Metabolic Fingerprinting of *Citrus* Cultivars and Related Genera Using HPLC and Multivariate Analysis

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Abstract

Citrus taxonomy is very complex and confusing, because of asexual reproduction and sexual compatibility between *Citrus* and related genera. Metabolic diversity was studied in *Citrus*, *Poncirus* and *Fortunella* cultivars by the high performance liquid chromatography technique combined with multivariate statistical analysis. Chromatograms obtained from cultivars of the same species showed similar elution profiles. These results suggested that metabolic profiles carry characteristics of hybrid origin. To confirm the similarities among the *Citrus* species and their cultivars, multivariate statistical analysis was applied to the chromatograms. According to hierarchical cluster analysis, all cultivars used in this study were divided into three major groups, which largely correspond to pummelo, mandarin and lemon. Hybrids were clustered together with their hybrid origin or their related cultivars. Our results indicated that the metabolic fingerprinting method provides an insight into the phylogenic relationships among *Citrus* species and cultivars.

Keywords: chemotaxonomy, *Citrus*, metabolic fingerprinting, secondary metabolite

1. Introduction

The true citrus fruit trees comprise six genera, i.e. *Fortunella, Poncirus, Citrus, Microcitrus, Eremocitrus* and *Clymenia.* Among these, the *Citrus* species is one of the most commercially important groups and has a wide diversity of related cultivars. However, *Citrus* taxonomy and phylogeny are very complex and confusing because of asexual seed reproduction and sexual compatibility between *Citrus* and related genera. Until the mid 1970s, studies on *Citrus* taxonomy were carried out based on morphological and geographical data, and numbers of classification systems were formulated. Among them, the classification systems formulated by Swingle (1943) and Tanaka (1969) have been widely accepted. However, these two widely accepted systems are based on different classification concepts. Swingle identified only 16 species, while Tanaka recognized 162 species. Later, Scora (1975) and Barret and Rhodes (1976) proposed that there are only three species in the subgenus *Citrus*, i.e. citron (*Citrus medica* L.), mandarin (*Citrus reticulata* Blanco) and pummelo (*Citrus grandis* (L.) Osbeck), and the other genotypes have originated by hybridization between these three true species. Recently, this three-species concept has been supported by many studies using biochemical and molecular markers, such as isozymes (Torres *et al*., 1978; Fang *et al*., 1993; Herrero *et al*., 1996), microsatellites (Fang and Roose, 1997; Fang *et al*., 1998) and nuclear (Ramadugu *et al*., 2013) and organellar genome analyses (Green *et al*., 1986; Yamamoto *et al*., 1993; Nicolosi *et al*., 2000; Carbonell-Caballero *et al*., 2015).

Since a variety of secondary metabolites present in higher plants apparently serve as defense compounds against environmental stresses, they are important for plant survival. Thus, the distribution of secondary metabolites appears to represent the adaptive characters that have been evolved through natural selection. Since secondary metabolites within the members of a family are often similar, their distribution of secondary metabolites could be used as one of the markers for phylogenetic and taxonomic classifications.

Early studies on *Citrus* chemotaxonomy based on secondary metabolites, were carried out using thin-layer chromatography (Albach and Redman, 1969; Tatum *et al*., 1974) and paper chromatography techniques (Dass *et al*., 1977, 1978; Grieve and Scora, 1980; Handa, 1988). These methods are effective for comparing patterns of pigment composition, but require visual estimation of the separated compounds for their quantification. Recently, high-performance liquid chromatography (HPLC) has been used for the separation and quantification of metabolites, and multivariate statistical methods have also been introduced for estimating similarities in chemical composition. Gayou *et al*. (1987) analyzed six polymethoxylated flavones in peel oils of six *Citrus*

cultivars and applied a multivariate statistical analysis to sample classification. Kawaii *et al.* (1999) also quantitatively identified 24 flavonoids in fruits in order to determine the relationships among 66 *Citrus* species.

