

Original Article

## Phylogeny and Classification of *Fortunella* (Aurantioideae) Inferred from DNA Polymorphisms.

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**Summary :** *Fortunella* are 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 RAPD and cytoplasmic CAPS analyses in order to understand the relationship and phylogeny of the genus *Fortunella*. Among the 6 *Fortunella* species examined, *F. hindsii* showed the most distant position on the dendrogram based on RAPD analysis. On the other hand, close relationships were found among the 3 species *F. margarita*, *F. japonica* and *F. crassifolia*. *F. obovata* was separated into the same cluster as *C. madurensis*, which was reported to be an intergeneric hybrid between *Fortunella* and *Citrus*, and the band patterns on CAPS analysis for the cpDNA regions were similar to other *Fortunella* species than *Citrus*. We concluded that there are only two true species for the genus *Fortunella*, *F. hindsii* and *F. margarita* complex, moreover, *F. obovata* should be provided a taxonomic rank as natural or horticultural intergeneric hybrids.

**Key words :** CAPS analysis, DNA polymorphisms, *Fortunella*, RAPD analysis

### Introduction

The genus *Fortunella* (kumquat) is one of the most important genera with *Citrus* and *Poncirus* in the sub family Aurantioideae (Citroideae), of the family Rutaceae. 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 in 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, 1967) and Tanaka

(1933), based mainly on morphological characteristics. According to the classification system of Tanaka (1933), this genus consists of six species, including the Hongkong kumquat (*Fortunella hindsii* var. *chintou* Swing.) as the subgenus *Protocitrus*, and the oval kumquat [*F. margarita* (Lour.) Swing.], round kumquat [*F. japonica* (Thunb.) Swing.], Meiwa kumquat (*F. crassifolia* Swing.), Malayan kumquat [*F. polyandra* (Ridl.) Tan.] and Changshou kumquat (*F. obovata* hort. ex Tan.) as the subgenus *Eufortunella*. On the other hand, Swingle (1967) 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 calamondin (*Citrus madurensis* Lour.) and *C. halimii* B. C. Stone were believed to be of hybrid origin (Handa & Oogaki 1985; Scora *et al.* 1988), and they have recently been proven to be intergeneric hybrids between *Fortunella* and *Citrus* (Cheng *et al.* 2005; Barkley *et al.* 2006; Pang *et al.* 2007). Although there has been argued for a long time on the correct classification of this genus with respect to the problem involved in the two different classification systems described above, and the origin of some species as the natural hybrids, there have been few reports providing compelling evidences to resolve these problems. Thus, an understanding of the classification and phylogeny surrounding the genus *Fortunella* are still controversial and confusing.

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 development of 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 been shown many evidences of evolution and heredity in higher plants (Marcon *et al.* 2005; Cai *et al.* 2006; Ansari *et al.* 2008). Molecular markers, such as random amplified polymorphic DNA (RAPD), cleaved amplified polymorphic sequence (CAPS), amplified fragment length polymorphism (AFLP) and simple sequence repeat (SSR), have been especially used for elucidating the level of genetic diversity and relationships in many taxonomic groups (Millan *et al.* 1996; Choi & Wen 2000; Xu & Ban 2004; Nicolosi *et al.* 2000; Weiguo *et al.* 2007).

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 & Rhodes 1976; Iwamasa *et al.* 1985; Iwamasa *et al.* 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 few detailed information have been described on the classification of *Fortunella*. The previous studies on *Fortunella* have been carried out on the morphological characters, the flavonoid characters and essential oils (Handa & 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 & Nito 1994), and the phylogeny of *Citrus*, *Fortunella* and *Poncirus* (Handa *et al.* 1986). More recently, Barkley *et al.* (2006) performed detailed research on the phylogenetic relationships among germplasm collections in three genera, *Citrus*, *Fortunella* and *Poncirus*, by SSR marker analysis. In this study, they described that *Fortunella* was clustered within the *Citrus* clade on the dendrogram, suggesting that *Fortunella* is not a distant relative of *Citrus*. 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 different analyses to get a comprehensive estimation on the phylogeny of this genus. In the present study, we investigated about RAPD and cytoplasmic CAPS polymorphism with 6 *Fortunella* species, in order to better understand the phylogeny and classification of the genus *Fortunella*.

