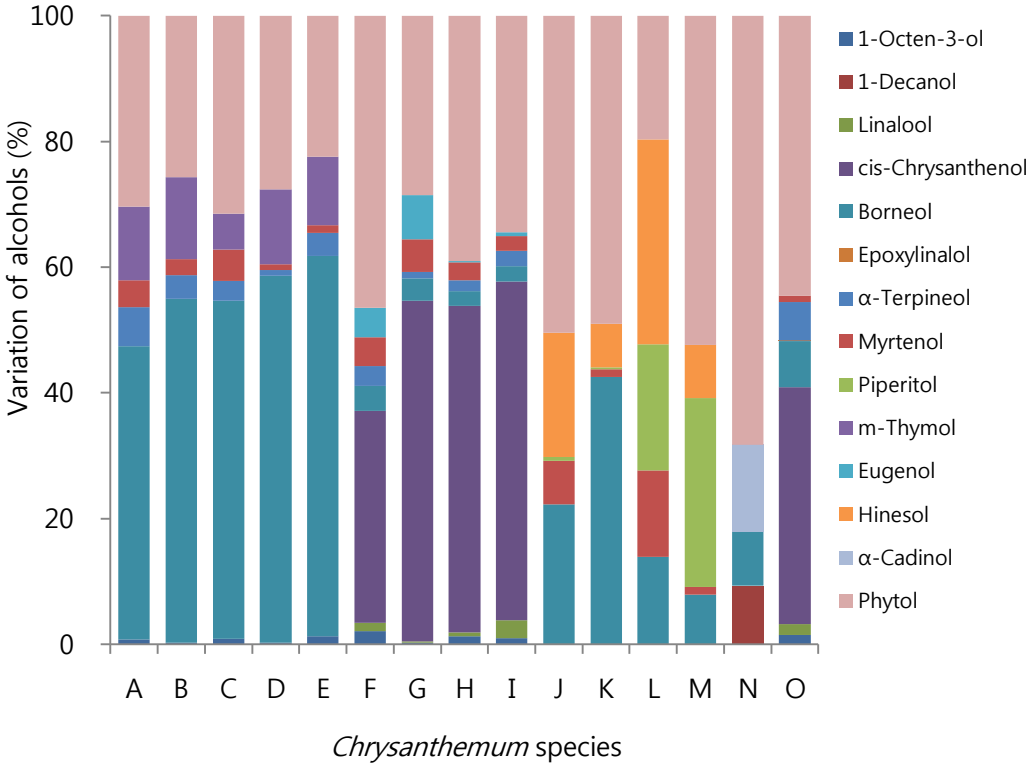

Fig. IV-1. Variation in the alcohols of volatile flavor compounds in 15 Korean *Chrysanthemum* taxa. *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (B), *C. zawadskii* ssp. *acutilobum* var. *alpinum* (C), *C. zawadskii* ssp. *lucidum* (D), *C. zawadskii* ssp. *coreanum* (E), *C. zawadskii* ssp. *naktongense* (F), *C. zawadskii* ssp. *yezoense* (G), *C. zawadskii* ssp. *latilobum* (H), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (I), *C. indicum* (J), *C. indicum* var. *albescens* (K), *C. indicum* var. *acuta* (L), *C. boreale* (M), N; *C. lineare* (N), *C. makinoi* (O).

Borneol was the most abundant alcohol compound in five *C. zawadskii* subspecies (ssp. *acutilobum*, ssp. *acutilobum* var. *tenuisectum*, ssp. *acutilobum* var. *alpinum*, ssp. *lucidum*, and ssp. *coreanum*) and *C. indicum* var. *albescens*, which had more than 11.5% of peak area (Table IV-4). Other four *C. zawadskii* species had less than 1.1% of peak area. In previous studies, Shunying et al. (2005) detected borneol (8.3-18.3% of peak area) from *C. indicum* flower, however this study showed borneol was detected at 5.4% of peak area in the leaves of the same species, likely due to different plant parts. Borneol was reported to have an antimicrobial activity (Shunying et al., 2005).

All nine *C. zawadskii* species and *C. makino* contained 1-octen-3-ol. Interestingly, *m*-thymol was only detected in the five *C. zawadskii* subspecies with high borneol contents, whereas eugenol was only detected in the other four *C. zawadskii* subspecies.

In this study, large quantities of *m*-thymol were also seen in *C. zawadskii* ssp. *lucidum* (4.3% of peak area) and *C. zawadskii* ssp. *coreanum* (3.4% of peak area), indicating potential as an ingredient for development of bactericidal drugs. Moreover, *m*-thymol has been reported to have similar medicinal odor as phenol, with antiseptic and disinfectant qualities (Pirbalouti et al., 2013).

Linalool and *cis*-chrysanthenol were also detected from the four *C. zawadskii* subspecies and *C. makinoi* which contained low borneol. Linalool was often used as a raw incense in cosmetics (Arctander, 1969), and was reported to have strong inhibitory effect against 17 bacteria and 10 fungi (Pattnaik et al., 1997).

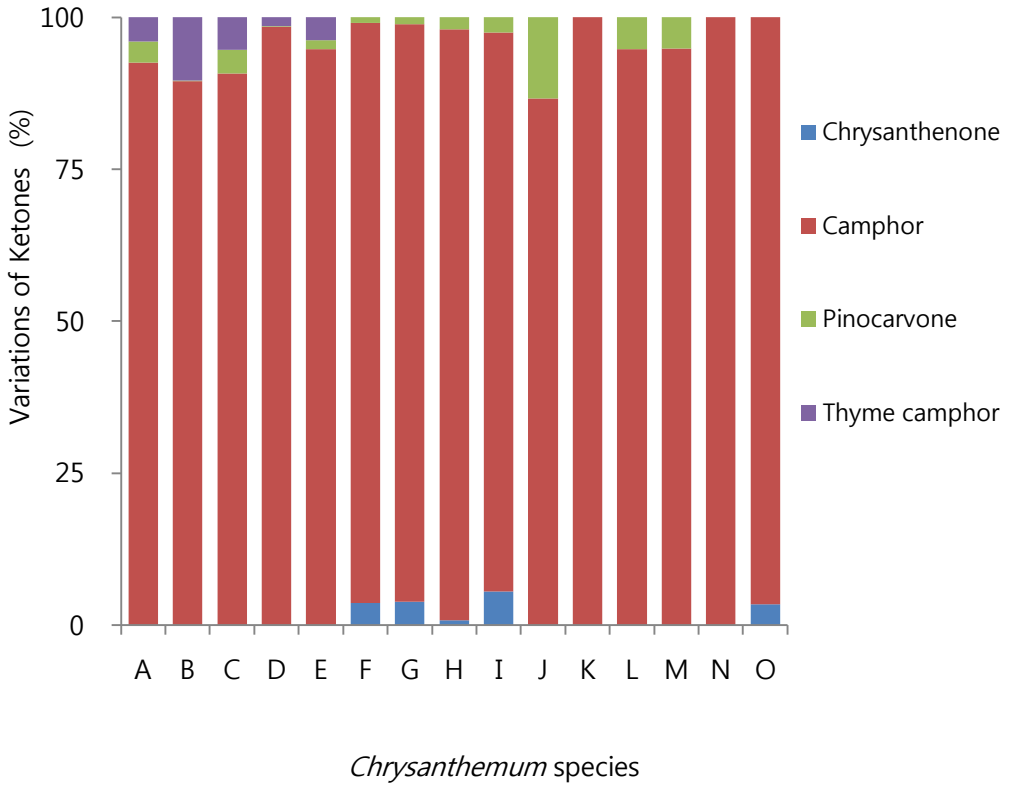

Fig. IV-2. Variation in the ketones of volatile flavor compounds in 15 Korean *Chrysanthemum* taxa. *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (B), *C. zawadskii* ssp. *acutilobum* var. *alpinum* (C), *C. zawadskii* ssp. *lucidum* (D), *C. zawadskii* ssp. *coreanum* (E), *C. zawadskii* ssp. *naktongense* (F), *C. zawadskii* ssp. *yezoense* (G), *C. zawadskii* ssp. *latilobum* (H), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (I), *C. indicum* (J), *C. indicum* var. *albescens* (K), *C. indicum* var. *acuta* (L), *C. boreale* (M), N; *C. lineare* (N), *C. makinoi* (O).

The *cis*-chrysanthenol was not common compound in some *C. zawadskii* and *C. indicum* species (Hong, 2002; Woo et al., 2008). It has only been reported that *C. indicum* flowers (Jung, 2009) had minor compound of *cis*-chrysanthenol, and aerial part from *C. boreale* had *cis*-chrysanthenol as a major compound (Hong, 2002). From the results of linalool and *cis*-chrysanthenol contents, there were two different subgroups within *C. zawadskii* subspecies with different compositions of alcohols.

Piperitol and hinesol were only detected in three *C. indicum* subspecies and *C. boreale*. These two compounds might be good flavor compounds to identify *C. indicum* or *C. boreale* from other species. While 1-decanol and α-cadinol were detected only in *C. lineare*, indicating very specific flavor compounds for *C. lineare*.

Ketones

Ketones were the most abundant volatile flavor compounds in seven *Chrysanthemum* taxa (13.1-36.1% of peak area), including six *C. zawadskii* subspecies, and *C. makinoi* (Table IV-4). Four ketones in *Chrysanthemum* were camphor, pinocarvone, thyme camphor, and chrysanthenone. Camphor was the most common and the most abundant (86.7-100.0% of total ketones) in these species (Fig. IV-2).

Camphor was detected in all 15 taxa investigated. It was the most abundant compound in most species. In particular, *C. zawadskii* ssp. *latilobum* and ssp. *latilobum* var. *leiophyllum* had high camphor contents with over 30% of peak area (Table IV-4). Camphor has been reported as an important substance for medicinal purposes (Arctander, 1969); it could alleviate itchiness, suppress cough or food poisoning bacteria (Jang et al., 2010), mostly due to its antimicrobial properties (Tzakou et al., 2001; Viljoen et al., 2003). Previously, several researchers (Chang and Kim, 2008) reported that the most predominant compounds of *Chrysanthemum* were camphor, although there were some variations in *Chrysanthemum* (Chang and Kim, 2008; Huang et al., 2001). These results correlate with the findings of previous research that *Chrysanthemum* contains high amounts of camphor.

Pinocarvone was also commonly detected in most *Chrysanthemum* except for three taxa: *C. indicum* var. *albescens*, *C. lineare*, and *C. makinoi*. Pinocarvone, a similar compound to *iso*-pinocamphone, was reported to be contained in a large amount in *Artemisia iwayomogi* (Choe et al., 2004).

Interestingly, thyme camphor was detected only in five *C. zawadskii* subspecies with *m*-thymol (also had high borneol contents), while chrysanthenone was detected only in four *C. zawadskii* subspecies and *C. makinoi*, which had linalool and *cis*-chrysanthenol. As previously discussed, nine *C. zawadskii* subspecies could be divided into two groups not only by its alcohols, but also by ketones.

Formerly, Chang and Kim (2009) identified six ketones (15.3% of peak area) in *C. indicum* flower, which included camphor, filifolone, chrysanthenone, menthone, pinocarvone, and carvone.

Initial researches revealed that chrysanthenone was not detected in *Artemisia asictica* or *Matricaria camomilla* in the same Asteraceae, and was detected only in *C. boreale* (Chang and Kim, 2009; Choi et al., 2006). However, this study revealed opposite results, suggesting that its content depend on the plant organs such as leaf, flower or aerial part. Therefore, further research with plant part specific comparative studies is needed.

Hydrocarbons

Hydrocarbons are the third most important functional groups in *Chrysanthemum*, which consisted of an average 13.2% of peak area (Table IV-4). Among 19 hydrocarbons, α-pinene, camphene, and germacrene-D were the only three compounds that were detected from all species (Fig. IV-3).