These studies focused on fruit metabolites because of their commercial importance. However, since leaves are a richer source of secondary metabolites in higher plants, metabolites in leaves should also be included in chemotaxonomic analysis. The previous authors purified metabolites, identified their chemical structures and determined their amounts. However, the requirement for the preparation of authentic compounds and baseline separation on HPLC limits the analysis to a narrow range of metabolites, and thus, restricsts their application for taxonomic analysis of a wide range of cultivars and species. Recently, metabolomic approaches using LC/MS and GC/MS techniques have been used for qualitative analysis (Sumner et al., 2003). These techniques have become powerful tool to gain comprehensive information of metabolites in plant materials. However, these techniques need expensive devises and specific software which handle a large amount of information produced by the devices. Moreover, since identification of metabolites depends on mass spectra databases, application of these techniques is limited to primary metabolites or secondary metabolites of model organisms. In order to provide the relationships between the different species based on secondary metabolites, we performed a comprehensive analysis of UV absorbing secondary metabolites such as flavonoids and coumarins, major compounds in citrus, in leaves by using conventional HPLC, and the elution profiles were applied to chromatographic pattern analysis based on multivariate statistical analysis.

2. Materials and Methods

2.1 Plant Material

The youngest and fully expanded leaves of 29 cultivars (Table 1) were harvested and collected from mature trees maintained in Yuasa Experimental Farm, Wakayama Prefecture, Japan. For minimize the environmental condition, all trees used in this study were grown in the same fields. Five biological replicates were collected for each species.
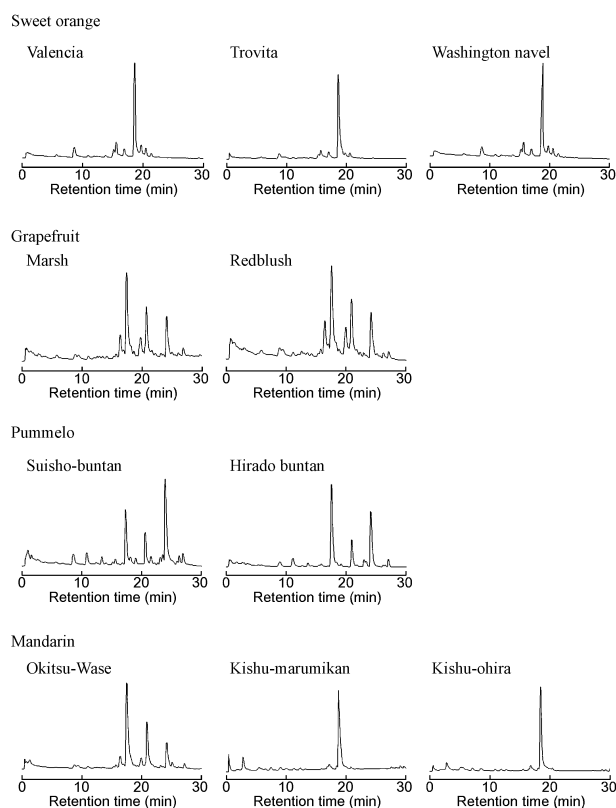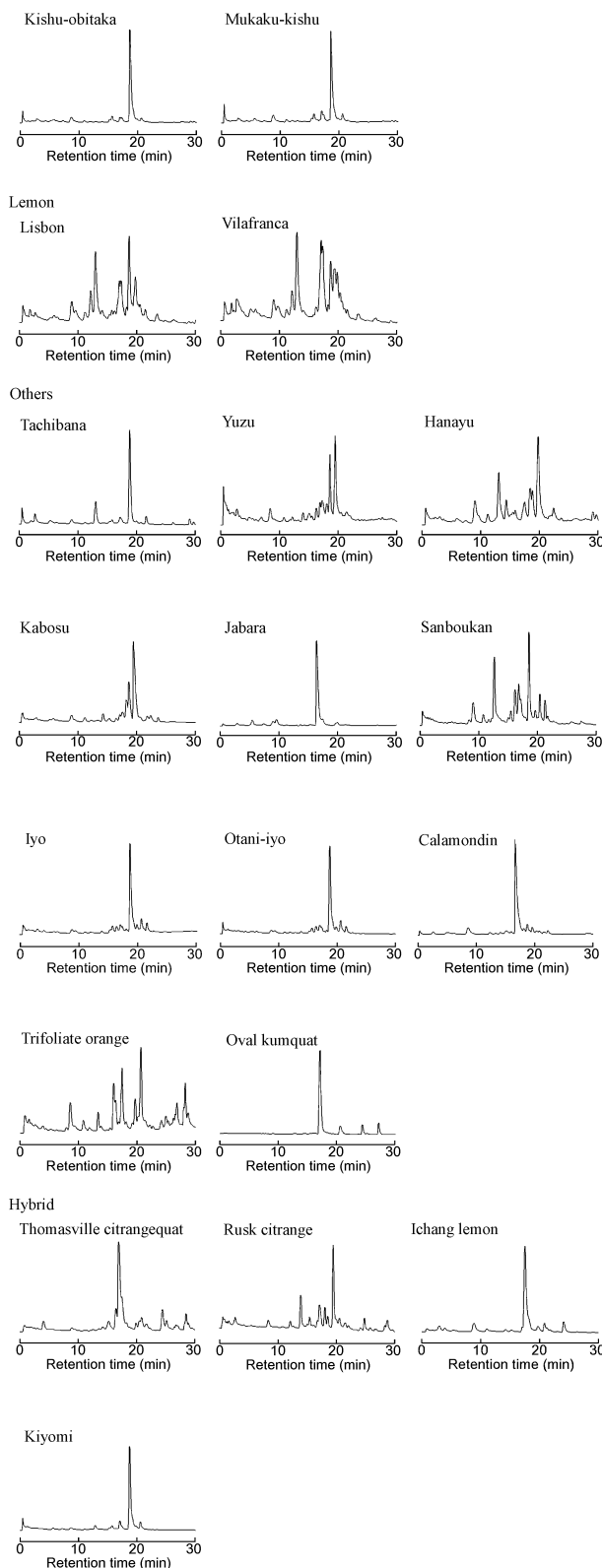
Figure 1. HPLC chromatograms of MeOH extracts obtained from mature leaves of *Citrus* and related genera. Elution of metabolites was monitored at 280 nm

