Phylogeny and Classification of Kumquats (*Fortunella* spp.) Inferred from CMA Karyotype Composition

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Kumquats (*Fortunella* spp.) is classified into the subfamily Aurantioideae (family Rutaceae). The taxonomy and phylogeny of this genus are complicated and controversial. Therefore, we carried out an estimation based on chromomycin A3 (CMA) karyotype composition in order to understand the cytogenetics and evolution of the genus *Fortunella*. Among the 6 *Fortunella* species examined, *Fortunella hindsii* var. *chintou* Swing. (Hongkong kumquat) showed the simplest CMA karyotype composition. On the other hand, close relationships were found among the 3 species *F. margarita* (Lour.) Swing. (Oval kumquat), *F. japonica* (Thunb.) Swing. (Round kumquat), *F. crassifolia* Swing. (Meiwa kumquat). *Fortunella polyandra* (Ridl.) Tan. (Malayan kumquat) and *F. obovata* hort. ex Tan. (Changshou kumquat) had type E chromosomes, which is an elemental chromosome type for *Citrus*. We concluded that there are only two true species for the genus *Fortunella*, *F. hindsii* and *F. margarita* complex, which includes *F. margarita*, *F. japonica*, and *F. crassifolia*, and that *F. polyandra* and *F. obovata* should be classified as natural or horticultural hybrids.

Key Words: chromosome evolution, *Citrus*, close relationship, GC-rich region, hybrid origin.

**Introduction**

The genus *Fortunella* (kumquat) is one of the most important genera with *Citrus* and *Poncirus* in the subfamily Aurantioideae (Citroideae), of the family Rutaceae (Swingle and Reece, 1967). This genus is distributed in only China, Japan, Indonesia and the Malay Peninsula. In general, *Citrus* is believed to have originated in Assam or Southeast Asia, whereas *Fortunella* is reported to have originated from the southeast part of China (Webber, 1967; Yin-Min, 1985). Although the genus *Fortunella* has important agronomic traits such as comparatively good cold tolerance, a small tree form and small fruit with an edible peel, only few studies have been undertaken for clarifying the phylogeny of this genus.

In the past, the genus *Fortunella* was classified by two taxonomists, Swingle (1915) and Tanaka (1933), based mainly on morphological characteristics. According to the classification system of Tanaka (1933), this genus consists of six species, including the *F. hindsii* var. *chintou* Swing. (Hongkong kumquat) as the subgenus *Protocitrus*, and the *F. margarita* (Lour.) Swing. (Oval kumquat), *F. japonica* (Thunb.) Swing. (Round kumquat), *F. crassifolia* Swing. (Meiwa kumquat), *F. polyandra* (Ridl.) Tan. (Malayan kumquat), and *F. obovata* hort. ex Tan. (Changshou kumquat) as the subgenus *Eufortunella*. On the other hand, Swingle (1915) eliminated two species, Meiwa kumquat and Changshou kumquat, from the 6 species of Tanaka, because he considered that these two species might be the hybrids that had arisen through intrageneric crosses in *Fortunella* or intergeneric crosses between *Fortunella* and *Citrus*, and that they should not be entitled to the rank of species. Similar taxonomic problems have also been pointed out in some species of *Citrus*. Namely, both *Citrus madurensis* Lour. (Calamondin) and *C. halimii* B. C. Stone were believed to be of hybrid origin (Handa and Oogaki, 1985; Scora et al., 1988),

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and they have recently been proven to be intergeneric hybrids between *Fortunella* and *Citrus* (Barkley et al., 2006; Cheng et al., 2005; Pang et al., 2007). Although the correct classification of this genus has long been argued with respect to the problem involved in the two different classification systems described above and on the origin of some species as the natural hybrids, there have been few reports providing compelling evidence to resolve these problems. Thus, an understanding of the classification and phylogeny of the genus *Fortunella* are still controversial.