Interestingly, α-thujene was detected only in four *C. zawadskii* subspecies such as ssp. *naktongense*, ssp. *yezoense*, ssp. *latilobum*, and ssp. *latilobum* var. *leiophyllum*, indicating specific flavor compounds in *Chrysanthemum*. Jang et al. (2010) reported that α-thujone had outstanding anti-bacterial, anti-cancer, anti-inflammatory, anti-ulcer, and anti-diabetic efficacies, and *C. zawadskii* subspecies may serve potential ingredient for medicinal products.

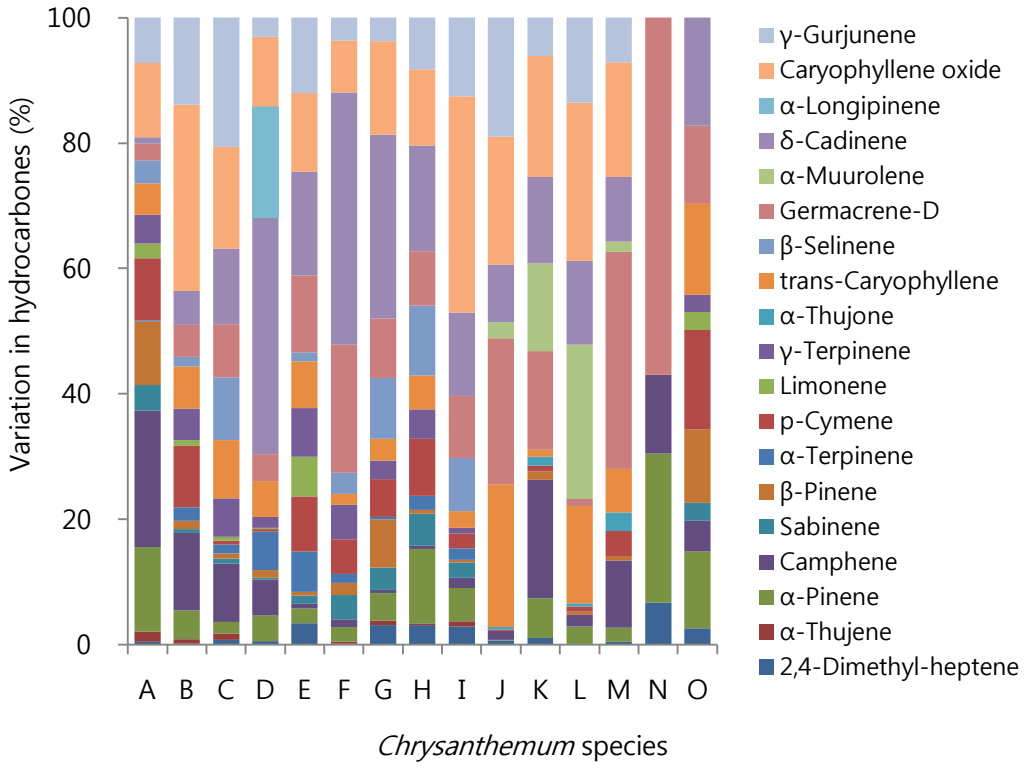

Fig. IV-3. Variation in the hydrocarbons of volatile flavor compounds in 15 Korean *Chrysanthemum* taxa. *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (B), *C. zawadskii* ssp. *acutilobum* var. *alpinum* (C), *C. zawadskii* ssp. *lucidum* (D), *C. zawadskii* ssp. *coreanum* (E), *C. zawadskii* ssp. *naktongense* (F), *C. zawadskii* ssp. *yezoense* (G), *C. zawadskii* ssp. *latilobum* (H), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (I), *C. indicum* (J), *C. indicum* var. *albescens* (K), *C. indicum* var. *acuta* (L), *C. boreale* (M), N; *C. lineare* (N), *C. makinoi* (O).

Amount of α-pinene content varied from 0.01% in *C. indicum* to 1.27% in *C. zawadskii* ssp. *acutilobum*, with an average of 0.7% of peak area (Table IV-3). Choi et al. (2006) noted that small amount of α-pinene was detected in *C. boreale*, which was consistent with this study (0.2% of peak area). The α-pinene was often used as a perfume ingredient (Arctander, 1969) and it was known to have anti-inflammatory effects (Jang et al., 2010). Since *C. zawadskii* ssp. *acutilobum*, *C. zawadskii* ssp. *latilobum* and *C. indicum* var. *albescens* were rich in α-pinene (peak area > 1%), they could be used as an ingredient for anti-inflammatory medicines. Although α-pinene content of *C. lineare* was the highest (23.8%), it only had four hydrocarbons since the percentage of total hydrocarbons of the species was relatively low (0.79% of peak area).

Camphene was detected in all taxa, even though its content is also dependent on taxa. In particular, *C. zawadskii* ssp. *acutilobum* and *C. indicum* var. *albescens* contained high camphene content (2.04 and 3.57% of peak area), and consisted more than 18% of hydrocarbons. Woo et al. (2008) reported that camphene content of *C. zawadskii* was five times higher than the quantity in *C. indicum*. However, this study showed that *C. indicum* var. *albescens* contained high camphene (3.57% of peak area), which was 21 folds of the amount detected in *C. indicum*. The *C. zawadskii* ssp. *latilobum* appeared to contain rather small amount of camphene (0.06% of peak area), indicating that the camphene contents of *C. zawadskii* were also dependent on the subspecies. These results were different from the previous report of Woo et al. (2008), and

this is likely due to the unclear distinction within *C. zawadskii* subspecies.

Furthermore, *trans*-Caryophyllene was detected in all taxa, except in *C. lineare*. This compound had been used in spices (Yeon et al., 2012) and detected in *Artemisia*, *Caryopteris*, *Cinnamonum*, *Citurs*, *Eucalyptus*, *Lavandula*, *Melissa*, *Menthe*, *Pinus*, *Salvia*, *Thymus*, and *Erigeron* (Yu et al., 2008).

Germacrene-D detected in all taxa was investigated. Its contents ranged from 0.18% in *C. indicum* var. *acuta* to 3.71% of peak area in *C. zawadskii* ssp. *naktongense*. Germacrene is a volatile hydrocarbon that exists in many plants and it was known to exist as five isomers (A, B, C, D, and E). Germacrene-D was found to be an initial substance from the biosynthesis of sesquiterpene derivative (Ahn et al., 2002), and mainly known as a compound with high antibacterial and antioxidant effects (Rivero-Cruz et al., 2006). Woo et al. (2008) reported that *C. zawadskii* species contained higher germacrene-D than *C. indicum* species or *C. morifolium*. Hong (2002) also reported that the germacrene-D of *C. boreale* has 2.69% of peak area, and this study also showed the high germacrene-D content in *C. zawadskii* ssp. *naktongense* at 3.71% of peak area. Within nine *C. zawadskii* subspecies, germacrene-D contents also varied (0.25-3.71% of peak area) depending on the subspecies. Among three *C. indicum* subspecies, two species had high content of germacrene-D (2.67% and 2.96% of peak area for *C. indicum* and *C. indicum* var. *albescens*, respectively), and *C. indicum* var. *acuta* had the lowest germacrene-D content at 0.18% of

peak area.

In this study, cadinene was detected in 14 taxa except in *C. lineare*. Among all the hydrocarbons, δ-cadinene content was the highest (16.6-40.3% of total hydrocarbons) in six taxa, including *C. zawadskii* ssp. *lucidum*, ssp. *coreanum*, ssp. *naktongense*, ssp. *yezoense*, ssp. *latilobum*, and *C. makinoi*. This compound was reported to display bioactivities such as insecticidal, antipyretic, anti-inflammatory, anti-bacterial, and anti-cancer (Lee et al., 2012).

Caryophyllene oxide content was the highest among hydrocarbons in four taxa, including *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (29.8%), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (34.5%), *C. indicum* var. *albescens* (19.3%), and *C. indicum* var. *acuta* (25.2%) of total hydrocarbons. The *C. indicum* had particularly high *trans*-caryophyllene (22.8%) as well.

Esters

Although esters were relatively few (an average of 3.8% of peak area) compared with other functional groups such as alcohols, ketones, and hydrocarbons, these were detected in all 15 taxa (Table IV-4 and Fig. IV-4).

Although 1,8-cineole was commonly detected in all taxa, it was relatively a small amount among esters. Previous research indicated that 1,8-cineole was found in *C. boreale* and *C. indicum*, but not in *Matricaria recutita*, which was another genus in Asteraceae, suggesting it as an index compound for the species classification of *Chrysanthemum* (Chang and Kim, 2008; Choi et al., 2006).

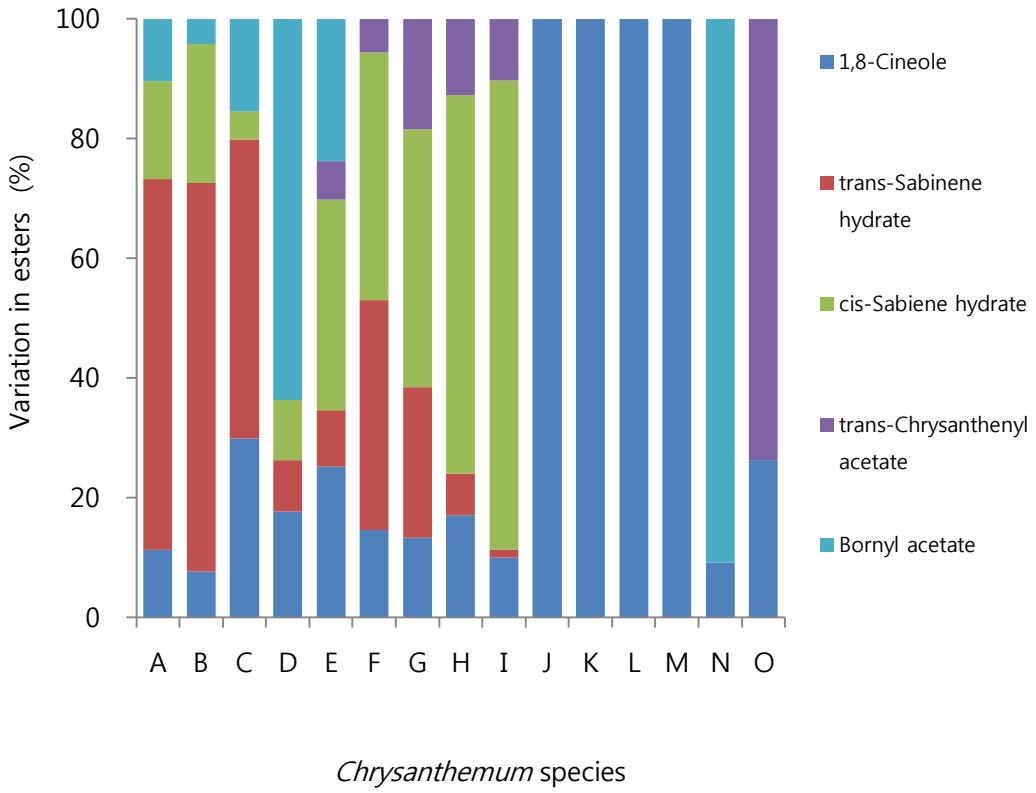

Fig. IV-4. Variation in the esters of volatile flavor compounds in 15 Korean *Chrysanthemum* taxa. *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (B), *C. zawadskii* ssp. *acutilobum* var. *alpinum* (C), *C. zawadskii* ssp. *lucidum* (D), *C. zawadskii* ssp. *coreanum* (E), *C. zawadskii* ssp. *naktongense* (F), *C. zawadskii* ssp. *yezoense* (G), *C. zawadskii* ssp. *latilobum* (H), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (I), *C. indicum* (J), *C. indicum* var. *albescens* (K), *C. indicum* var. *acuta* (L), *C. boreale* (M), N; *C. lineare* (N), *C. makinoi* (O).