## Materials and Methods

The six *Fortunella* species based on the classification systems of Swingle (1967) and Tanaka (1933) were used for this study: Hongkong kumquat [*Fortunella hindsii* var. *chintou* Swing.], oval kumquat [*F. margarita* (Lour.) Swing.], round kumquat [*F. japonica* (Thunb.) Swing.], Meiwa kumquat (*F. crassifolia* Swing.), Malayan kumquat [*F. polyandra* (Ridl.) Tan.] and Changshou kumquat (*F. obovata* hort. ex Tan.) (Fig. 1). As controls, calamondin (*C. madurensis* Lour.) (Fig. 2), 'Aoshima-unshiu' satsuma mandarin (*C. unshiu* Marcow.) and trifoliolate orange [*Poncirus trifoliata* (L.) Raf.] were used. In addition, yuzu (*C. juno* Siebold ex Tan.), 'Eureka' lemon [*C. limon* (L.) Burm.f.], fingered citron (*C. medica* L. var. *sarcodactylis* (Hoola van Nooten) Swing.), 'Marsh' grapefruit (*C. paradise* Macfad.) and 'Tarocco' sweet orange [*C. sinensis* (L.) Osb.] were used as the controls for the RAPD analysis. These samples were obtained from mature trees preserved at the Japan Mandarin Center (Kagoshima, Japan), Saga University (Saga, Japan) and the Kumamoto Prefectural Research Center (Kumamoto, Japan).

The total DNA was extracted from young leaves of each plant according to the method of Doyle and Doyle (1987). The total DNA was used for RAPD and CAPS analyses.

RAPD analysis of the nuclear DNA was



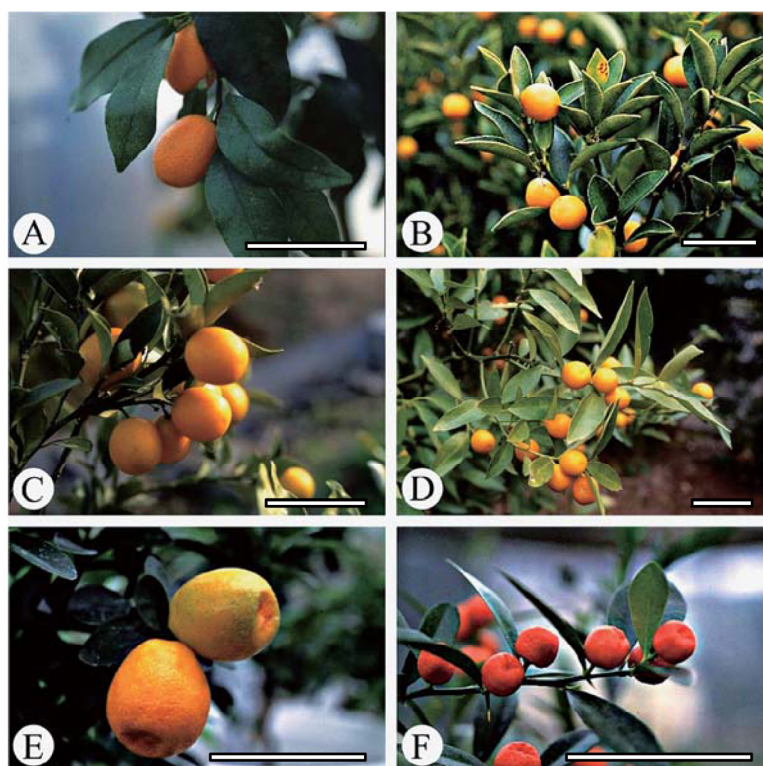


Fig. 1. Morphological characteristics of fruits in 6 *Fortunella* species. (Bars = 5 cm)



Fig. 2. Morphological characteristics of fruits in *C. madurensis* considered as intergeneric hybrid between *Citrus* and *Fortunella*. (Bar = 5 cm)