The classification of plants has mainly been based on morphology, anatomy, topographic distribution and cross compatibility (Kress, 1983; Smith, 1972; Thoday, 1925). In addition, recent developments in the novel technologies in cytogenetics and molecular biology enabled us to utilize them to clarify the taxonomic relationships in various living organisms based on the genetic homology. Chromosomes analyses, characterized by banding techniques with fluorochrome or fluorescence *in situ* hybridization with a labeled DNA fragment such as 5S and 18S-5.8S-26S rDNAs, have shown abundant evidence of evolution and heredity in higher plants (Ansari et al., 2008; Cai et al., 2006; Marcon et al., 2005; Xu et al., 2013; Yamamoto, 2012). Molecular markers, such as random amplified polymorphhic DNA (RAPD), cleaved amplified polymorphic sequence (CAPS), amplified fragment length polymorphism (AFLP), simple sequence repeat (SSR), and single nucleotide polymorphism (SNP) have been especially used for elucidating the level of genetic diversity and relationships in many taxonomic groups (Choi and Wen, 2000; Garcia-Lor et al., 2013; Millan et al., 1996; Weiguo et al., 2007; Xu and Ban, 2004). The fluorochrome chromomycin A3 (CMA) banding technique has been especially used in the classification and phylogeny research of *Citrus* (Cornelio et al., 2003; Moraes et al., 2007; Yamamoto et al., 2008). CMA exhibits preferential staining for GC-rich DNA sequences allowing the identification of different positions of heterochromatin. This technique is useful and suitable for the karyotype analysis of *Citrus* species, which have small-sized chromosomes and a stable chromosome number of 2n = 18.

In taxonomic research of *Fortunella* and the related genera, a variety of molecular markers have also been used in addition to the traditional means (Barrett and Rhodes, 1976; Iwamasa et al., 1985, 1988; Nicolosi et al., 2000; Pang et al., 2007). However, these studies were mostly concentrated on the genus *Citrus* or the subfamily Aurantioideae, and only a little detailed information has been described on the classification of *Fortunella*. The previous studies on *Fortunella* have been carried out on the morphological characteristics, the flavonoid characteristics and essential oils (Handa and Oogaki, 1985; Katayama et al., 1994; Nito et al., 1996; Ogawa et al., 2001). Isozyme analysis has also been used to estimate the relationships among the six *Fortunella* species (Rahman and Nito, 1994), and the phylogeny of *Citrus, Fortunella* and *Poncirus* (Handa et al., 1986). More recently, Garcia-Lor et al. (2013) performed detailed research on the phylogenetic relationships among germplasm collections in the “true citrus fruit trees” group by SSR and SNP analysis. They suggested that *Fortunella* is not a distant relative of *Citrus* since it was clustered within the *Citrus* clade on the dendrogram. Although the taxonomic data of *Fortunella* have been accumulated as described above, the results from these examinations were not sufficient for reconsidering the classification of the genus *Fortunella*, because they often showed contradictory results. Therefore, it is necessary to accumulate more useful information by conducting several different analyses to obtain a comprehensive estimation of the phylogeny of this genus. We have estimated phylogeny of *Fortunella* based on DNA polymorphisms used by RAPD and cytoplasmic CAPS (Yasuda et al., 2010). In the present study, we investigated the CMA karyotype composition of the genus *Fortunella* to obtain additional significant phylogenetic information of this genus.

**Materials and Methods**

The six *Fortunella* species based on the classification systems of Tanaka (1933) were used for this study: *F. hindsii*, *F. margarita*, *F. japonica*, *F. crassifolia*, *F. polyandra*, and *F. obovata*. As controls, *C. madurensis*, *C. unshiu* Marcow. ‘Aoshima-unshiu’ (Satsuma mandarin), and *Poncirus trifoliata* (L.) Raf. (trifoliare orange) were used. These samples were obtained from mature trees at the Japan Mandarin Center (Kagoshima, Japan), Saga University (Saga, Japan), and the Kumamoto Prefectural Research Center (Kumamoto, Japan) (Table 1).