In particular, *C. indicum* var. *acuta* had the highest 1,8-cineole content at 3.50% of peak area. While 1,8-cineole has been reported to have a suppressive effect on occurrences of mutations (Kim et al., 1992) with the recognition of actual chemical treatment effect on breast cancer in mice (Kubo et al., 1992), it was suggested for a possible potential ingredient for medicinal use.

Moreover, *trans*-Sabinene hydrate and *cis*-sabiene hydrate were detected only in nine *C. zawadskii* subspecies. Also, *trans*-Chrysanthenyl acetate and bornyl acetate were detected in a little amount on *C. zawadskii* subspecies. The *C. lineare* contained bornyl acetate at 2.0% of peak area.

Esters synthesized by short-chain acids and alcohols of a large group of flavor and fragrance compounds which are used extensively in food, cosmetic, beverage, and pharmaceutical industries (Shu et al., 2011). Although one ester from *C. indicum* was detected in this study, four esters in the same species were identified as *trans*-sabinene hydrate, *cis*-sabinene hydrate, *trans*-chrysanthenyl acetate, and bornyl acetate (Chang and Kim, 2009).

Acids

Acids were detected in all *C. indicum* subspecies, *C. boreale*, *C. lineare*, and *C. makinoi*, but not in all *C. zawadskii* subspecies. Although acid content was low in most of *Chrysanthemum* (average 2.1% of peak area), *C. indicum* had hexadecanic acid at 5.0% of peak area and *C. lineare* had pentadecanoic acid at 10.9% of peak area (Table IV-4). In particular, only *C. lineare* contained

pentadecanoic acid compound whereas other species contained hexadecanoic acid. This specific compound was very unique to *C. lineare*, and this species had also unique morphological characteristics with different leaf and seed shapes compared to the other 14 *Chrysanthemum* taxa (Kim et al., 2011). Woo et al. (2008) reported that amount of acids detected in *C. indicum*, but it was not detected in *C. zawadskii*. However, results of the study showed that four *C. zawadskii* subspecies contained hexadecanoic acid indicating subspecies specific volatile flavor compound contents.

Aldehyde

Aldehyde was detected in all *Chrysanthemum* except *C. lineare*, and only one type (*trans*-2-hexenal) was identified (Table IV-4). All the *Chrysanthemum* contained very little amount of aldehyde (less than 0.3% of peak area). Choi et al. (2006) also reported that aldehyde content in *Chrysanthemum* was negligible, which was consistent with this study. Chang and Kim (2009) identified five aldehydes from *C. indicum* flowers with major compounds being 2-hexanal, safranal, benzealdehyde, and phenylacetaldehyde. However, this study found only one aldehyde from *C. indicum* leaves, indicating difference in flavor compounds from different part of organs.

Principal component analysis (PCA) of volatile compounds

Volatile compounds were evaluated and coded by each graded value to classify 15 *Chrysanthemum* taxa. Table IV-5 showed the eigenvalues and their contribution through PCA using 45 volatile compounds. Data analyzed with all variables, found the principal component and understood that main component. It showed the eigenvalues of the matrix consisting of component of the variance and convariance parameter. In case of the first Principal component (PC 1), the eigenvalue of each of the characteristics was 10.47, thereby showing 23.28% contribution on the total variation. The second and third PCs had eigenvalues of 6.76 and 5.82, respectively, and the degree of contribution on the total variation was 51.25%. The first 10 PCs in Table IV-5 showed 91.33% cumulative proportion by PCA. Each of the 10 PCs was presented in one characteristic because the eigenvalues were over 1.68, which was more informative than any single variable alone (Iezzoni and Pritts, 1991).

In addition, to estimate the characteristics contained in the various volatile flavor compounds, correlations between the individual PCs were analyzed (Table IV-6). The eigenvalues of each PC were the total number of hypothetic variables analyzed by PCA, indicating that the first, second, and third PCs represented approximately 10, 7, and 6 variables among the 45 volatile flavor compounds of *Chrysanthemum*, respectively.

Table IV-5. Eigenvalues and contributions of the first 10 principal components using 45 volatile compounds found in Korean *Chrysanthemum*.

Principal component	Eigenvalue	Difference	Contribution (%)	Cumulative contribution (%)
PC 1	10.47	3.71	23.28	23.28
PC 2	6.76	0.93	15.03	38.30
PC 3	5.82	2.22	12.95	51.25
PC 4	3.60	0.48	8.00	59.25
PC 5	3.11	0.25	6.92	66.17
PC 6	2.86	0.39	6.36	72.53
PC 7	2.47	0.18	5.50	78.03
PC 8	2.29	0.28	5.10	83.13
PC 9	2.01	0.33	4.47	87.60
PC 10	1.68	0.42	3.73	91.33

Table IV-6. List of three principal components among 45 volatile compounds in Korean *Chrysanthemum*.

Fuctional group	Volatile flavor compounds	Loading of principal component		
		PC 1	PC 2	PC 3
Alcohols	1-Octen-3-ol	**0.232682**	0.031638	0.028810
	1-Decanol	-0.070376	-0.010000	**-0.326521**
	Linalool	0.179901	-0.165416	-0.016956
	cis-Chrysanthenol	**0.246447**	-0.203264	-0.005911
	Borneol	-0.087005	**0.299217**	0.057413
	Epoxylinalol	0.056441	-0.038169	-0.101769
	α-Terpineol	0.188122	0.186852	0.040418
	Myrtenol	-0.057896	-0.082279	**0.319047**
	Piperitol	-0.134099	-0.130623	0.036826
	m-Thymol	0.008844	**0.370277**	0.037989
	Eugenol	0.169627	-0.121044	0.019953
	Hinesol	**-0.232142**	-0.157992	0.177239
	α-Cadinol	-0.070376	-0.010000	**-0.326521**
	Phytol	-0.109881	-0.161784	-0.150459
Ketones	Chrysanthenone	**0.205682**	-0.183751	0.000285
	Camphor	**0.259927**	-0.022520	0.006863
	Pinocarvone	-0.009726	-0.104409	0.191614
	Thyme camphor	0.018116	**0.255284**	0.105630
Hydrocarbones	2,4-Dimethyl-heptene	0.151169	-0.036358	-0.081854
	α-Thujene	0.156667	0.073682	0.133573
	α-Pinene	0.107073	0.033887	-0.131258
	Camphene	-0.112005	0.115246	0.005350
	Sabinene	**0.274976**[a]	-0.077003	0.031517
	β-Pinene	0.148728	0.005741	-0.017851
	α-Terpinene	0.042363	**0.246840**	-0.019599
	Cymene	**0.217660**	0.078302	0.023357
	Limonene	0.050832	**0.208936**	0.009159
	γ-Terpinene	0.186282	0.191088	0.087366
	α-Thujone	-0.187067	-0.147448	0.017357
	trans-Caryophyllene	-0.137563	0.018842	0.232685
	β-Selinene	**0.207477**	-0.071048	0.104086
	Germacrene-D	-0.040842	-0.169127	-0.085989
	α-Muurolene	-0.172210	-0.094069	0.155605
	δ-Cadinene	0.089344	0.032327	0.016232
	α-Longipinene	-0.038363	0.176341	-0.070457
	Caryophyllene oxide	-0.059811	-0.016015	**0.275720**
	γ-Gurjunene	-0.082181	0.068966	0.316431
Esters	1,8-Cineole	-0.057421	0.059248	**0.236369**
	trans-Sabinene hydrate	0.078373	**0.212838**	0.092349
	cis-Sabiene hydrate	**0.234741**	0.024207	0.071986
	trans-Chrysanthenyl acetate	**0.243119**	-0.130490	0.014068
	Bornyl acetate	-0.050397	**0.269687**	-0.209525
Acids	Pentadecanoic acid	-0.070376	-0.010000	**-0.326521**
	Hexadecanoic acid	-0.134942	-0.131552	0.061932
Aldehydes	*trans*-2-Hexenal	0.131332	-0.185657	0.096000

[z]Underlined loading was the trait that had higher correlation with principal component of column.

The first PC showed highly correlated eigenvalue with 10 volatile flavor compounds including sabinene, cymene, β-selinene, 1-octen-3-ol, *cis*-chrysanthenol, hinesol, chrysanthenone, camphor, cis-sabinene hydrate, and *tans*-chrysanthenyl acetate. Among these sabinene showed the highest correlation based on loading of PC at 0.274976 followed by camphor at 0.259927 and *cis*-chrysanthenol at 0.246447. In the case of second PC, *m*-thymol showed the highest correlation based on loading of PC at 0.370277 followed by borneol. The third PC was closely related with alcohols including 1-decanol, pentadecanoic acid, and α-cadinol.

As a result of arranging the values of first and second PCs on two-dimensional scatter diagram for 15 *Chrysanthemum* taxa, these were categorized into three groups (Fig. IV-5). Five *C. zawadskii* subspecies having with main volatile flavor compounds of D-limonene and *m*-thymol were located in the middle part of plot 1 and upper part of plot 2 (Fig. IV-5; Group I). This study corresponded that D-limonene was reported as the main compound in *Ligularia fischeri* var. *spiciformis* and *Ligularia fischeri* var. *spiciformis* in Asteraceae (Lee et al., 2012). However, this compound was neither detected in *Aster scaber* nor in *Synurus deltoid* (Lee et al., 2012). When the genus *Thymus* was analyzed, it was further observed that even though the main compound of *T. quinquecostatus* var. *quinquecostatus* was cymene, while that of *T. quinquecostatus* var. *japonica* was *m*-thymol. These results showed that each species has different chemotypes, making it possible to completely distinguish

the species (Kwon et al., 2006). Thus, the main compounds of *m*-thymol and limonene in 15 taxa corresponded to *Thymus* based on classification results.

In the result of PCA, four *C. zawadskii* subspecies and *C. makinoi*, having main volatile flavor compounds of linalool, *cis*-chrysanthenol, eugenol, and chrysanthenone, were located in the right part of plot 1 and lower part of plot 2 (Fig. IV-5; Group II). Using plants as basic materials in perfumes and cosmetics, among others (Arctander, 1969), linalool was contained in *Matricaria recutita* flower, *C. boreale* flower and *Camellia sinensis* leaf (Choi et al., 2006). It was further determined to be an index compound where taxa could be identified only in Group II. In addition, most compounds in Group II were detected quite similarly to the volatile flavor compounds of Group I.

Five taxa were located in the left part of plot 1 and lower part of plot 2 (Fig. IV-5; Group III). The *C. indicum*, *C. indicum* var. *albescens*, *C. indicum* var. *acuta*, *C. boreale*, and *C. lineare* had mainly α-thujene, α-muurolene, piperitol, and hinesol due to the different volatile compounds of Groups I and II. *C. lineare* contained 1-decanol, α-cadinol, pentadecanoic acid compounds which were detected specifically in Group III, showed different pattern. Even though α-terpineol was identified in Groups I and II, it was determined to be an index compound among taxa, because it was not identified in Group III.