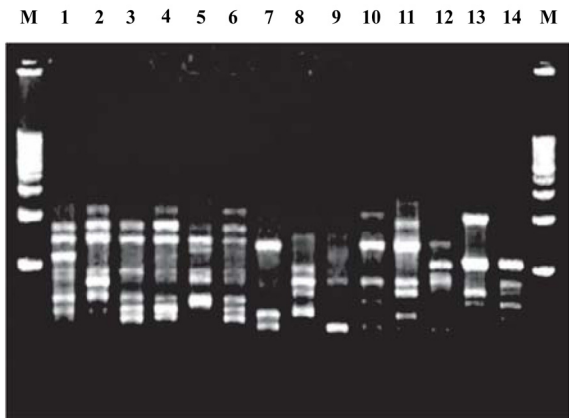
performed by modified methods of Williams *et al.* (1990). PCR was performed with Operon random 10-mer primers OPA1-20 (Operon Technology Inc., CA, USA) by ASTEC Program Control System PC-700 (ASTEC Co., Ltd., Fukuoka, Japan). DNA amplification reactions were performed in volumes of 25  $\mu$ l with 10 mM Tris-HCl (pH 8.0), 80 mM KCl, 1.5 mM MgCl<sub>2</sub>, 100  $\mu$ M dNTPs, 0.3  $\mu$ M primers, 2.5 unit of Tth DNA Polymerase (TOYOBO Co., Ltd., Osaka, Japan) and 10 ng DNA. The thermocycler was programmed for 30 sec at 94°C for 1 cycle, followed by 30 sec at 94°C, 2 min at 37°C, 3 min at 72°C for 45

cycle. The reaction products were electrophoresed on 1.5% agarose gels containing 25  $\mu$ l/l SYBR Safe™ (Life Technologies Japan Ltd., Tokyo, Japan) and subsequently photographed under ultraviolet light (360 nm). For each combination of sample and primers, PCR was carried out twice, and only stable polymorphisms were analyzed; 335 out of 461 RAPD polymorphisms obtained by PCR were used for statistical analyses. Based on RAPD polymorphism data, a dendrogram and a diagram were prepared using UPGMA and quantas type 3 analyses, respectively. CAPS analysis was performed for several chloroplastic (cp) and mitochondrial (mt) non-coding regions. For analysis of cpDNA, two primer pairs of *rbcl-psaI* (F: 5'-TTTGGTGGAGGAACCTTTAGGACACCCTTGGGG-3', R: 5'-GCAATTGCCGAAA TACTAAGC-3') and *trnD-trnT* (F: 5'-ACCAATTG AACTACAATCCC-3', R: 5'-CTACCACTGAGTTA AAAGGG-3') were used for amplification according to the methods of Cheng *et al.* (2002) and Ureshino and Miyajima (2002). For analysis of mtDNA, two primer pairs of *18SrRNA-5SrRNA* (F: 5'-GTGTTGC TGAGACATGCGCC-3', R: 5'-ATATGGCGCAAG ACGATTCC-3') and *nad5/1-nad5/2r* (F: 5'-TTTTT TCGGACGTTTTCTAG-3', R: 5'-TTGGCCAAGTA TCCTACAA-3') were used for amplification according to the methods of Cheng *et al.* (2002) and

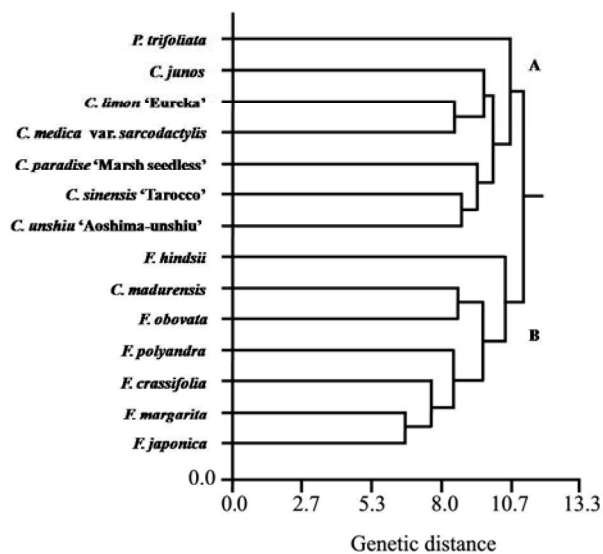
Dumolin-Lapegue *et al.* (1997). The PCR products were digested with three restriction endonucleases of *Hap* II, *Hinf* I, *Mbo* I (TOYOBO Co., Ltd., Osaka, Japan) and electrophoresed.

