Phylogenetic Relationships of Citrus and Its Relatives Based on matK Gene Sequences

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Abstract

The genus Citrus includes mandarin, orange, lemon, grapefruit and lime, which have high economic and nutritional value. The family Rutaceae can be divided into 7 subfamilies, including Aurantioideae. The genus Citrus belongs to the subfamily Aurantioideae. In this study, we sequenced the chloroplast matK genes of 135 accessions from 22 genera of Aurantioideae and analyzed them phylogenetically. Our study includes many accessions that have not been examined in other studies. The subfamily Aurantioideae has been classified into 2 tribes, Clauseneae and Citreae, and our current molecular analysis clearly discriminate Citreae from Clauseneae by using only 1 chloroplast DNA sequence. Our study confirms previous observations on the molecular phylogeny of Aurantioideae in many aspects. However, we have provided novel information on these genetic relationships. For example, inconsistent with the previous observation, and consistent with our preliminary study using the chloroplast rbcl genes, our analysis showed that Feronia oblata is not nested in Citrus species and is closely related with Feronia limonia. Furthermore, we have shown that Murraya paniculata is similar to Merrillia caloxylon and is dissimilar to Murraya koenigii. We found that “true citrus fruit trees” could be divided into 2 subclusters. One subcluster included Citrus, Fortunella, and Poncirus, while the other cluster included Microcitrus and Eremocitrus. Compared to previous studies, our current study is the most extensive phylogenetic study of Citrus species since it includes 93 accessions. The results indicate that Citrus species can be classified into 3 clusters: a citron cluster, a pummelo cluster, and a mandarin cluster. Although most mandarin accessions belonged to the mandarin cluster, we found some exceptions. We also obtained the information on the genetic background of various species of acid citrus grown in Japan. Because the genus Citrus contains many important accessions, we have comprehensively discussed the classification of this genus.

Introduction

The genus Citrus, which includes mandarin, orange, lemon, grapefruit and lime, has high economic and nutritional value. This genus belongs to the subfamily Aurantioideae, which is one of the 7 subfamilies of the family Rutaceae. Therefore, phylogenetic study of both the genus Citrus and of the subfamily Aurantioideae is important.

The Aurantioideae consists of 2 tribes with 33 genera [1]. These 2 tribes are each composed of 3 subtribes: the tribe Clauseneae, which includes Micromelinae, Clauseninae, and Merrillinae; and the tribe Citreae, which includes Triphasiinae, Citrinae, and Balsamocitrinae. None of the Clauseneae species develop axillary spines, and the odd-pinnate leaves have alternately attached leaflets. The fruits are usually small and carry semi-dry or juicy berries, except in Merrillia. In contrast, nearly all the species develop axillary spines in the Citreae. The simple leaves are easily distinguished from those of the tribe Clauseneae. The subtribe Citrinae, in the tribe Citreae, is distinct from all the other subtribes in the Aurantioideae because of the presence of pulp vesicles in the fruit. In this subtribe, “true citrus fruit trees” are considered the most advanced genera based on morphological traits [1]. The genus Citrus belongs to the “true citrus fruit trees.” The characteristics of Citrus species include asexual reproduction, high mutation frequency, and cross compatibility between species. Because of these characteristics, there is great morphological and ecological diversity among Citrus species.

Since the 1970s, morphological [2–4] and biochemical studies [5–9] have been conducted to elucidate the phylogeny of Aurantioideae, especially of Citrus and its close relatives. Because of improved DNA analysis, these relationships have been studied extensively. Several techniques, such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), simple sequence repeat (SSR), and sequence-related amplified polymorphism (SRAP) have been commonly used in taxonomic studies [10–14]. Recent progress in DNA sequencing techniques has allowed the extensive use of short DNA fragments, especially those of the chloroplast genome, in the study of phylogenetic relationships.
Phylogenetic analyses based on the various regions of the chloroplast genome have been conducted in the family Rutaceae and the subfamily Aurantioideae [15–24].

We have also previously reported the phylogenetic relationships among the Aurantioideae, including Citrus and its relatives, based on rbcL gene sequences [25]. The rbcL gene, located on the chloroplast DNA (cpDNA), encodes the large subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase, an enzyme that catalyzes carbon fixation in photosynthesis. Compared to most genes encoded in the cpDNA, the rbcL gene has a relatively slow nucleotide substitution rate [26–28]. A characteristic feature of our previous study [25] is that it included several accessions, which had not been examined in other studies [15–24]. However, the power of discrimination in our previous study was not high.

The matK gene is also located on the cpDNA and encodes a maturase involved in splicing type II introns from RNA transcripts. The matK gene is encoded by the chloroplast dnaK intron. Since matK has a relatively fast mutation rate, it evolves faster than the rbcL gene [26–28]. Therefore, matK analysis should be useful for studying the phylogeny of the genera included in Aurantioideae.