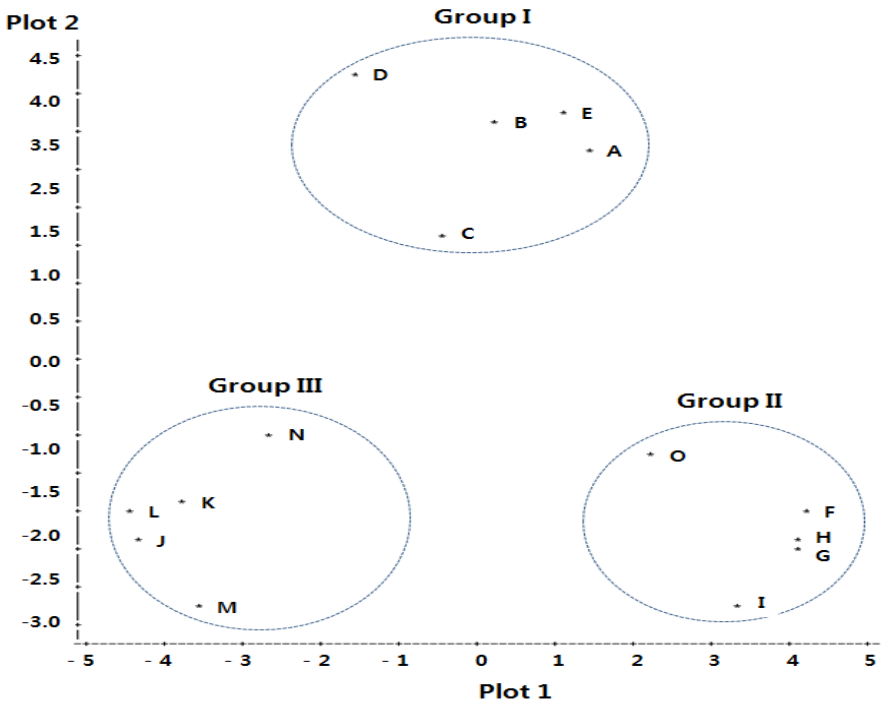

Fig. IV-5. Plot showing the principal component analysis in 15 Korean *Chrysanthemum* taxa. *C. zawadskii* ssp. *acutilobum* (A), *C. zawadskii* ssp. *acutilobum* var. *tenuisectum* (B), *C. zawadskii* ssp. *acutilobum* var. *alpinum* (C), *C. zawadskii* ssp. *lucidum* (D), *C. zawadskii* ssp. *coreanum* (E), *C. zawadskii* ssp. *naktongense* (F), *C. zawadskii* ssp. *yezoense* (G), *C. zawadskii* ssp. *latilobum* (H), *C. zawadskii* ssp. *latilobum* var. *leiophyllum* (I), *C. indicum* (J), *C. indicum* var. *albescens* (K), *C. indicum* var. *acuta* (L), *C. boreale* (M), N; *C. lineare* (N), *C. makinoi* (O).

Cluster analysis of compositions of volatile compounds

Cluster analysis was performed based on 45 volatile compounds and showed the dendrogram of relationships among 15 *Chrysanthemum* taxa (Fig. IV-6). When the volatile compounds were subjected to cluster analysis using average distance of 0.15 semi-partial R square as the criterion, it was possible to classify volatile compounds into three groups based on the cluster history. Group I included *C. zawadskii* ssp. *acutilobum*, ssp. *acutilobum* var. *tenuisectum*, ssp. *acutilobum* var. *alpinum*, ssp. *lucidum*, and ssp. *coreanum*. In the case of *C. zawadskii* ssp. *lucidum*, it was distinguished within Group I due to the presence of α-longipinene. This seemed to be attributed to the unique volatile flavor compounds generated in the leaf, because *C. zawadskii* ssp. *lucidum* grew only in the habitual environment of island in the East Sea of Korea.

Group II included five taxa that were mostly distributed geographically in the southern regions of the Korean Peninsula. These taxa were *C. zawadskii* ssp. *naktongense*, ssp. *yezoense*, ssp. *latilobum*, ssp. *latilobum* var. *leiophyllum*, and *C. makinoi*. However, *C. makinoi* was rather distant in terms of phylogenetic relationship with the other taxa in Group II.

Group III consisted of five taxa of *C. indicum*, var. *albescens*, var. *acuta*, *C. boreale*, and *C. lineare*. On the other hand, *C. lineare* was detected with 1-decanol, α-cadinol, and pentadecanoic acid which had not been seen in other taxa. δ-Cadinene was detected in 14 *Chrysanthemum* taxa except for *C. lineare*.

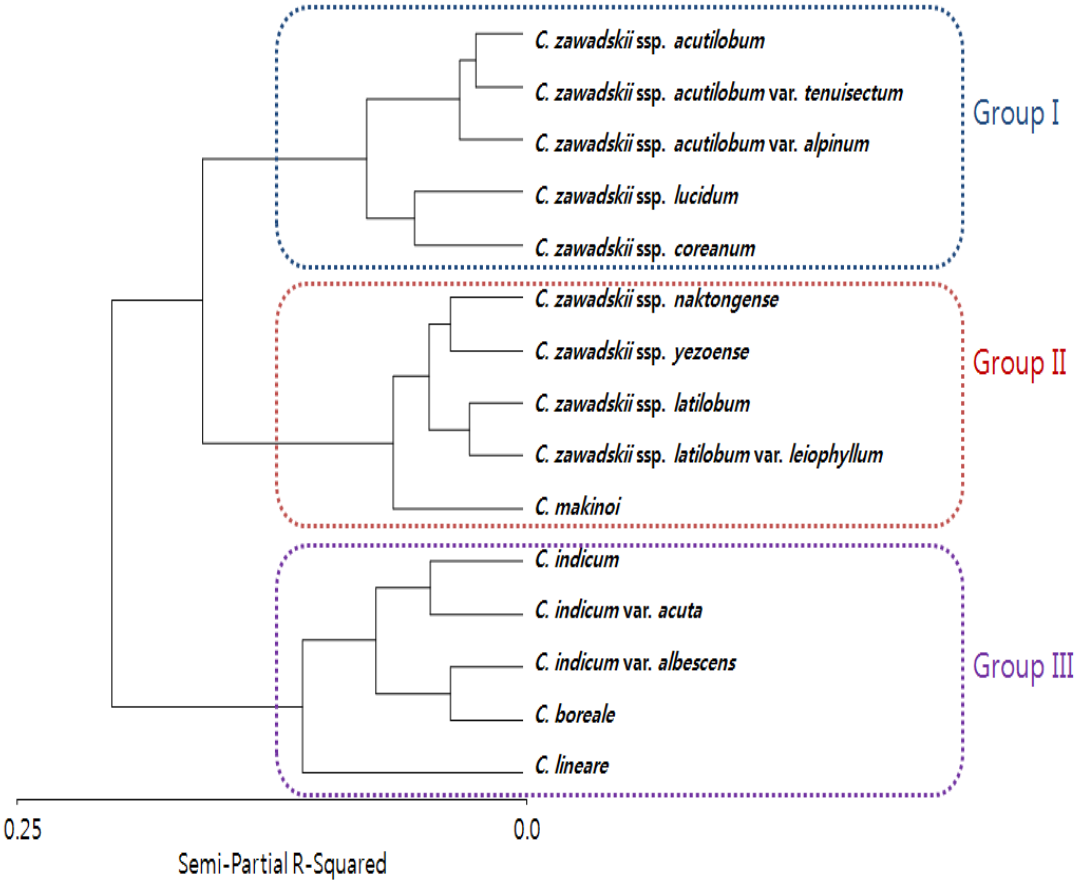

Fig. IV-6. Dendrogram of 15 Korean *Chrysanthemum* taxa classified by Ward's minimum variance clustering method.

Comparison of the inter-group characteristics of *Chrysanthemum* through cluster analysis, functional group of volatile compounds such as hydrocarbons, alcohols, aldehydes, ketones, acids, and esters showed differences (Fig. IV-7). In Group I, the composition of aldehyde was relatively low, whereas the compositions of alcohols and esters were high. In Group II, the composition of ketones was high whereas the compositions of alcohols and acids were relatively low. In Group III, the compositions of alcohols and acids were higher in relation to Groups I and II, whereas the compositions of ketones were lower than Groups I and II. The present study also corresponded to the previous report that mainly hydrocarbons and alcohols contained volatile flavor compounds in *C. boreale* in large quantities (Choi et al., 2006).

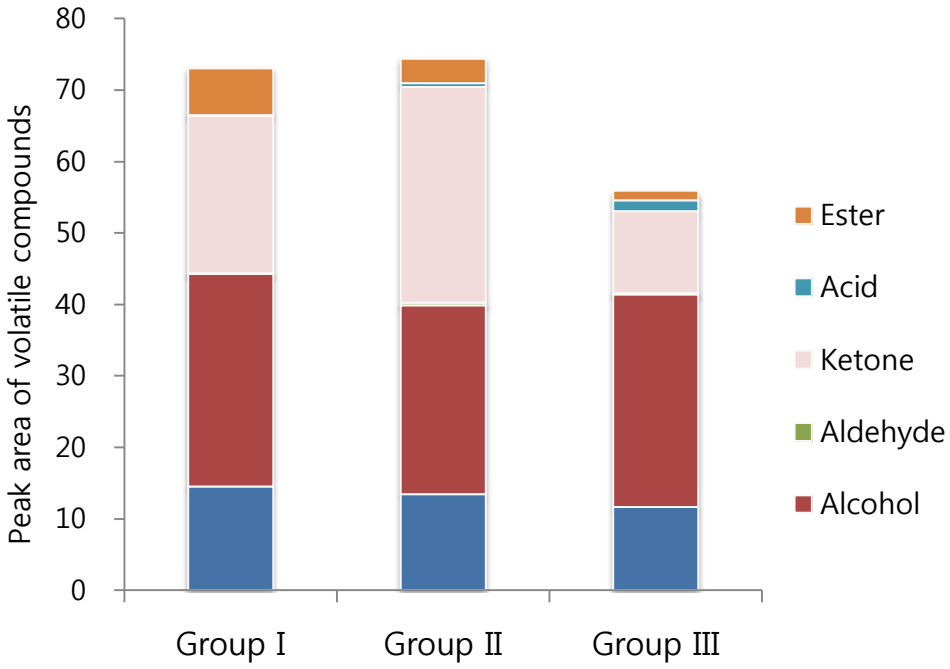

Fig. IV-7. Variation in the functional group of volatile flavor compounds in 15 Korean *Chrysanthemum* taxa classified by cluster analysis.

DISCUSSION

This study was conducted to investigate taxanomic relationship among 15 Korean *Chrysanthemum* taxa by analyzing and comparing specific morphological, molecular, flavonoid, and flavor compounds, which could be used as baseline data for the germplasms and potential ingredient for functional foods, medicines, and cosmetics.

Korean *Chrysanthemum* showed considerable variation in volatile flavor compounds in their leaves, and this study might provide good indication of species specific potential usage for various applications. Most *C. zawadskii* subspecies had the most abundant borneol and camphor, which was reported as volatile flavor compound with antimicrobial properties. In particular, *C. indicum* var. *albescens* has high utility in making perfumes, since it showed camphene content that was 21 times higher than *C. indicum*. Since *C. indicum* var. *albescens* also contains α-pinene and offers anti-inflammatory effect, its value in cosmetics is expected to increase. *C. indicum* var. *acuta* contained fairly high content of 1,8-cineole, which may have great potential as medicinal plant material. *C. lineare* also had unique morphological characteristics from other 14 *Chrysanthemum* taxa.

In this study, volatile compounds of 15 taxa were analyzed with GC/MSD as well as through multivariate analysis techniques of PCA and cluster analysis, thereby confirming the possibility of classifying 15 taxa into three groups. Thus,

it was determined that the chemo-taxonomy technique using the compositions of the volatile flavor compounds of *Chrysanthemum* would be useful in the classification and identification of the verified inter-species differences. Finally, this study provided the basic materials needed for species selection and cultivation of *Chrysanthemum* species, which are useful for food, cosmetics, and medicine.

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CONCLUSIONS

This taxonomic study was focused on identifying 15 Korean *Chrysanthemum* taxa based on the morphological, genetic and chemotaxonomical characteristics.