## Results and Discussion

We made a dendrogram by UPGMA based on RAPD polymorphism data (Fig. 3) to clarify the genetic homology among the species of the 3 genera, *Fortunella*, *Citrus* and *Poncirus* (Fig. 4). The quantas

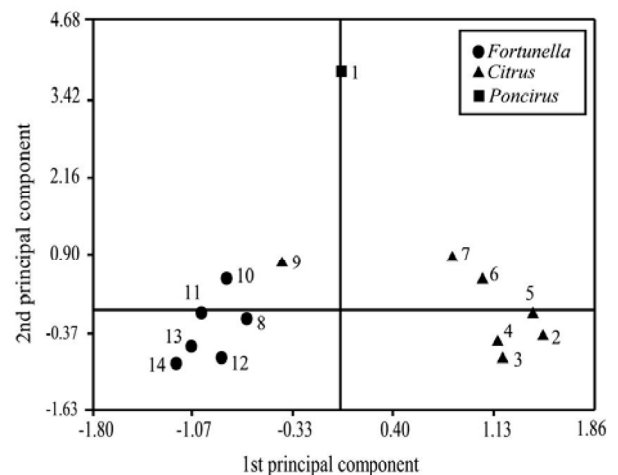


**Fig. 3.** RAPD polymorphisms in *Fortunella* 6 species, *Citrus* 7 species and *Poncirus* 1 species. (1) *P. trifoliata*, (2) *C. junos*, (3) *C. limon* 'Eureka', (4) *C. medica* var. *sarcodactylis*, (5) *C. paradise* 'Marsh seedless', (6) *C. sinensis* 'Tarocco', (7) *C. unshiu* 'Aoshima-unshiu', (8) *F. hindsii*, (9) *C. maudurensis*, (10) *F. obovata*, (11) *F. polyandra*, (12) *F. crassifolia*, (13) *F. margarita*, (14) *F. japonica*, (M) 1 kb ladder marker.



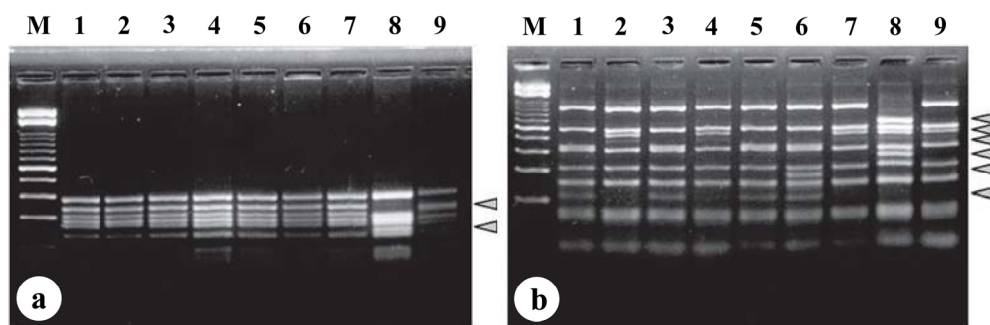
**Fig. 4.** Dendrogram of *Fortunella* 6 species, *Citrus* 7 species and *Poncirus* 1 species based on the cluster analysis from RAPD analysis with UPGMA.

type 3 analysis was also used to produce another diagram for clustering the genera (Fig. 5). Based on the data of these two classification methods, these species were clustered into two main groups: group A consisted of *Poncirus* and *Citrus*, and group B mostly consisted of *Fortunella*, which was distinctly separated from *Citrus* and *Poncirus* in the diagram of the quantas type 3 analysis. In group B, *F. hindsii* was distantly placed from the other *Fortunella* species. *F. margarita* and *F. japonica* were clustered in the same group, and *F. crassifolia* was also placed in a comparatively close position to this group. Interestingly, the dendrogram and diagram of the quantas type 3 analysis showed that *C. madurensis* and *F. obovata* were clustered together in the same group as *Fortunella*. As another approach, we performed CAPS analysis for several cp- and mt- non-coding regions to clarify the genetic homology in cytoplasmic DNA using the *Fortunella* 6 species, *C. unshiu*, *C. madurensis* and *P. trifoliata*. Consequently, polymorphic bands were obtained with the primer/restriction enzyme combinations of *psal-rbcI*/*Mbo* I and *trnD-trnT*/*Hinf* I for the cpDNA regions, while no polymorphic bands were detected in the mtDNA regions (Fig. 6). *F. margarita*, *F. japonica*, *F. crassifolia*, and *F. obovata* had three specific bands obtained with *psal-rbcI*/*Mbo* I that were similar to each other. *C. unshiu* was the only species which showed many bands, which were different from the other species and their primer / re-