Table 1. Plant materials used in this study

| Cultivar | Scientific name[a] | |
	Swingle's system	Tanaka's system
Sweet orange	*Citrus sinensis* (L.) Osbeck	*Citrus sinensis* (L.) Osbeck
Trovita		
Valencia		
Washington		
Navel		
Grapefruit	*C. paradisi* Macf.	*C. paradisi* Macf.
Marsh		
Redblush		
Pummelo	*C. grandis* (L.) Osbeck	*C. grandis* (L.) Osbeck
Suisho buntan		
Hirado buntan		
Mandarin		
Okitsu Wase	*C. reticulata* Blanco	*C. unshiu* Marc.
Kishu	-[b]	*C. kinokuni* Hort. ex. Tan.
marumikan		
Kishu ohira	-	*C. kinokuni* Hort. ex. Tan.
Kishu obitaka	-	*C. kinokuni* Hort. ex. Tan.
Mukaku kishu	-	*C. kinokuni* Hort. ex. Tan.
Lemon	*C. limon* Osbeck	*C. limon* Osbeck
Lisbon		
Villafranca		
Others		
Tachibana	-	*C. tachibana* (Mak.) Tan.
Yuzu	-	*C. junos* Sieb. ex. Tan.
Hanayu	-	*C. hanayu* Sieb. ex. Tan.
Kabosu	*C. aurantium*	*C. sphaernocarp* Hort. ex. Y. Tan.
Jabara	-	*C. jabara* Hort. ex. Y. Tanaka
Samboukan	-	*C. sulcata* Hort. ex. Takahashi
Iyo	-	*C. iyo* Hort. ex. Tan.
Ohtani iyo	-	*C. iyo* Hort. ex. Tan.
Calamondin	-	*C. madurensis* Lour.
'Oval' kumquat	*Fortunella margarita* Swingle	*Fortunella margarita* Swingle
Trifoliate orange	*Poncirus trifoliata* Raf.	*Poncirus trifoliata* Raf.
Hybrid		
Thomasville	*F. margarita* × (*P. trifoliata* × *C. sinensis*)	
Rusk	*C. sinensis* × *P. trifoliata*	
Ichang lemon	*C. ichangensis* × *C. grandis*	
Kiyomi	*C. unshiu* × *C. sinensis*	

[a] Botanical names are based on Swingle's system. In addition to this system, the botanical names of Tanaka's system are mentioned for species indigenous to Japan.