Young leaves (approximately 3–5 mm long) were excised from each plant, immersed in 2 mM 8-hydroxyquinoline for 10 hr at 4°C, and fixed in a mixed solution of ethanol and acetic acid (3:1) for 12 hr at 4°C. Enzymatic maceration and air-drying were performed according to the method of Fukui (1996) and Yahata et al. (2015) with some modifications. The young leaves were washed in distilled water to remove the fixative, and then macerated in an enzyme mixture containing 2.0% (w/v) Cellulase Onozuka RS (Yakult Pharmaceutical Ind. Co. Ltd., Tokyo, Japan), 1.0% (w/v) Macerozyme (MP Biomedicals, Inc., Santa Ana, CA, USA), 0.3% Pectolyase Y-23 (w/v) (KYOWAKASEI Co. Ltd., Saitama, Japan) and 200 mM EDTA at 37°C for 40 min. The chromosomes were stained with 2.0% Giemsa solution (Merck KGaA, Darmstadt, Landkreis Darmstadt-Dieburg, Germany) in 1/30 M phosphate buffer (pH 6.8) for 30 min. They were then rinsed with distilled water, air dried, and observed under an optical microscope. After confirming the number and position on the slide, the chromosomes...
were de-stained with 70% methanol, re-stained with 0.1 mg·L⁻¹ CMA and 0.1 mg·L⁻¹ Distamycin A hydrochloride (Sigma-Aldrich Co. St. Louis, MO, USA) according to Befu et al. (2000) with some modification, and observed under a fluorescence microscope with a BV filter cassette (Olympus Co. Ltd., Tokyo, Japan).

### Results and Discussion

All *Fortunella* species were confirmed diploid (2n = 2x = 18) by counting the chromosome numbers. Furthermore, when we performed CMA banding, the chromosomes of *Fortunella* were classified into the following six types based on the number and position of the CMA banding pattern according to Yamamoto and Tominaga (2003) and Yamamoto et al. (2007): A = two telomeric bands and one proximal band, B = one telomeric band and one proximal band, C = two telomeric bands, D = one telomeric band, E = no band, F = one proximal band and Dst = type D with a satellite chromosome (Fig. 1). The karyotype compositions of the genus *Fortunella* are shown in Table 1 and Figure 2. Almost of the *Fortunella* species had either one or two characteristic type F chromosomes. *Fortunella hindsii* had the simplest karyotype without types A, E, and Dst chromosomes (1B + 1C + 14D + 2F). In addition, a characteristic type Dst chromosome was observed in *F. margarita* (1A + 1B + 2C + 13D + 1F + 1Dst), *F. japonica* (2A + 2C + 12D + 2Dst), *F. crassifolia* (2A + 2C + 12D + 1F + 1Dst), and *F. polyandra* (1A + 2C + 11D + 1E + 2F + 1Dst). Of the 6 *Fortunella* species, the type E chromosomes were found only in *F. polyandra* and *F. obovata* (1A + 1B + 2C + 10D + 3E + 1F). As a whole, the type D chromosomes were predominant in this genus, and 10 to 14 of the 18 chromosomes belonged to this type. In previous studies, the karyotype compositions were revealed in three species of the genus *Fortunella*, *F. hindsii*, *F. margarita*, and *F. crassifolia* to be 2B + 16D, 1A + 1B + 2C + 13D + 1F, and 2A + 2C + 14D, respectively (Abkenar et al., 2007; Miranda et al., 1997). Our present results showed different karyotype compositions from those in these previous reports. The type F chromosomes in citrus were first reported by Yamamoto and Tominaga (2003), and therefore, this chromosome type might have been confused with the type D chromosomes due to overlooking the telomeric negative band before this report was made. In the same way, the type Dst chromosomes in citrus were also first described by Yamamoto et al. (2007). Therefore, the chromosome type might have been misidentified with type B and D chromosomes, which has a similar CMA banding pattern. The karyotype compositions of the *Citrus* and *Poncirus* species used as the controls were 1A + 1C + 13D + 3E in *C. madurensis*, 1A + 1C + 8D + 8E in *C. unshiu ‘Aoshima-unshiu’*, and 4B + 8D + 6E in *P. trifoliata*,