**Fig. 5.** Scatter diagram of *Fortunella* 6 species, *Citrus* 7 species and *Poncirus* 1 species projected on the first and second axes of quantification theory III based on RAPD analysis. (1) *P. trifoliata*, (2) *C. junos*, (3) *C. limon* 'Eureka', (4) *C. medica* var. *sarcodactylis*, (5) *C. paradise* 'Marsh seedless', (6) *C. sinensis* 'Tarocco', (7) *C. unshiu* 'Aoshima-unshiu', (8) *F. hindsii*, (9) *C. maudurensis*, (10) *F. obovata*, (11) *F. polyandra*, (12) *F. crassifolia*, (13) *F. margarita*, (14) *F. japonica*.





**Fig. 6.** Restriction pattern of the *Mbo*I and *Hinf*I digested respectively *trnD* - *trnT* (a) and *psal* - *rbcL* (b) regions of chloroplast genomes in *Fortunella*. Letters of lanes show follow, (1) *F. hindsii*, (2) *F. margarita*, (3) *F. japonica*, (4) *F. crassifolia*, (5) *F. polyandra*, (6) *F. obovata*, (7) *C. madurensis*, (8) *C. unshiu* 'Aoshima-unshiu', (9) *P. trifoliata*, (M) 100 bp ladder marker. Allows indicate polymorphic fragments.

striction enzymes. Of the many molecular markers, RAPD and CAPS analyses are more commonly used for the phylogenetic systematics of plants because of their convenience (Badenes & Parfitt 1995; Lim *et al.* 1999; Tercek *et al.* 2003; Yonemori *et al.* 1998). In summary, we were able to reveal the genetic relationship in the genus *Fortunella* by RAPD analysis in the present study. Although no polymorphism was obtained in the mtDNA regions, CAPS analysis provided some information for understanding the homology of the cpDNA regions.

According to both the Swingle (1967) and Tanaka (1933) classification systems, only *F. hindsii* was determinately separated from other *Fortunella* species as the subgenus *Protocitrus* because of the unique morphological characters of this species. In the present study, *F. hindsii* showed the most distant cluster from the other species of the genus *Fortunella* based on RAPD analysis, supporting the theory established by the previous taxonomic studies that only *F. hindsii* could be separated as a primitive species from the other five species.

In the genus *Fortunella*, *F. margarita*, *F. japonica* and *F. crassifolia* showed comparatively close genetic distance on the dendrogram by RAPD analysis, and the same specific bands in CAPS analysis for the cpDNA regions. Swingle (1967) also described how *F. crassifolia* might have resulted from chance hybridization between *F. margarita* and *F. japonica*, or a backcross of the *Citrus-Fortunella* hybrid with *Fortunella*. The present results suggest that the three species have a close relation to each other, although we were not able to understand the process of derivation in these species.

Although *F. obovata* were classified into the genus *Fortunella* by Tanaka (1933), this species was separated into the same cluster as *C. madurensis* on the dendrogram in the present study. It was reported

that *C. madurensis* was an intergeneric hybrid between *Fortunella* and *Citrus* (Swingle 1967; Handa & Oogaki 1985; Cheng *et al.* 2005). Therefore, it is natural to consider that *F. obovata* are also intergeneric hybrids. On the other hand, *F. polyandra* was separated into the same cluster as *F. margarita*, *F. japonica* and *F. crassifolia* not *C. madurensis* on the dendrogram and scatter diagram in the present study. These results suggest that *F. polyandra* might have a genetic background which is more related to *Fortunella* than *Citrus*. Swingle (1967) proposed that *F. polyandra* might be a limequat, i.e., a hybrid of *Fortunella* and some variety of the lime (*C. aurantifolia*), but also stated that most of morphological characters resembled those of *Fortunella*. This inconsistency could be explained by the genetic background presumed in the present study. The maternal ancestors of *F. obovata* would probably be *Fortunella* species from the results on CAPS analysis for the cpDNA regions. Swingle (1967) also described how *F. obovata* may be an intrageneric chance hybrid between two *Fortunella* species. From molecular biological point of view, our results showed that *F. obovata* was not an intrageneric hybrid but an intergeneric hybrid with *Citrus*.