[b] not identified

2.2 Extraction of Secondary Metabolites

Citrus leaves were frozen in liquid nitrogen and ground to a fine powder. The powder was extracted with 5 volumes of methanol, a suitable solvent for extraction of semi-polar metabolites (Ballester *et al*., 2016), and centrifuged at 12,000 *g* for 15 min. An aliquot of the supernatant was filtered, and the filtrate was subjected to HPLC analysis.

2.3 HPLC Condition

The extract was analyzed by an Shimadzu 10A system (Shimadzu, Kyoto, Japan) with a 4.6 i.d. × 50 mm Cosmosil 3C18 AR-II column (Nacalai tesque, Kyoto, Japan). A two solvent system was used to generate the mobile phase—solvent A was 0.1% acetic acid solution and solvent B was MeOH. The flow rate was 1.2 ml/min.

The mobile phase at the beginning of the analysis was 18% B in A. After the injection of the extract, the ratio was maintained for 5 min, followed by a linear gradient from 18% to 38% B in A in 15 min. Subsequently, a 10 min linear gradient from 38% to 58% B in A was applied. The elution of secondary metabolites was monitored at 286 nm.

2.4 Statistical Analysis

To apply a multivariate analysis to the chromatogram profile, raw chromatograms obtained from each run were scanned and transformed to digital (x, y) data by a digitizing program Un-Scan-It (Silk Scientific, Inc., UT). All the transformed data were imported to the multivariate data analysis program Pirouette 3.11 (Infometrix, Inc., WA). The elution profiles of five replicates were averaged, and the differences in the elution time of each run were adjusted and aligned with reference to the co-chromatogram data of each sample. The aligned data set was used for hierarchical cluster analysis (HCA). The multivariate measure of the distance between each cluster was based on Euclidean distance, and the cluster was linked based on the incremental method.

3. Results and Discussion

3.1 HPLC Chromatograms

All the leaf samples used in this study were harvested from the same field on the same day, and thus, were grown under the same climatic conditions. The leaf samples were extracted with methanol and subjected to reverse phase HPLC analyses. The applied gradient system covers the major UV active compounds, including flavonoids, coumarins and phenylpropanoids.

As shown in Figure. 1, the chromatograms obtained from cultivars of the same species showed largely similar elution profiles. In the chromatograms of sweet orange (*C. sinensis*) cultivars, all the major peaks were common to the cultivars and were of a similar size. Almost all peaks were common to the two cultivars of grapefruit (*C. paradisi*), and in pummelos (*C. grandis*), the three major peaks detected in 'Suisho buntan' were also detected in 'Hirado buntan'. In contrast, mandarin cultivars showed heterogeneity in the elution profiles. 'Okitsu Wase' showed three major peaks, which were not detected in the other mandarin cultivars. On the other hand, the largest peak commonly detected in 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' was absent in 'Okitsu Wase'. 'Okitsu Wase' are considered to belong to satsuma mandarins, while others are kishu mandarins, and thus, the differences in elution profiles reflect the difference between satsuma and kishu. In the chromatograms of the two lemon (*C. limon*) cultivars, many peaks overlapped each other. The similarity between the elution profiles of the two cultivars was observed, although the intensity of each peaks was different among two species.

On comparing the elution profiles of the species, a similarity was detected between the elution profiles of grapefruit and pummelo cultivars. The retention times of the three major peaks in the grapefruit cultivars were completely consistent with those of the two pummelo cultivars, although the relative sizes of the peaks in the two species were different. The elution profile of the mandarin cultivar 'Okitsu Wase' (*C. unshiu*) also resembled those of the other species.