A total of 35 morphological characteristics of *Chrysanthemum* were considered as identification keys for classification. Variance analysis of morphological characteristics showed that 23 variables were significantly different among the taxa, whereas 12 variables were not. Of the 23 significant variables, 12 variables were highly significant at $p < 0.001$ which included plant type, basal and cauline leaf length, basal and cauline *L/W* ratio, basal and cauline petiole length, flower head diameter, ligulate length, seed length, seed width, and seed color. *Chrysanthemum* was classified into three groups according to the multivariate analysis using qualitative and quantitative morphological characteristics. This the study provided a clear diagnostic key for species-specific classification based on leaf, branch type, plant height, flower size, and flowering type.

Chrysanthemum was diploid, tetraploid, hexaploid, and octoploid with basic number x = 9 in Asteraceae. To date, polyploid (4x, 6x, 8x) in *C. zawadskii*, polyploid (4x, 6x) in *C. indicum*, and diploid (2x) in *C. boreale*, *C. lineare*, and *C. makinoi*, respectively, have been found.

The ITS approach did not demonstrated effectiveness on the phylogenetic analysis. The strict consensus phylogenetic tree supported the taxa organization

in three main clusters. The size of ITS sequences aligned was 663-664 bp, and the lengths of ITS 1 and ITS 2 regions were 268-269 bp and 231 bp, respectively. The chromosome analysis and ITS sequence analysis of *Chrysanthemum* used in this study provided new information on the phylogenetic relationship among taxa. The information gathered will provide useful data for the identification and breeding of *Chrysanthemum* in future.

In this study, flavonoids and anti-inflammatory activities of Korean *Chrysanthemum* were analyzed. Leaf extracts from *Chrysanthemum* had five flavonoids. Four flavonoids such as luteolin-7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, and acacetin-7-*O*-rutinoside were detected in taxa with effective anti-inflammatory activities. Selected *Chrysanthemum* can be used as functional material and as traditional herbs due to these properties.

Chrysanthemum was classified into three groups of volatile flavor compounds based on the cluster history. Functional group compositions of hydrocarbons, alcohols, aldehydes, ketones, acids, and esters showed differences according to each group. Group I included *C. zawadskii* ssp. *acutilobum*, ssp. *acutilobum* var. *tenuisectum*, ssp. *acutilobum* var. *alpinum*, ssp. *lucidum*, and ssp. *coreanum*. In the case of *C. zawadskii* ssp. *lucidum*, it was distinguished within Group I due to the presence of α-longipinene. In Group I, the composition of aldehyde was relatively low, while the compositions of alcohols and esters were high. Group II included five taxa that were mostly

distributed geographically in the southern regions of Korean Peninsula. These taxa were *C. zawadskii* ssp. *naktongense*, ssp. *yezoense*, ssp. *latilobum*, ssp. *latilobum* var. *leiophyllum*, and *C. makinoi*. In Group II, the composition of ketones was high, while the compositions of alcohols and acids were relatively low. Group III consisted of five taxa of *C. indicum*, var. *albescens*, var. *acuta*, *C. boreale*, and *C. lineare*. In Group III, the compositions of alcohols and acids were higher in relation to Groups I or II, whereas the compositions of ketones were lower than Groups I and II.

In summary, to establish the proper classification and usage of Korean *Chrysanthemum*, better identification, understanding of the species, and clear classification and grouping would be needed for leaf morphological characteristics. The number of somatic chromosomes in *Chrysanthemum* was investigated to determine the following numbers of basic chromosomes: 2n = 4x = 36, 6x = 54, and 8x = 72 instead of the normal 2n = 2x = 18, ranging from diploid to octoploid. Overall, each *C. zawadskii* subspecies could not be easily identified based on the ITS sequence. Moreover, genetic relationships among *Chrysanthemum* could not be easily determined based on the comparison of ITS sequences within subspecies and varieties. In the present study, the volatile compounds of 15 taxa were analyzed with GC/MSD as well as through multivariate analysis techniques of PCA and cluster analysis, thereby confirming the possibility of classifying 15 *Chrysanthemum* taxa into three groups. Thus, the chemo-taxonomy technique using the compositions of the

volatile flavor compounds of *Chrysanthemum* would be useful in the classification and identification of the verified inter-species differences. These findings lead us to the understanding of the phylogeny, origin, and classification of *Chrysanthemum*. Finally, this study provided the basic information needed for species selection and cultivation of *Chrysanthemum* species which is highly useful for food, cosmetics, and medicine.

APPENDIX

Table 1. Comparison of various classification systems of Korean *Chrysanthemum*.

Name	Palibin	Nakai	T.B. Lee	Bremer and Humphries	W.-T. Lee	Y.-N. Lee
Year	(1896)	(1911,1918,1921)	(1979,1993)	(1993)	(1996)	(2006)
Species	*C. sibiricum* (산구절초)	*C. sibiricum* var. *acutilobum* (산구절초)	*C. zawadskii* var. *zawadskii* (산구절초)	*D. zawadskii* (산구절초)	*C. zawadskii* var. *zawadskii* (산구절초)	*C. zawadskii* ssp. *acutilobum* (가는잎구절초)
						C. intermedium (이화구절초)
		var. *alpinum* (바위구절초)	var. *alpinum* (바위구절초)	*D. maximowiczii* (바위구절초)	var .*alpinum* (바위구절초)	ssp. *acutilobum* var .*alpinum* (바위구절초)
			var. *latilobum* (구절초)	*D. chanetii* (구절초)	var. *latilobum* (구절초)	ssp. *latilobum* (넓은잎구절초)
		C. leiophyllum (서흥구절초)	var. *leiophyllum* (서흥구절초)			ssp. *latilobum* var. *leiophyllum* (서흥넓은잎구절초)
		C. naktongense (낙동구절초)		*D. naktongense* (낙동구절초)		ssp. *naktongense* (낙동구절초)
			var. *tenuisectum* (포천구절초)		var. *tenuisectum* (포천구절초)	ssp. *acutilobum* var .*tenuisectum* (포천가는잎구절초)
		C. lucidum (울릉국화)	var. *lucidum* (울릉국화)	*D. littorale* (울릉국화)	var. *lucidum* (울릉국화)	ssp. *lucidum* (울릉국화)
	C. coronarium (한라구절초)	*C. coreanum* (한라구절초)		*D. coreana* (한라구절초)		ssp. *coreanum* (한라구절초)
						ssp. *yezoense* (남구절초)
	C. indicum (감국)	*C. indicum* var. *indicum* (감국)		*D. indica* (감국)	*C. indicum* (감국)	*C. indicum* (감국)
					subsp. *albescence* (흰감국)	*C. indicum* var. *albescens* (흰감국)
		var. *procumbens* (가는잎감국)			var .*acutum* (가는잎감국)	
		var. *lavandulaefolium* (산국)	*C. boreale* (산국)	*D. boreale* (산국)	*C. boreale* (산국)	*C. boreale* (산국)
		var. *leucanthum* (신창구절초)		*D. sinchangense* (신창구절초)	*C. sinchangense* (신창구절초)	
	C. sinense (국화)	*C. morifolium* var. *morifolium* (국화)	*C. morifolium* (국화)	*D. grandiflora* (국화)	*C. morifolium* (국화)	*C. morifolium* (국화)
		var. *japonicum* (일본국화)				
		C. coronaries (쑥갓)	*C. coronarium* var. *spatiosum* (쑥갓)	*C. coronarium* (쑥갓)	*C. coronarium* var. *spatiosum* (쑥갓)	*C. coronarium* var. *spatiosum* (쑥갓)
			C. pallasianum (솔인진)	*Ajania pallasiana* (솔인진)	*C. pallasianum* (솔인진)	*C. pallasianum* (솔인진)
				Tanacetum cinerariefolium (제충국)	*C. cinerariefolium* (제충국)	
		C. lineare (키큰산국)	*C. lineare* (키큰산국)	*Leucanthemella linearis* (키큰산국)	*C. lineare* (키큰산국)	*C. lineare* (키큰산국)
						C. makinoi (마키노국화)
Total	4	14	11	14	15	18

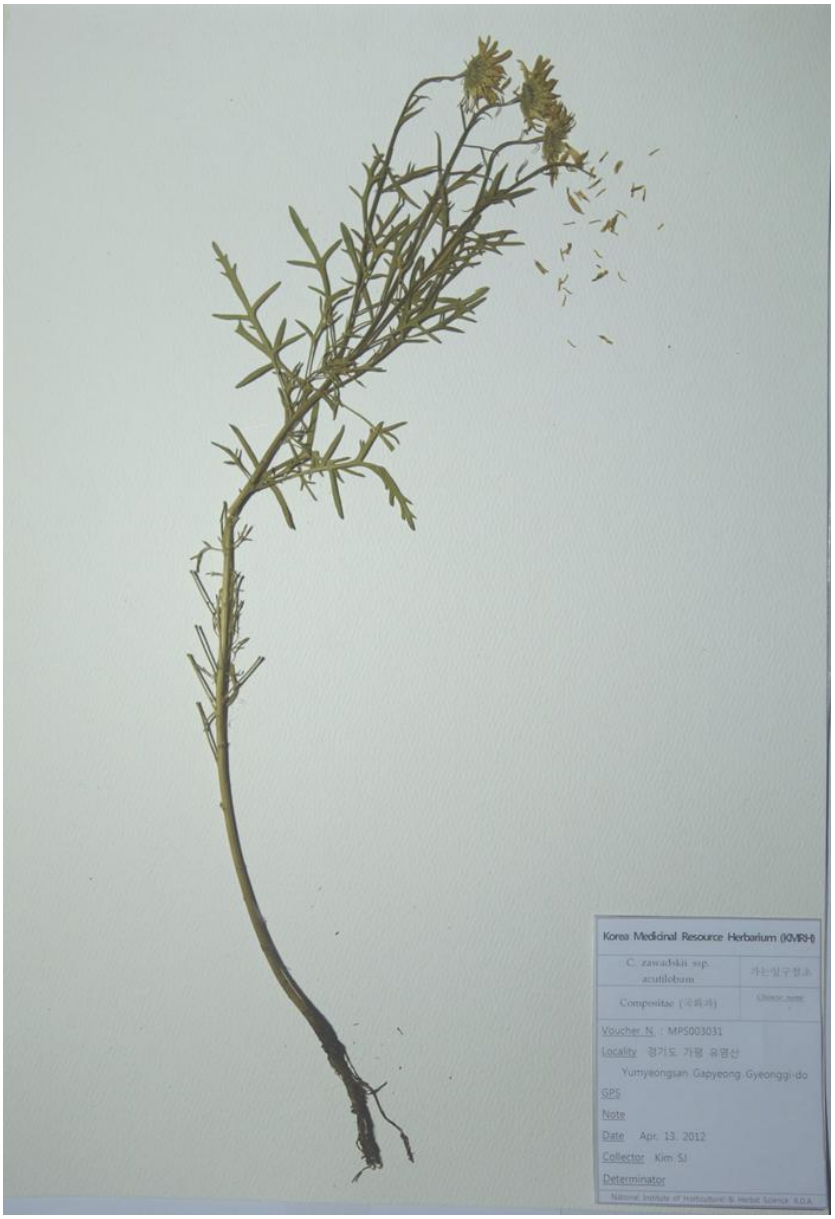

Fig. 1. *C. zawadskii* Herbich ssp. *acutilobum* (DC.) Kitagawa deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (가는잎구절초, voucher MPS003031).