To comprehensively analyze the phylogenetic relationships of the superfamily Aurantioideae, we determined the matK sequences of 155 accessions from 22 genera of the Aurantioideae. In this study, we used matK sequences derived from basic and major species of the Aurantioideae. Similar to our previous study [25], our current study included several accessions that had not been examined in other studies [15–24]. Furthermore, we increased the number of accessions that were analyzed, in order to focus on interspecific relationships among the mandarin varieties of the Citrus genus because several studies have suggested the existence of great genetic variation among mandarin varieties [5,6,29,30]. Our study included many kinds of mandarin varieties grown in Japan, many of which have not been studied previously at the DNA level. The genetic background of various species of acid citrus (considered to be of hybrid origin) grown in Japan has also been investigated.

Materials and Methods

Plant Materials

The 135 accessions from 22 genera of the Rutaceae subfamily Aurantioideae that were used in this study as well as the sources of the materials are shown in Tables 1 and 2. The materials have been preserved at the Faculty of Agriculture, Saga University, the Saga Prefectural Fruit Tree Experimental Research Station, the Faculty of Agriculture, Kagoshima University, and the National Institute of Fruit Tree Science.

Polymerase Chain Reaction Amplification and DNA Sequencing

Crude extracts from approximately 10-mm² regions of leaves were prepared by incubating the leaf tissue with 100 μl of a solution containing 100 mM Tris-HCl (pH 9.5), 1 M KCl, and 10 mM EDTA, at 95°C for 20 min [31]. The primers used for polymerase chain reaction amplification of the matK gene were matK1F (5′-ACCGTATCGCAATGTAGTATC-3′) and matK1R (5′-GAAACTGTCCGATGAGTAG-3′). Using the crude extract as template, the matK gene was amplified by PCR with proofreading KOD FX or KOD FX Neo DNA polymerase (Toyobo, Osaka, Japan). The amplified DNA fragments were purified using the MonoFas DNA Purification Kit 1 (GL Sciences, Tokyo, Japan). The primers used for sequencing of the matK gene were matK1F, matK2F (5′-ACGGTCTTTTCTCTCCACGGAT-3′), matK3F (5′-GGTCCGATTCTCCTGATTCT-3′), matK1R, matK2R (5′-AGAACAGAGAATCGGGGCAG-3′), and matK3R (5′-ACTCGGAGAAGAAACCGGT-3′). The purified DNA fragments were sequenced in both directions in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems) with a BigDye Terminator Cycle Sequencing Ready Reaction Kit v. 3.1 (Applied Biosystems), as described previously [32]. Sequence data were submitted to DDBJ/GenBank/EBI and were assigned accession numbers ranging from AB626749 to AB626802, and from AB762316 to AB762396. The DNA templates used in this study are distinct from those used in the previous study [25].

Phylogenetic Analyses

The maximum likelihood (ML) and neighbor-joining (NJ) methods from the MEGA (version 5.05) program [33] were used to create phylogenetic trees. The reliability of each branch was tested by bootstrap analysis with 1,000 replications. The sequences of Zanthoxyllum sp, Claytonia 15 or Triphasia trifolia were used as an outgroup.

Results and Discussion

We constructed multiple sequence alignments of DNA sequences containing the matK gene from different accessions. The typical length of the protein-coding sequences and 3′ UTRs was 1,530 bases and 100 bases, respectively. However, some indels were present in several accessions. Because no indel was observed in the rbcL gene of this subfamily [25], we concluded that matK had a relatively fast mutation rate. The matK sequence used in this study was different from the partial matK sequence used in other published studies [16,34,35]. It is also different from the partial matK sequence used in the unpublished DNA barcoding projects. In addition, although other studies [24,36] used the entire matK sequence, the number of accessions tested was small.

Phylogenetic trees of Aurantioideae were created using the ML (Figure 1) and NJ (Figure 2) methods. Except for 7 species (Glycosmis citrifolia, Glycosmis pentaphylla, Murraya koenigii, Microsmum minutum, Clausena anisata, Clausena harmandiana, and Clausena laniana), both phylogenetic trees showed the same topology. Among these 7 species, 3 species of genus Clausena belonged to the same cluster, and 2 species of the genus Glycosmis belonged to the same cluster. A characteristic feature of both trees is that the “true citrus fruit trees” were clearly distinguished from other species. Another feature is that the “wood apple group” of Balsamocitrinae (Feronia limonia and Feroniella oblate), “primitive citrus fruit trees” (Securinega Variegata) and “near citrus fruit trees” (5 species of the genus Atalantia) also belonged to the same cluster in both trees. The remaining species were divided into a few groups in both phylogenetic trees. One group contained “primitive citrus fruit trees” (Hesperethusa crenulata), “near citrus fruit trees” (3 species of Citropolis), and the tabog group (Swinglea glutinosa). Another group contained 2 species of the Bael fruit group (Aegle marmelos and Afroanga paniculata), and yet another group contained Murraya paniculata and Merrillia calyxon. Members of the tribe Citreae belonged to the same large cluster, whereas members of the tribe Clauseneae did not.