The elution profile of 'Kiyomi' was quite similar to those of the sweet orange cultivars. 'Kiyomi' is the artificial hybrid of 'Miyagawa Wase' (*C. unshiu*) and 'Trovita' (*C. sinensis*) (Nishiura *et al.*, 1983). Thus, it was found that the metabolic character of 'Trovita' was dominantly inherited through the screening and breeding program.

Other cultivars showed cultivar-specific chromatograms. Many of these cultivars are thought to be indigenous to Japan and their origin is still obscure. Among them, cultivars classified as *C. iyo* ('Iyo' and 'Ohtani iyo') showed similar elution profiles. Their profiles were also similar to those of the four mandarin cultivars 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu', suggesting that these two groups might have originated from the same ancestor.

3.2 Hierarchical Cluster Analysis

To confirm the similarities among the *Citrus* species and their cultivars, the elution profiles were subjected to the hierarchical cluster analysis. Chromatograms were converted to 1000 data points, and the data was clustered by the application of an incremental linkage method based on Euclidean distance. Figure. 2 shows the dendrogram constructed by using the hierarchical cluster analysis.

Based on this dendrogram, the analyzed cultivars were divided into three major groups. The first group contained three cultivars of sweet orange (*C. sinensis*), and the four mandarin cultivars namely, 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' (*C. kinokuni*). In addition, the two *C. iyo* cultivars, 'Iyo' and 'Ohtani iyo' as well as the other *Citrus* cultivars 'Tachibana' (*C. tachibana*), 'Kabosu' (*C.

sphaernocarp), 'Yuzu' (*C. junos*), 'Hanayu' (*C. hanayu*), 'Calamondin' (*C. madurensis*) and the two hybrids 'Kiyomi' and 'Rusk' citrange were included in this group. The second group consisted mainly of the grapefruit and pummelo cultivars. 'Oval' kumquat (*F. margarita*) and trifoliate orange (*C. trifoliata*) were also clustered in the second group. The third group contained cultivars of lemon (*C. limon*) and 'Jabara' (*C. jabara*). Barret and Rhodes (1976) postulated that the diverse genotypes in the genus *Citrus* originated from only three species, namely, mandarin (*C. reticulata*), pummelo (*C. grandis*) and citron (*C. medica*). The three groups observed in our dendrogram largely corresponded to the mandarin, pummelo and citron groups, supporting the three species concept (Scora, 1975; Barret and Rhodes, 1976; Nicolosi *et al*., 2000; Carbonell-Caballero *et al*., 2015).
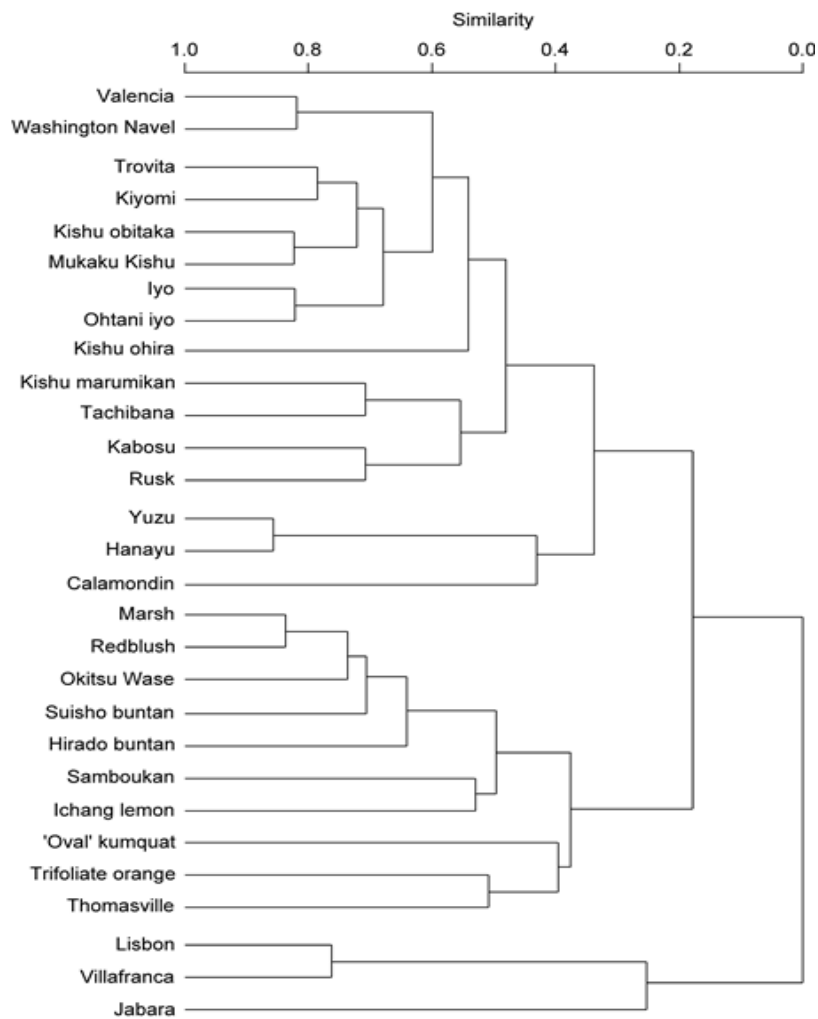