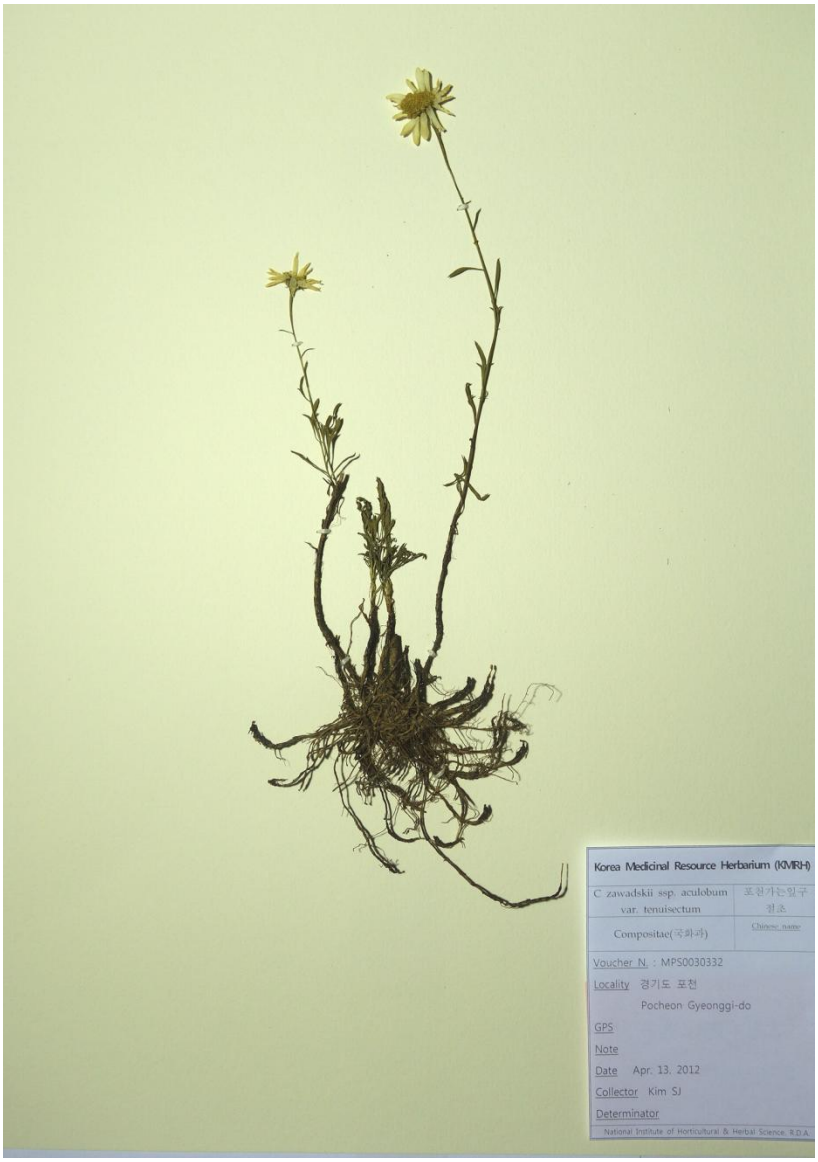

Fig. 2. *C. zawadskii* Herbich ssp. *acutilobum* (DC.) Kitagawa var. *tenuisectum* (Kitagawa) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (포천가는잎구절초, voucher MPS003032).

Fig. 3. *C. zawadskii* Herbich ssp. *acutilobum* (DC.) Kitagawa var. *alpinum* (Nak.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (바위구절초, voucher MPS003033).

Fig. 4. *C. zawadskii* Herbich ssp. *lucidum* (NAK.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (울릉국화, voucher MPS003034).

Fig. 5. *C. zawadskii* Herbich ssp. *coreanum* (NAK.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (한라구절초, voucher MPS003035).

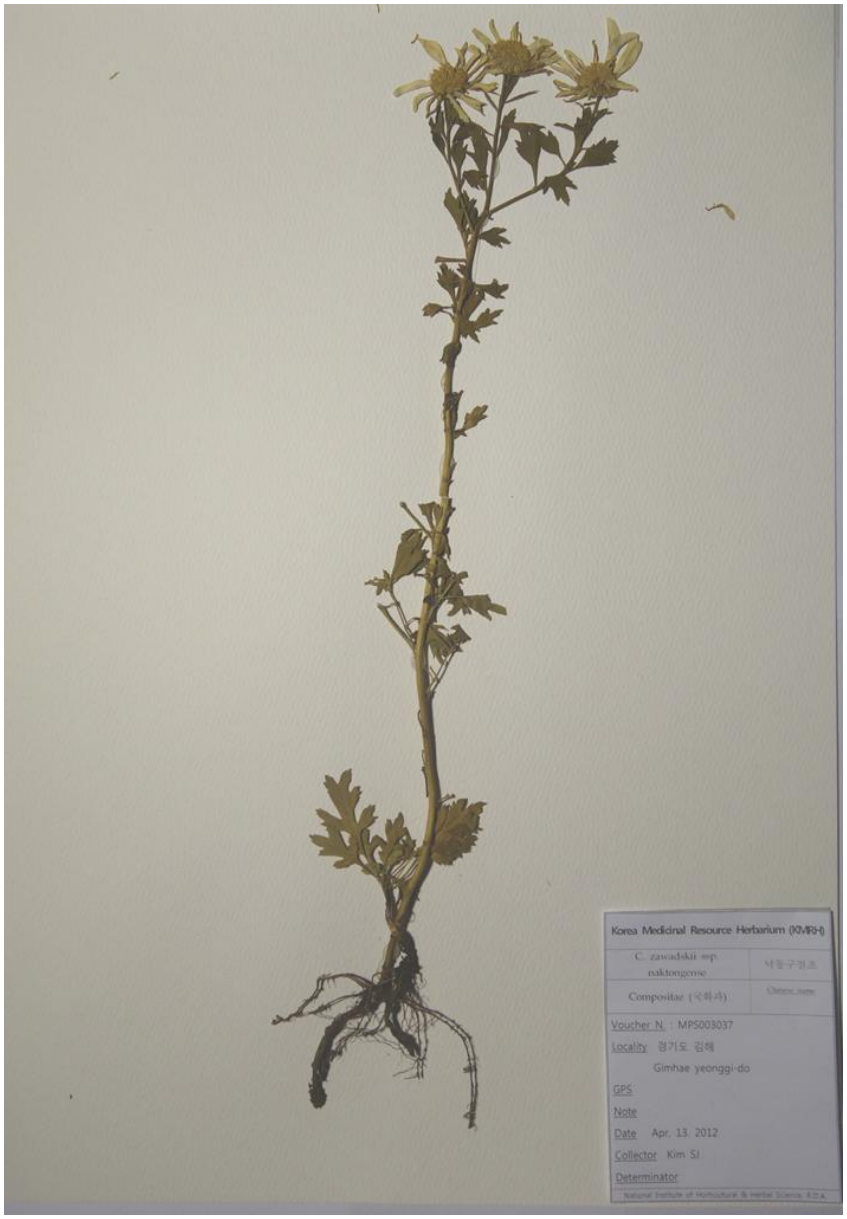

Fig. 6. *C. zawadskii* Herbich ssp. *naktongense* (NAK.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (낙동구절초, voucher MPS003037).

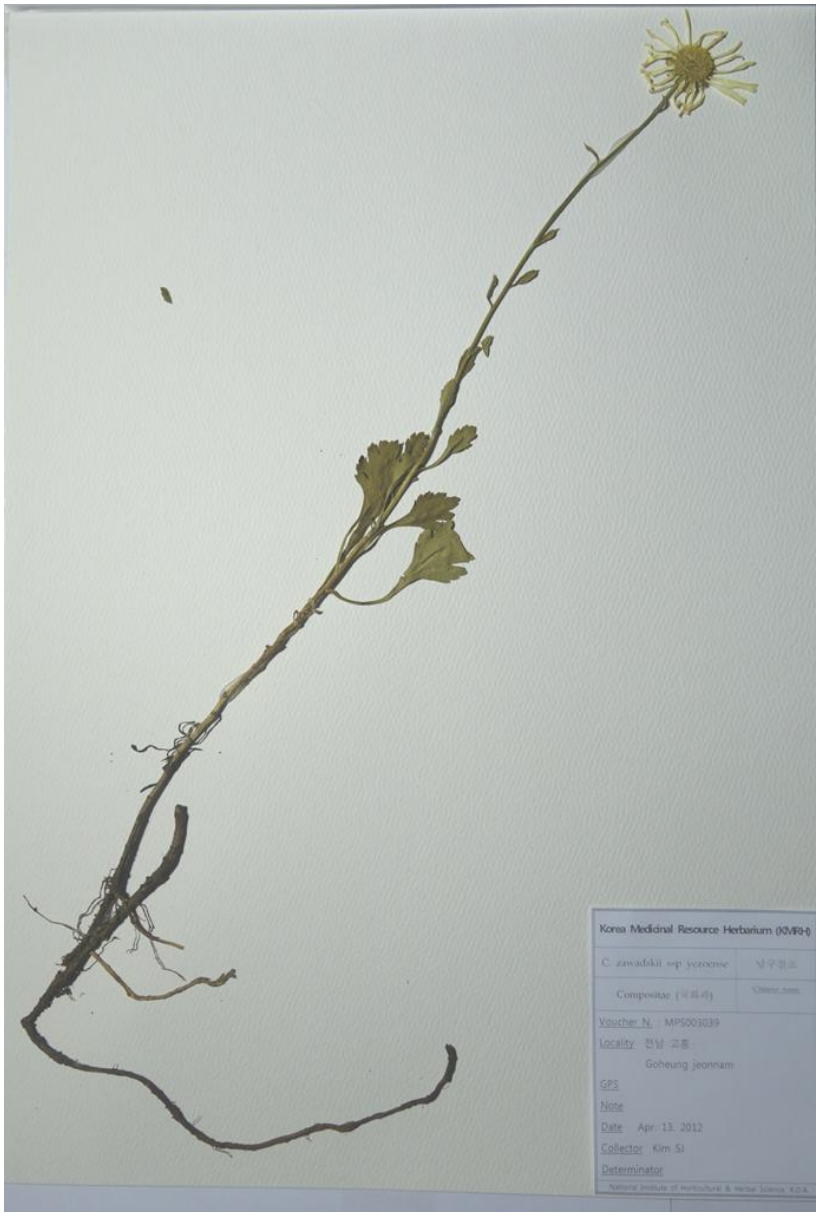

Fig. 7. *C. zawadskii* Herbich ssp. *yezoense* (Maekawa.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (남구절초, voucher MPS003039).

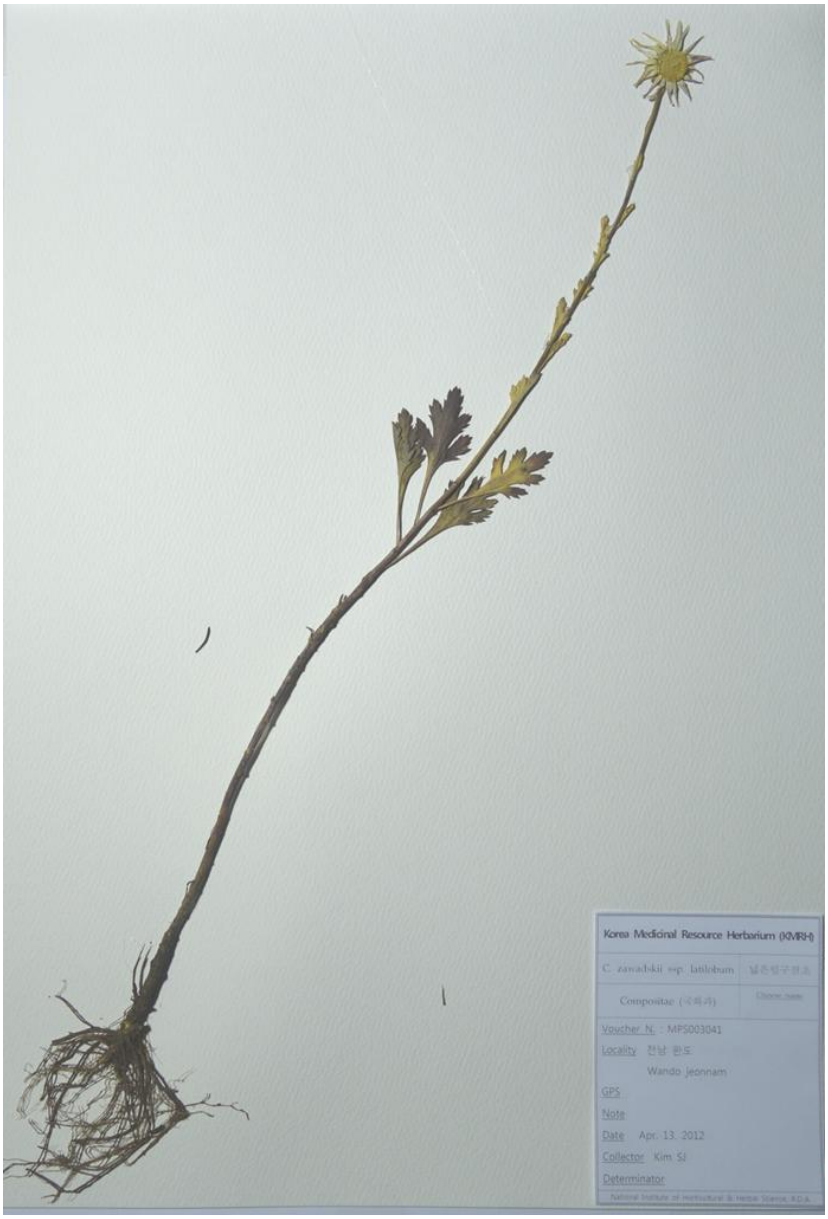

Fig. 8. *C. zawadskii* Herbich ssp. *latilobum* (Maxim.) Kitagawa deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (넓은잎구절초, voucher MPS003041).