The trees created in the present study (Figures 1 and 2) supported Swingle and Reece’s [1] classification of the subfamily Aurantioideae as monophyletic. These results are also consistent with those of Bayer et al. [16], Chase et al. [17], Groppo et al. [18], Morton et al. [20], Salvo et al. [24] and Tshering Penjor et al. [25].

The trees showed that the tribe Citreae is monophyletic, that is, they clearly discriminated Citreae from Clauseneae. This result
supports Swingle and Recce’s system of tribes. Previously, Morton et al. [20] and our group [25] reported the difficulty encountered in discriminating Citreae from Clauseneae in the analyses of rps19 and rbcL sequences, respectively. Our current results may be attributable to the fact that the matK data have high discrimination power. Although a previous study using 9 cpDNA sequences also discriminated Citreae from Clauseneae [16], we succeeded in this discrimination by using only 1 chloroplast DNA sequence. In contrast, the members of the tribe Clauseneae did not belong to the same cluster, i.e., Clauseneae is not monophyletic. Rather, the

| Tribe     | Subtribe | Group                  | Latin name (Common name)     | No.*  |
|-----------|----------|------------------------|-------------------------------|-------|
| Clauseneae| Micromelinae |                       | Micromelum minutum (Forst.) Wt. & Am. | 8650  |
|           | Clauseninae     |                        | Clausena anisata (Willd.) Hook. f. | 8612  |
|           |                     |                        | C. harmandiana (Pierre) Guill. | 8613  |
|           |                     |                        | C. iansium (Lour.) Skeels | 8611  |
|           |                     |                        | Glycosmis citrifolia (Willd.) Lindl. | 8601  |
|           |                     |                        | G. pentaphylla (Retz.) Correa | 8600  |
|           |                     |                        | Murraya koenigii (L.) Spreng. | 8622  |
|           |                     |                        | M. paniculata (L.) Jack. | 8621  |
|           |                     |                        | Merrillia caloxylon (Ridl.) Swing. | 8640  |
| Clauseneae|           |                       |                              | 8640  |
| Citreae   | Triphasinae | Triphasis group        | Paramignya lobata Burkhill | 8350  |
|           |          |                        | Triphasis trifoliata (Burm. f) P. Wils. f. | 8500  |
| Citrinae  | Primitive citrus fruit trees | | Hesperethusa crenulata (Roxb.) Roem. | 8320  |
|           |          |                        | Severinia buxfolia (Poir.) Tenore | 8340  |
|           | Near citrus fruit trees |                        | A. ceylanica (Am.) Oliv. | 8310  |
|           |          |                        | A. monophylla DC. | 8314  |
|           |          |                        | A. roxburghiana Hook. f. | 8316  |
|           |          |                        | A. spinosa (Willd.) Tanaka | 8315  |
|           |          |                        | Citropsis gabunensis (Engl.) Swing. & M. Kell. | 8300  |
|           |          |                        | C. gilletiana Swing. & M. Kell. | 8302  |
|           |          |                        | C. schweinfurthii (Engl.) Swing. & M. Kell. | 8301  |
|           |          |                        | Clymenia polyandra (Tan.) Swing. | 8280  |
|           |          |                        | Eremocitrus glauca (Lindl.) Swing. | 8251  |
|           |          |                        | Fortunella crassifolia Swing. (meiwa kumquat) | 8000  |
|           |          |                        | F. hindii (Champ.) Swing. ‘Diploid (2x)’ (Hongkong wild kumquat) | 8004  |
|           |          |                        | F. hindii (Champ.) Swing. ‘Tetraploid (4x)’ | 8005  |
|           |          |                        | F. japonica (Thunb.) Swing. | 8002  |
|           |          |                        | F. margarita (Lour.) Swing. (oval kumquat) | 8001  |
|           |          |                        | F. obovata hort. ex Tanaka (changshou kumquat) | 8003  |
|           |          |                        | F. polyandra (Rindl.) Tanaka (Malayan kumquat) | 8006  |
|           |          |                        | Microcitrus australasica (F. Muell.) Swing. | 8203  |
|           |          |                        | M. australis (Planch.) Swing. | 8201  |
|           |          |                        | M. inodora (F. M Bail.) Swing. | 8209  |
|           |          |                        | M. papuana H. F. Winters | 9210  |
|           |          |                        | M. warburgiana (F. M. Bail.) Tanaka | 8206  |
|           |          |                        | Poncirus trifoliata (L.) Raf. ‘Flying Dragon’ (trifoliate orange) | 8103  |
|           |          |                        | P. trifoliata (L.) Raf. ‘Standard’ (trifoliate orange) | 8100  |
| Balsamocitrinae | Tabog group |                        | Swinglea glutinosa (Blanco) Merr | 8420  |
|           |          |                        | Aegle marmelos (L.) Corr. | 8400  |
|           |          |                        | Afraegle paniculata (Schum.) Engl. | 8411  |
|           |          |                        | Feronia limonia (L.) Swing. | 8411  |
|           |          |                        | Feroniella oblata Swing. | 8460  |
| Outgroup  |          |                        | Zanthoxylum sp. Clayton 15 | –     |