Figure 2. Dendrogram of 29 cultivars of *Citrus* and related genera obtained by hierarchical cluster analysis of HPLC chromatogram data

The first group was divided into two subgroups; the first subgroup was composed mainly of sweet orange cultivars and four mandarin cultivars, and the other subgroup was composed of the cultivars 'Yuzu', 'Hanayu' and 'Calamondin'. 'Iyo' and 'Ohtani iyo' were included in the first subgroup together with the sweet orange cultivars. Since it has been suggested that 'Iyo' and 'Ohtani iyo' are natural tangors (Tanaka, 1954), their close relationship with sweet oranges is reasonable. 'Kishu marumikan', 'Kishu ohira', 'Kishu obitaka' and 'Mukaku Kishu' (*C. kinokuni*) were also clustered in the first subgroup. The phylogenic relationship between *C. kinokuni* and the other cultivars is unclear. The close relationship between *C. kinokuni* and *C. sinensis* suggests that *C. reticulata* was involved in the differentiation of *C. kinokuni* because *C. sinensis* have been thought to be of predominantly the *C. reticulata* genotype introgressed with genes from *C. grandis* (Barrett and Rhodes, 1976; Nicolosi *et al*., 2000). Moreover, *C. sinensis* is now thought to originate from backcross hybrid between pummelo and mandarin (Xu *et al*., 2013). The phylogenic relationship between 'Tachibana' and 'Kabosu' and sweet oranges may suggest the involvement of *C. reticulata* in the differentiation of these cultivars. 'Yuzu',

'Hanayu' and 'Calamondin' were clustered as the second subgroup in the first group, suggesting the introgression of traits of *C. reticulata* into the cultivars.

'Kiyomi' was closely related to orange cultivars, such as 'Valencia', 'Trovita' and 'Washington Navel'. 'Kiyomi' is the artificial hybrid of 'Miyagawa Wase' unshu (*C. unshiu*) and 'Trovita' orange (*C. sinensis*) (Nishiura *et al*., 1983). Thus, the clustering of 'Kiyomi' with 'Trovita' reflects the origin of 'Kiyomi' and the metabolic characteristics of 'Trovita' may be predominantly inherited in this hybrid. Among sweet orange cultivars 'Kiyomi' with 'Trovita' showed the closest relationship, which indicates the utility of the hierarchical cluster analysis of elution profiles of the *Citrus* cultivars.