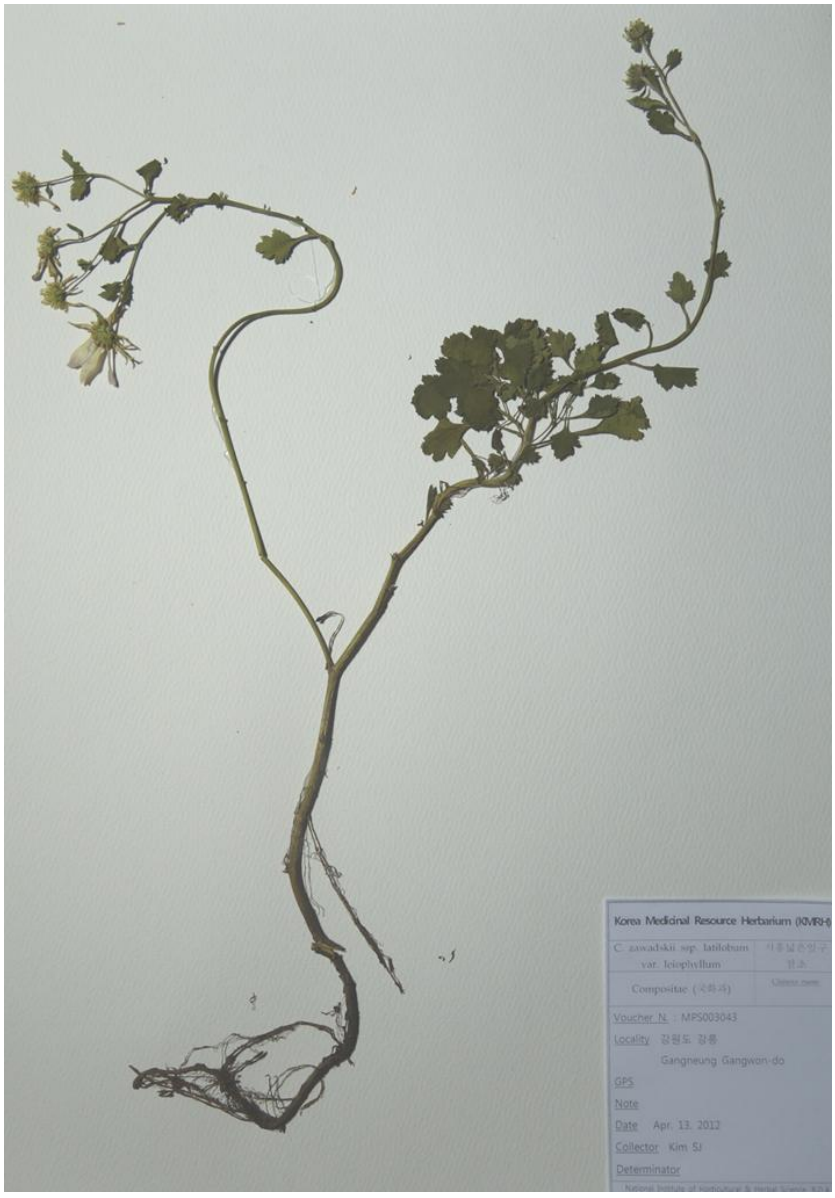

Fig. 9. *C. zawadskii* Herbich ssp. *latilobum* (Maxim.) Kitagawa var. *leiophyllum* (Nak.) Y. Lee deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (서흥넓은잎구절초, voucher MPS003043).

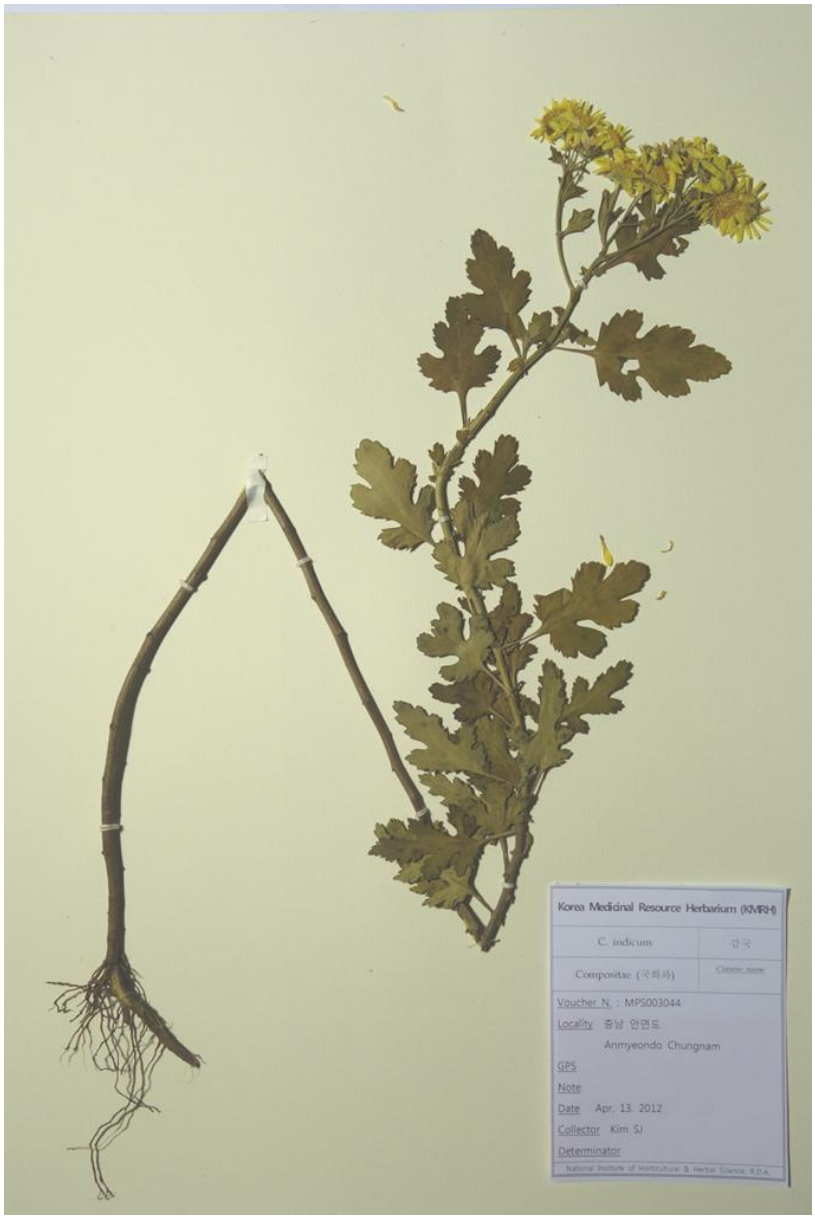

Fig. 10. *C. indicum* Linné deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (감국, voucher MPS003044).

Fig. 11. *C. indicum* Linné var. *albescens* Makino deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (흰감국, voucher MPS003046)

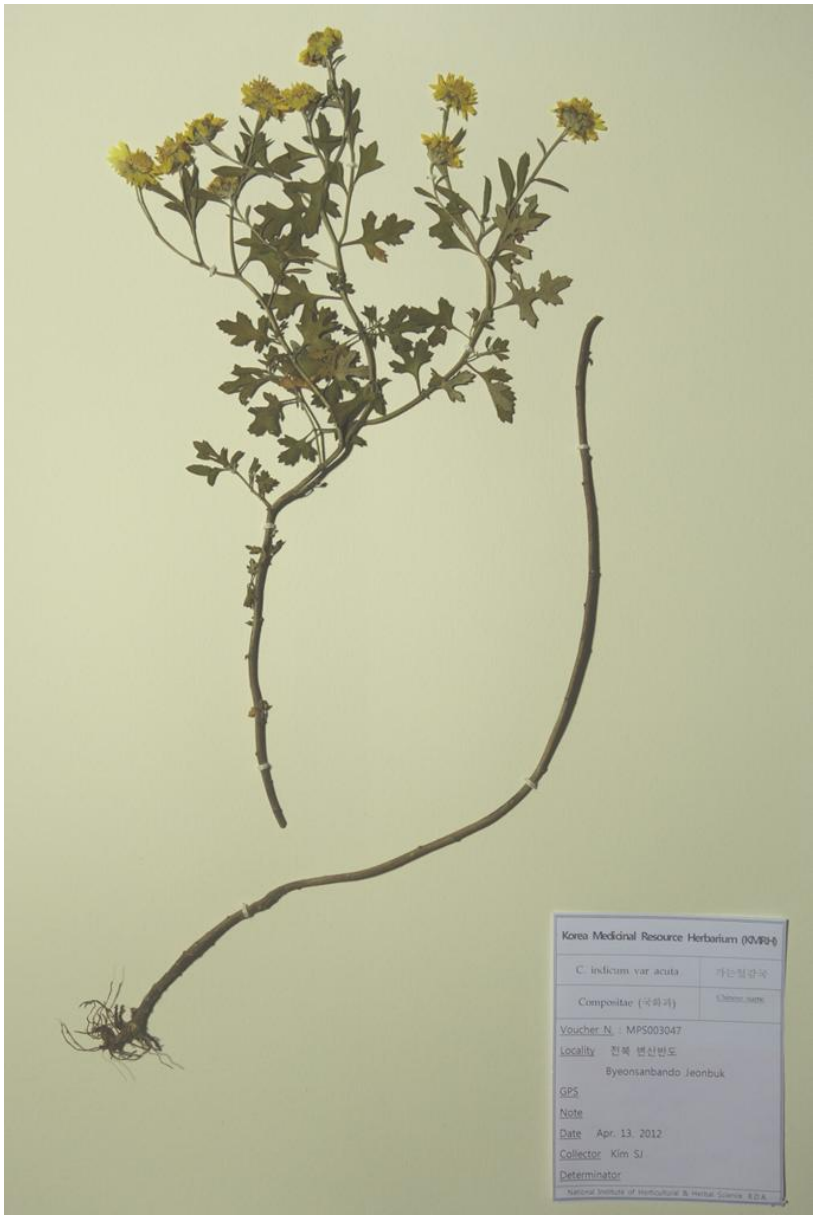

Fig. 12. *C. indicum* Linné var. *acuta* (Uyeki) Kitam. deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (가는잎감국, voucher MPS003047).