In conclusion, we propose the following hypothesis about the *Fortunella* phylogeny. *F. hindsii*, which belongs to the subgenus *Protocitrus*, is a surviving ancestor for 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 *Protocitrus* species. *F. obovata* is the results of later hybridization between *Fortunella* and *Citrus*. Therefore, we think that there are only two true species for the genus *Fortunella*, *F. hindsii* and another species including the *F. margarita* complex. Moreover, *F. obovata* should be provided a taxonomic

rank as natural or horticultural hybrids. This study provided us with important information for reconsidering the classification and phylogeny of the genus *Fortunella*. However, we were not able to clearly understand the process of derivation in *F. polyandra* and three closely related species, *F. margarita*, *F. japonica* and *F. crassifolia*. To clarify these issues, we have been attempting to collect more information with the latest analytical techniques, including the CMA banding techniques combined with fluorescence *in situ* hybridization or genomic *in situ* hybridization, and AFLP and SSR (Barkley *et al.* 2006 ; Kitajima *et al.* 2007 ; Moraes *et al.* 2007 ; Pang *et al.* 2007).

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### Literature Cited

- Ansari, H. A., N. W. Ellison, W. M. Williams (2008) Molecular and cytogenetic evidence for an allotetraploid origin of *Trifolium dubium* (Leguminosae). *Chromosoma* **117**, 159-167.
- Badenes, M. L. & D. E. Parfitt (1995) Phylogenetic relationships of cultivated *Prunus* species from an analysis of chloroplast DNA variation. *Theor. Appl. Genet.* **90**, 1035-1041.
- Barkley, N. A., M. L. Roose, R. R. Krueger, C. T. Federici (2006) Assessing genetic diversity and population structure in a citrus germplasm collection utilizing simple sequence repeat markers (SSRs). *Theor. Appl. Genet.* **112**, 1519-1531.
- Barrett, H. C. & A. M. Rhodes (1976) A numerical taxonomic study of affinity relationships in cultivated *Citrus* and its close relatives. *Syst. Bot.* **1**, 105-136.
- Cai, Q., D. Zhang, Z. L. Liu, X. R. Wang (2006) Chromosomal localization of 5S and 18S rDNA in five species of subgenus *Strobilus* and their implications for genome evolution of Pinus. *Ann. Bot.* **97**, 715-722.
- Cheng, Y. J., W. W. Guo, X. X. Deng (2002) Inheritance of organelle genomes of the somatic hybrid between Cleopatra mandarin (*Citrus reticulata*) and Flying dragon (*Poncirus trifoliata*). *Acta Genet. Sin.* **29**, 364-369.
- Cheng, Y., M. C. Vicente, H. Meng, W. Guo, N. Tao, X. Deng (2005) A set of primers for analyzing chloroplast DNA diversity in *Citrus* and related. *Tree Physiol.* **25**, 661-672.
- Choi, H. K. & J. Wen (2000) A phylogenetic analysis of *Panax* (Araliaceae) : integrating cpDNA restriction site and nuclear rDNA sequence data. *Plant Syst. Evol.* **224**, 109-120.
- Doyle, J. & J. L. Doyle (1987) A rapid DNA isolation procedure for small quantities fresh leaf tissue. *Phytochemistry Bull.* **19**, 11-15.
- Dumolin-Lapegue, S., M. H. Pemonge, R. J. Petit (1997) An enlarged set of consensus primers for the study of organelle DNA in plants. *Molec. Ecol.* **6**, 393-397.
- Handa, T., Y. Ishizawa, C. Oogaki (1986) Phylogenetic of fraction I protein in the genus *Citrus* and its close related genera. *Japan. J. Genet.* **61**, 15-24.
- Handa, T. & C. Oogaki (1985) Numerical taxonomic study of *Citrus* L. and *Fortunella* Swingle using morphological characters — Application of multivariate analysis —. *J. Japan. Soc. Hort. Sci.* **54**, 145-154.
- Iwamasa, M., N. Nito, J. T. Ling (1988) Intra- and intergeneric hybridization in the orange subfamily, Aurantioideae. *In Proc. Int. Soc. Citriculture*, vol 1. (Eds. R. Goren & K. Mendel) Balaband, Rehovot, Israel and Margrat Publishers, Weikersheim, Germany. pp. 123-130.
- Iwamasa, M., N. Nito, Y. Katayama, T. Yamaguchi, S. Matsunaga (1985) Cross-compatibility between Aurantioideae plants. *Bull. Fac. Agr., Saga Univ.* **59**, 57-69.
- Katayama, Y., N. Nito, J. Machino (1994) Species identification of genus *Fortunella* by essential oil analysis. *Bull. Fac. Agr., Saga Univ.* **77**, 37-45.
- Kitajima, A., A. Yamasaki, T. Habu, B. Preedasuttijit, K. Hasegawa (2007) Chromosome identification and karyotyping of Satsuma mandarin by Genomic *in situ* hybridization. *J. Amer. Soc. Hort. Sci.* **132**, 836-841.
- Kress, W. J. (1983) Crossability Barriers in Neotropical Heliconia. *Ann. Bot.* **52**, 131-147.
- Lim, S. H., P. C. P. Teng, Y. H. Lee, C. J. Goh (1999) RAPD analysis of some species in the genus *Vanda* (Orchidaceae). *Ann. Bot.* **83**, 193-196.
- Marcon, A. B., I. C. L. Barros, M. Guerra (2005) Variation in chromosome number, CMA bands and 45S rDNA sites in species of *Selaginella* (Pteridophyta). *Ann. Bot.* **95**, 271-276.
- Millan, T., F. Osuna, S. Cobos, A. M. Torres, J. I. Cubero (1996) Using RAPDs to study phylogenetic relationship in *Rosa*. *Theor. Appl. Genet.* **92**, 273-277.
- Moraes AP, Soares Filho WS, Guerra M (2007)