The second group consists of 11 cultivars and is divided into two subgroups. The first subgroup includes grapefruit (*C. paradisi*) cultivars, the mandarin cultivar 'Okitsu Wase' (*C. unshiu*), pummelo (*C. grandis*) cultivars, 'Ichang lemon' (*C. ichangensis* × unknown cultivar of *C. grandis*) and 'Samboukan' (*C. sulcata*). It has been suggested that grapefruit is derived from a sweet orange and pummelo hybrid (Scora, 1975; Barrett and Rhodes, 1976; Scora *et al*., 1982 Scora and Kumamoto, 1983; Nicolosi *et al*., 2000). The strong similarity between grapefruit cultivars and those of pummelo was indicated in our analysis, whereas no similarity was observed between grapefruit and sweet oranges. This is consistent with previous studies that indicate the close relationship between grapefruit and pummelo (Swingle and Reece, 1967; Handa, 1988). The close similarity between satsuma mandarin (*C. unshiu*) and pummelos and grapefruits was an unexpected result. Satsuma mandarin is believed to have originated in Japan as a chance seedling from a fruit or to have been imported from China (Swingle and Reece, 1967). Swingle (1943) identified it as one of the *C. reticulata* hybrids, while Tanaka (1977) recognized it as one of the *Citrus* species. In this study, metabolic patterns and statistical analysis showed a high similarity between satsuma mandarin and *C. grandis*, suggesting that some pummelo-type species were involved in the origin of satsuma mandarin. Tanaka (1932) named Ichang lemon *C. wilsonii* and stated that this species unquestionably had *C. junos* as one parent and that it appeared to be a cultigen that had originated as a chance seedling. On the contrary, Swingle and Reece (1967) stated that there was no valid reason for considering this plant as a botanical species since it is undoubtedly a chance hybrid of *C. ichangensis*. Based on our results, the characteristics of *C. grandis* might be strongly appeared. 'Samboukan' is indigenous to Japan, and its origin and its related species or cultivars are unknown. Our results suggest that pummelos were involved in the origin or differentiation of this cultivar.

The second subgroup contains 'Oval' kumquat (*F. margarita*), trifoliate orange (*P. trifoliata*) and 'Thomasville' citrangequat. However, 'Oval' kumquat cluster showed low similarity to the *Poncirus* cluster. 'Thomasville' citrangequat is an artificial trigeneric hybrid of 'Oval' kumquat (*F. margarita*) and 'Wilts' citrange (*P. trifoliata* × *C. sinensis*). Our results indicate that the metabolic profile of 'Thomasville' citrangequat is closer to *P. trifoliata*, suggesting that the metabolic character of *P. trifoliata* is dominantly inherited.

The last group is composed of lemon cultivars (*C. limon*) and 'Jabara' (*C. jabara*). Based on morphological characteristics, lemon was supposed to be a hybrid of citron (*C. medica*) and lime (*C. aurantifolia*) (Swingle, 1943; Malik *et al*., 1974; Scora, 1975). Barrett and Rhodes (1976) reported that lemon is a trihybrid of citron, pummelo and *Microcitrus* and had a higher proportion of citron genes. Torres *et al*. (1978) suggested that sour orange (*C. aurantium*) and lime are of hybrid origin. Based on the molecular marker data, Yamamoto *et al*. (1993) suggested that lemon is a hybrid of pummelo and citron, while Nicolosi *et al*. (2000) reported that lemon originated from citron and lime. Other report suggested that lemon originated from the complex hybridization of *C. maxima, C. reticulata* and *C. medica* (Clurk *et al*., 2014). Lemon cultivars clustered separately from the mandarin and pummelo clusters because of the strong influence of citron. 'Jabara' is indigenous to Japan and is cultivated in a limited area. Its origin is obscure and is thought to be a cultivar related to 'Yuzu' or 'Kunembo' mandarin. In order to determine the origin of this unique cultivar, the analysis of lime and citron will be necessary.

In summary, our chemotaxonomic results were broadly in accordance with the three-species concept. Although our method cannot provide the structural information of individual peaks, the method using conventional HPLC combined with multivariate statistical analysis was proven to be a simple and powerful tool for providing new insights into the phylogenic relationship among the *Citrus* cultivars and hybrids. Several questions and unexpected results have to be investigated. Further studies with a large number of cultivars and species would provide us with a deeper understanding of *Citrus* phylogeny and taxonomy.

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