### Table 1. Composition of CMA Karyotype in Genus *Fortunella*.

<table>
<thead>
<tr>
<th>Taxon</th>
<th>Source</th>
<th>Chromosome number</th>
<th>CMA karyotype composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genus <em>Fortunella</em> Subgenus <em>Protocitrus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. hindsii</em> var. chintou Swingle.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>1B + 1C + 14D + 2F</td>
</tr>
<tr>
<td>Genus <em>Fortunella</em> Subgenus <em>Eufortunella</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>F. margarita</em> (Lour.) Swingle.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>1A + 2C + 13D + 1F + 1Dst</td>
</tr>
<tr>
<td><em>F. japonica</em> (Thumb.) Swingle.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>2A + 2C + 12D + 2Dst</td>
</tr>
<tr>
<td><em>F. crassifolia</em> Swingle.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>2A + 2C + 12D + 1F + 1Dst</td>
</tr>
<tr>
<td><em>F. polyandra</em> (Ridl.) Tan.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>1A + 2C + 11D + 1E + 2F + 1Dst</td>
</tr>
<tr>
<td><em>F. obovata</em> hort. ex Tan.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>1A + 1B + 2C + 10D + 3E + 1F</td>
</tr>
<tr>
<td>Genus <em>Citrus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>C. madurensis</em> Lour.</td>
<td>MC</td>
<td>2n = 2x = 18</td>
<td>1A + 1C + 13D + 3E</td>
</tr>
<tr>
<td><em>C. unshiu</em> Marcow. ‘Aoshima-unshiu’</td>
<td>KP</td>
<td>2n = 2x = 18</td>
<td>1A + 1C + 8D + 8E</td>
</tr>
<tr>
<td>Genus <em>Poncirus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. trifoliata</em> (L.) Raf.</td>
<td>SU</td>
<td>2n = 2x = 18</td>
<td>4B + 8D + 6E</td>
</tr>
</tbody>
</table>

z MC: Japan Mandarin Center, SU: Saga University, KP: Kumamoto Prefectural Research Center.

y As shown in Figure 2.
respectively. The CMA fluorochrome CMA banding technique was successfully applied to characterize the chromosomes of all *Fortunella* species as well as those of *Citrus*.

According to both the Swingle (1915) and Tanaka (1933) classification systems, only *F. hindsii* was separated from other *Fortunella* species as the subgenus *Protocitrus* because of the unique morphological characteristics of this species. In the present study, *F. hindsii* showed the simplest CMA karyotype composition, indicating that *F. hindsii* was the most distantly related to the other species of the genus *Fortunella*. Handa and Oogaki (1985) revealed tetraploid *F. hindsii* into distant relation to *F. margarita* and *F. japonica* by multivariate analysis of morphological characteristics. In a similar way, Katayama et al. (1994) also reported *F. hindsii* is distinctly independent in the genus *Fortunella* by essential oil analysis and morphological characteristics. In addition, the complete absence of type E chromosomes, which are predominant in *Citrus* (Yamamoto and Tominaga, 2003), suggests that *F. hindsii* has a different origin and process of derivation from *Citrus*. Thus, these data support the hypothesis established by the previous taxonomic studies in which only *F. hindsii* could be separated as a primitive species from the other five species.

![Fig. 2. Photographs and idiograms of the somatic chromosome stained with CMA. Bars = 5 μm.](image-url)
Interestingly, CMA banding revealed the presence of backcross of the type Dst chromosome and the deletion of type E chromosomes. Swingle and Reece (1967) also described that F. crassifolia might have resulted from chance hybridization between F. margarita and F. japonica, or a backcross of the Citrus-Fortunella hybrid with Fortunella. Although we were not able to clarify the process of derivation in these species, the present results at least suggest that these three species have a close relationship to each other.