- Karyotype diversity and origin of grapefruits. *Chromosome Res.* **15**, 115-121.
- Nicolosi, E., Z. N. Deng, A. Gentile, S. L. Malfa, G. Continella, E. Tribulato (2000) Citrus phylogeny and genetic origin of important species as investigated by molecular marker. *Theor. Appl. Genet.* **100**, 1155-1166.
- Nito, N., Y. Katayama, S. Yamaguchi (1996) Characterization of pollen grains of orange subfamily, Aurantioideae, plants. In Proc. Int. Soc. Citriculture, vol 1. (Eds. R. Goren & K. Mendel) Balaband, Rehovot, Israel and Margrat Publishers, Weikersheim, Germany. pp. 212-215.
- Ogawa, K., A. Kawasaki, M. Omura, T. Yoshida, Y. Ikoma, M. Yano (2001) 3', 5'-Di-C- $\beta$ -glucopyranosylphloretin, a flavonoid characteristic of the genus *Fortunella*. *Phytochemistry* **57**, 737-742.
- Pang, X.M., C.G. Hu, X.X. Deng (2007) Phylogenetic relationships within *Citrus* and its related genera as inferred from AFLP markers. *Genet. Resour. Crop Evol.* **54**, 429-436.
- Rahman, M.M. & N. Nito (1994) Phylogenetic relationships in the kumquat (*Fortunella*) as revealed by isozyme analysis. *Sci. Hort.* **57**, 17-28.
- Scora, R.W., T.E. Williams, S.Y. Wan (1988) Intra- and intergeneric relationships of *Citrus* halimii, Aurantioideae: investigation of leaf isozymes. *Punjab Fruits J.* **41**, 22-30.
- Smith, P. M. (1972) Serology and species relationships in annual bromes (*Bromus* L. sect. *Bromus*). *Ann. Bot.* **36**, 1-30.
- Swingle, W. T. (1915) A new genus, *Fortunella*, comprising four species of kumquat oranges. *J. Washington Acad. Sci.* **5**, 165-176.
- Swingle, W.T. & P.C. Reece (1967) The botany of *Citrus* and its wild relatives in the orange subfamily. In: The citrus industry, Vol. 1. (Eds. W. Reuther, H.J. Webber & L.D. Bachelor) Berkeley: Division of Agricultural Science, University of California, pp. 128-474.
- Tanaka, T. (1933) General remarks on the genus *Fortunella*, (2). *Stud. Citrol.* **6**, 19-40.
- Tercek, M. T., D. P. Hauber, S. P. Darwin (2003) Genetic and historical relationships among geothermally adapted *Agrostis* (Bentgrass) of North America and Kamchatka: evidence for a previously unrecognized, thermally adapted taxon. *Amer. J. Bot.* **90**, 1306-1312.
- Thoday, D. (1925) The geographical distribution and ecology of passerine. *Ann. Bot.* **39**, 175-208.
- Ureshino, K. & I. Miyajima (2002) The study on the relationship between leaf colors and ptDNA inheritance in inter-sectional cross of *Rhododendron kiusianum*  $\times$  *R. japonicum* f. *flavum*, resulting in an unexpected triploid progeny. *J. Japan. Soc. Hort. Sci.* **71**, 214-219.
- Webber, H. J. (1967) History and development of the citrus industry. In The citrus industry, Vol. 1. (Eds. W. Reuther, H. J. Webber & L. D. Bachelor) Berkeley: Division of Agricultural Science, University of California, pp. 1-39.
- Weiguo, Z., Z. Zhihua, M. Xuexia, Z. Yong, W. Sibao, H. Jianhua, X. Hui, P. Yile, H. Yongping (2007) A comparison of genetic variation among wild and cultivated *Morus* Species (Moraceae: *Morus*) as revealed by ISSR and SSR markers. *Biodivers. Conserv.* **16**, 275-290.
- Williams, J.G.K., A.R. Kubelik, K.J. Lival, J.A. Rafalski, S.V. Tingey (1990) DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucl. Acids Res.* **18**, 6531-6535.
- Xu, D.H. & T. Ban (2004) Phylogenetic and evolutionary relationships between *Elymus humidus* and other *Elymus* species based on sequencing of non-coding regions of cpDNA and AFLP of nuclear DNA. *Theor. Appl. Genet.* **108**, 1443-1448.
- Yin-Min, Y. (1985) The status of *Fortunella* genetic resources in China. *Fruit Varieties J.* **39**, 17-20.
- Yonemori, K., S. Kanzaki, D. E. Parfitt, N. Utsunomiya, S. Subhadrabandhu, A. Sugiura (1998) Phylogenetic relationship of *Diospyros kaki* (persimmon) to *Diospyros* spp. (Ebenaceae) of Thailand and four temperate zone *Diospyros* spp. from an analysis of RFLP variation in amplified cpDNA. *Genome* **41**, 173-182.