Fig. 13. *C. boreale* (Mak.) Makino deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (산국, voucher MPS003049).

Fig. 14. *C. lineare* Matsumura deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (키큰산국, voucher MPS003050).

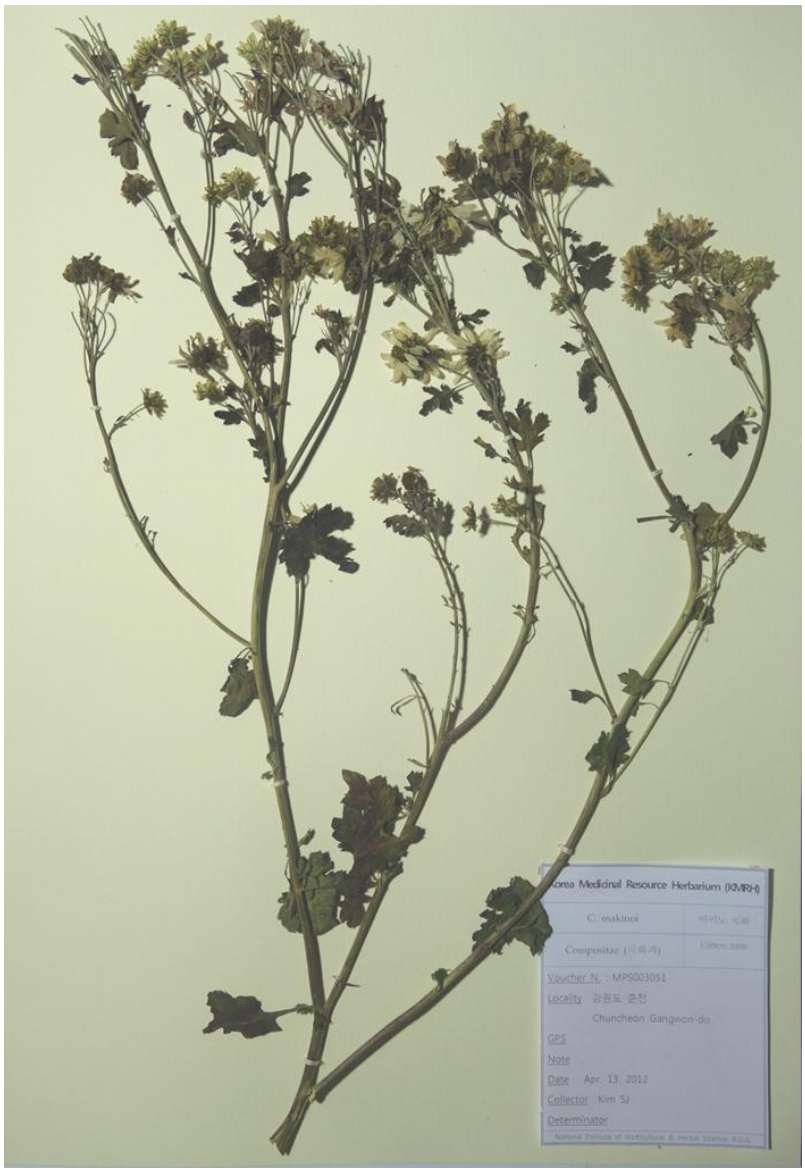

Fig. 15. *C. makinoi* Matsumura et Nakai deposited in Korean Medicinal Resource Herbarium at Rural Development Administration, Korea (마키노 국화, voucher MPS003051).

GRAPHIC ABSTRACT

Korean name	Scientific name	Morphological characteristics				Molecular characteristics		Chemotaxonomical characteristics	
		Seed	Flower	Leaf	Group	Ploidy	Group	flavor	Group
가는잎구절초	***C. zawadskii* ssp. *acutilobum***				I	6x	I	D-limonene, *m*-thymol	I
포천가는잎구절초	***C. zawadskii* ssp. *acutilobum* var. *tenuisectum***				I	6x	I	D-limonene, *m*-thymol	I
바위구절초	***C. zawadskii* ssp. *acutilobum* var. *alpinum***				I	6x	II	D-limonene, *m*-thymol	I
울릉국화	***C. zawadskii* ssp. *lucidum***				I	4x	I	D-limonene, *m*-thymol	I
한라구절초	***C. zawadskii* ssp. *coreanum***				I	8x	I	D-limonene, *m*-thymol	I
낙동구절초	***C. zawadskii* ssp. *naktongense***				I	6x	I	Linalool, *cis*-chrysanthenol	II
남구절초	***C. zawadskii* ssp. *yezoense***				I	6x	I	Linalool, *cis*-chrysanthenol	II
넓은잎구절초	***C. zawadskii* ssp. *latilobum***				I	6x	I	Linalool, *cis*-chrysanthenol	II
서흥넓은잎구절초	***C. zawadskii* ssp. *latilobum* var. *leiophyllum***				I	4x	I	Linalool, *cis*-chrysanthenol	II
감국	***C. indicum***				III	4x	I	α-Thujone, Hinesol	III
흰감국	***C. indicum* var. *albescens***				III	6x	I	α-Thujone, Hinesol	III
가는잎감국	***C. indicum* var. *acuta***				III	4x	I	α-Thujone, Hinesol	III
산국	***C. boreale***				III	2x	I	α-Thujone, Hinesol	III
키큰산국	***C. lineare***				II	2x	III	α-Thujone,Hinesol	III
마키노 국화	***C. makinoi***				III	2x	I	Linalool, *cis*-chrysanthenol	II

ABSTRACT IN KOREAN

한국산 국화속 식물은 우리나라 들국화로 알려진 자생식물로 조경용 또는 약용으로 유용한 유전자원이다. 본 논문은 자생국화 15분류군을 대상으로 형태적 특성, 유전적 특성 그리고 생화학적 특성을 주성분 분석과 군집분석을 통해 분류학적 유연관계를 구명하여 그 분류 기준을 제시하고 조경용 및 약용 소재로 이용하기 위한 기초자료로 활용하고자 본 연구를 수행하였다.

한국산 국화속 식물 유전자원은 2006년부터 2009년동안 한반도에 각 지역에서 수집된 총 15종 분류군을 대상으로 하였다. 실험재료는 화분에 심어 강원도 평창군에 위치한 농촌진흥청 국립식량과학원 고령지농업연구센터에서 2006년부터 2014년 현재까지 유지 관리해 온 식물체를 사용하였다.

한국산 국화속의 형태적 특성 분류를 위해 외부형태학적, 해부학적, 화분학적 그리고 종자 형태적 특성을 비교하여 주성분분석과 군집분석을 수행하였다. 분석결과 국화속 식물은 잎의 모양, 잎의 길이 및 잎의

비율(엽장/엽폭)이 형태 분류에 가장 중요한 형질로 나타났다. 제 1그룹에서는 대부분 구절초류가 포함되었으며, 다른 그룹보다 초장이 평균 18.5cm 로 작고, 흰색 또는 핑크색 꽃이 동시에 오랫동안 개화하는 특성을 보여 조경용으로 적합하다고 판단되었다. 그 중에서 구절초가 가을에 개화하였다. 이에 반해 바위구절초는 여름에 개화하므로 다양한 개화시기 조절을 위한 육종 소재로 이용 가능하다. 제 2그룹에서는 키큰산국만이 속하였는데 엽병이 없으면서 독특한 잎모양을 가졌고, 종자가 비교적 크고, 연노란색을 나타내었다. 제 3그룹에서는 감국류와 감국류 근연종이 속하였는데 다른 두 그룹과 달리 개화기에 줄기가 목질화 되고, 꽃(직경 15.1-27.3 mm)이 작고, 꽃송이(80-223개)가 많으면서 다양한 기능성을 가지고 있어 국화차 또는 화장품과 같은 약용자원으로서 이용가능할 것으로 판단된다.

한국산 국화속의 세포학적 · 분자생물학적 유연관계를 알아보고자 염색체 및 ITS(internal transcribed spacer) 분석을 통해 분류군을 비교 분석하였다. 분류군의 염색체 수를 검토한 결과, 기본 염색체수는 x=9를 기원으로 2배체, 4배체, 6배체, 및 8배체로 다양하게 분화된 것으로 나타났다. 국화속

15분류군의 ITS 염기서열을 결정하고 이를 통한 분류군간의 유연관계를 분석하고자 계통분석을 실시하였다. ITS 1의 길이는 268-269bp로, ITS 2의 길이는 231bp로 나타났으며, 염기 치환율은 한 사이트 당 ITS 1.5% 이내로 분류군 간에 차이가 거의 없는 것으로 나타났다. 계통분석 결과 키큰산국이 가장 먼저 분지되었으며, ITS 분석으로는 아종이나 변종의 구분이 되지 않았다.

마지막으로 한국산 국화속 잎의 플라보노이드 성분과 휘발성 향기 성분을 분석하여 화학적 분류를 통해 분류군간의 유연관계를 밝혔다. 한국산 국화속 15분류군의 잎을 대상으로 플라보노이드 성분을 HPLC/MS로 분석한 결과 luteolin 7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, apigenin, 및 acacetin-7-*O*-rutinoside을 포함하여 총 5종류가 동정되었다. 항염증 효과가 뛰어난 4분류군(가는잎구절초, 남구절초, 넓은잎구절초, 가는잎감국)에서 주로 4종류의 플라보노이드(luteolin 7-*O*-rutinoside, luteolin-7-*O*-glucosede, apigenin-7-*O*-glucoside, 및 acacetin-7-*O*-rutinoside)가 확인되었다.

또한, 한국산 국화속 15분류군의 잎을 대상으로 GC/MS 분석 결과, 총

45종의 휘발성 향기성분이 함유된 것으로 분석되었다. 검출된 향기성분 중 camphor, borneol, phytol, α-pinene, camphene, 1,8-cineole 및 germacrene-D 는 자생국화에 공통적으로 함유된 성분으로 밝혀졌는데 분류군에 따라 함량에서 다소 차이를 보였다. 항균효과가 탁월한 것으로 알려진 camphor의 함량 범위는 6.44-28.61%이었으며, 특히 넓은잎구절초와 서흥넓은잎구절초에 많이 함유되었다. 항암과 항염증 효과에 뛰어난 α-thujone은 감국류와 산국에서 많았다. 특히, 흰감국의 경우 항균효과가 뛰어난 camphene 함량이 감국보다 21배 높게 나타났다. 항돌연변이 효과가 있는 1,8-cineole은 가는잎감국에 3.50%로 함량이 많았다. 본 연구를 통하여 나타난 한국산 국화속 분류군별 휘발성 향기성분 조성은 신품종 육종이나 약용소재 개발에 기초자료로 활용될 수 있을 것으로 기대된다.

이상의 결과를 통해, 한국산 국화속 15분류군의 형태적으로 분류에서 잎의 형질이 주요한 분류기준이 되었다. 세포학적 유전적 분류에서 염색체수는 2, 4, 6, 8배체로 다양하였고 ITS를 기반으로 한 유전적 차이에 근거한 구분에서는 아종이나 변종의 차이는 거의 없었다. 화학적 분류에서 플라보노이드보다 휘발성 향기 성분에서 종간 차이를 구분할 수 있었다.

향기성분을 바탕으로 주성분 분석과 군집 분석결과 한국산 국화속은 주요 데이터를 근거로 각기 3그룹으로 구분할 수 있었으며 이는 자원활용적인 면에서 이용 목적에 맞는 분류군을 활용할 수 있다고 판단된다. 이 결과를 바탕으로 향후 한국산 국화속 식물의 품종육성 및 자원활용의 기초자료로 활용될 수 있으리라 판단된다.

주요어: 국화, 분류, 배수성, 플라보노이드, 향기,

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