Fortunella polyandra and F. obovata were classified into the genus Fortunella by Tanaka (1933). In the past, it was reported that C. madurensis is an intergeneric hybrid between Fortunella and Citrus (Cheng et al., 2005; Handa and Oogaki, 1985; Swingle and Reece, 1967). Interestingly, CMA banding revealed the presence of type E chromosomes in the karyotype compositions of F. polyandra and F. obovata as C. madurensis. The E chromosomes were not found in any other Fortunella species, but the karyotype consisting mainly of E type chromosomes was an elemental chromosome type for most of the Citrus species, according to previous studies (Yamamoto and Tominaga, 2003; Yamamoto et al., 2007). Miranda et al. (1997) also described Citrus and Fortunella karyotypes seemed to be discontinuous from the result of Fortunella had relatively large CMA-positive regions. Therefore, it can be considered that the E chromosomes observed in the karyotype compositions of F. polyandra and F. obovata are derived from Citrus, and so it is natural to consider that these species are also intergeneric hybrids between Fortunella and Citrus. If this hypothesis would be right, it could be explained for a conclusion derived from essential oil analysis as F. polyandra and F. obovata are independent and are different from F. margarita, F. japonica, and F. crassifolia (Katayama et al., 1994). In these two species, F. polyandra had more than one of each of the type F and Dst chromosome than F. obovata in the present study. Therefore, F. polyandra might have a genetic background which is more related to Fortunella than Citrus. Swingle and Reece (1967) proposed that F. polyandra might be a limequat, i.e., a hybrid of Fortunella and some variety of the C. aurantifolia (Cristm.) Swingle (lime), although most of the morphological characteristics of F. polyandra resembled those of other Fortunella. This inconsistency could be explained by the genetic background which is more related to Fortunella than Citrus presumed in the present study. Swingle and Reece (1967) also described another possibility that F. obovata may be an intrageneric chance hybrid between two Fortunella species. From the cytological point of view, our results showed that F. obovata was not an intrageneric hybrid but an intergeneric hybrid with Citrus.

Three points about the phylogeny and classification of Fortunella discussed based on karyotype composition in this study, (i) the independence of F. hindsii, (ii) the close relationship among F. margarita, F. japonica and F. crassifolia, and (iii) the possibility of intergeneric hybrids of F. polyandra and F. obovata, are completely consistent with the similarity classification of DNA polymorphisms by Yasuda et al. (2010).

In conclusion, we propose the following hypothesis about the Fortunella phylogeny. Fortunella hindsii, which belongs to the subgenus Protocitrus, is a surviving ancestor of the other Fortunella species. Three Eufortunella, F. margarita, F. japonica, and F. crassifolia, might have derived from numerous mutations and crossings involving F. hindsii or other extinct Fortunella species. Fortunella polyandra and F. obovata are the results of later hybridization between Fortunella and Citrus. From the results of analyses with CMA karyotype composition, the genetic background of F. polyandra is more related to Fortunella than Citrus as compared with F. obovata. Therefore, we think that there are only two true species for the genus Fortunella, F. hindsii and F. margarita complex which includes F. margarita, F. japonica, and F. crassifolia. Moreover, F. polyandra and F. obovata should be classified as natural or horticultural hybrids. This study provided us important information for reconsidering the classification and phylogeny of the genus Fortunella. However, we were not able to clearly determine the process of derivation in three closely related species involved in F. margarita complex, F. margarita, F. japonica, and F. crassifolia. Furthermore, more distinct information is needed about derivation of each karyotype chromosomes including the characteristic type A, F and Dst chromosomes and immediate ancestor of F. polyandra and F. obovata as putative hybrids crossed with Citrus to establish our hypothesis. To clarify these issues, we have been attempting to collect more information by using the CMA banding techniques combined with fluorescence in situ hybridization or genomic in situ hybridization, as well as other analytical techniques such as AFLP, SSR, and SNP (Barkley et al., 2006; Garcia-Lor et al., 2013; Kitajima et al., 2007; Moraes et al., 2007; Pang et al., 2007).

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