## DNA多型に基づいたキンカン属植物の系統発生と分類

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### 要 約

キンカン属植物 (*Fortunella*) は、ミカン科ミカン亜科植物に分類されているが、この属における分類と系統発生は、未だ混乱している。そこで、我々は、キンカン属の類縁関係と系統発生を理解するために、RAPD分析とオルガネラDNA領域のCAPS分析に基づいて評価を行った。試験に用いられたキンカン属6種のうち、マメキンカンは、RAPD分析により作成した系統樹において最も離れた位置を示した。一方、ナガキンカン、マルキンカンおよびニンポウキンカンの3種は、近い類縁関係を有していた。フクシュウキンカンは、キンカン属植物とカンキツ属植物との属間雑種と報告されているシキキツと同じクラスターに分類され、オルガネラDNA領域のCAPS分析におけるバンドパターンは、カンキツ属植物よりも他のキンカン属植物に類似していた。本研究の結果より、我々は、キンカン属の種が、マメキンカンとナガキンカンコンプレックス (ナガキンカン、マルキンカンおよびニンポウキンカン) の2つのみであり、フクシュウキンカンは自然的もしくは園芸的に派生した属間雑種として分類すべきであると結論付けた。

キーワード: CAPS分析, DNA多型, *Fortunella*, RAPD分析