*Accession number at the Saga University.

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Table 2. *Citrus* species and accessions used in this study.

| No. | Latin name | Common name | Accession | Source | Accession No. |
|-----|------------|-------------|-----------|--------|---------------|
| 1   | *C. macroptera* Montr. | *C. macroptera* Montr. | Melanesian papeda | A 7006 |  
| 4   | *C. micrantha* Wester. | *C. micrantha* Wester. | Biasong | A 7004 |  
| 7   | *C. hystrix* DC. | *C. hystrix* DC. | Mauritius papeda | A 7003 |  
| 10  | *C. latipes* (Swingle) Tanaka | *C. latipes* (Swingle) Tanaka | Khasi papeda | A 7000 |  
| 13  | *C. aurantifolia* (Cristm.) Swing. | *C. aurantifolia* (Cristm.) Swing. | Lime Mexican | A 5115 |  
| 15  | *C. latifolia* Tanaka | *C. aurantifolia* (Cristm.) Swing. | Bearss lime | B 9432 |  
| 16  | *C. limettioides* Tanaka | *C. aurantifolia* (Cristm.) Swing. | Sweet lime | B 5638 |  
| 31  | *C. medica* L. | *C. medica* L. | Citron | Maru Buishukan | A 5001 |  
| 31  | *C. medica* var. sarcodactylyis Swing. | *C. medica* var. sarcodactylyis Swing. | Citron | Fingered Citron | A 5008 |  
| 36  | *C. limon* (L.) Burm. f. | *C. limon* (L.) Burm. f. | Lemon | Eureka | C – |  
| 36  | *C. limon* (L.) Burm. f. | *C. limon* (L.) Burm. f. | Lemon | Villafranca | A 5207 |  
| 39  | *C. jambhiri* Lush | *C. limon* relative | Rough lemon | B – |  
| 56  | *C. maxima* (Burm.) Merr. | *C. maxima* (Burm.) Merr. | Pummelo | Mato Buntan | A 3202 |  
| 56  | *C. maxima* (Burm.) Merr. | *C. maxima* (Burm.) Merr. | Pummelo | Suisho Buntan | A 3301 |  
| 56  | *C. maxima* (Burm.) Merr. | *C. maxima* (Burm.) Merr. | Pummelo | Banpeiyu | A 3206 |  
| 78  | *C. natsudaidai* Hayata | *C. maxima* hybrid | Natsudaidai | Kawano | B – |  
| 79  | *C. obovoides* Hort. ex Takahashi | *C. maxima* hybrid | Kinkoji |  |  
| 93  | *C. aurantium* L. | *C. aurantium* L. | Sour orange | Kabusu | D JP117365 |  
| 93  | *C. aurantium* L. | *C. aurantium* L. | Sour orange | Zadaidai | C – |  
| 94  | *C. myrtifolia* Rafin | *C. aurantium* L. | Myrtle-leaf orange | C – |  
| 99  | *C. canalliculata* Hort. ex Y. Tanaka | *C. aurantium* relative | Kikudaizai | B – |  
| 100 | *C. sinensis* (L.) Osbeck | *C. sinensis* (L.) Osbeck | Sweet orange | Fukuhara | A 2100 |  
| 100 | *C. sinensis* (L.) Osbeck | *C. sinensis* (L.) Osbeck | Sweet orange | Valencia | A 2200 |  
| 103 | *C. tankan* Hayata | *C. sinensis* hybrid | Tankan | Tarumizu 1-Gou | C – |  
| 103 | *C. hybrid* cultivar | *C. aurantium* relative | Fuiri daidai | B – |  
| 112 | *C. ichangensis* Swing. | *C. ichangensis* Swing. | Ichang papeda | A 7009 |  
| 113 | *C. junos* Siebold ex Tanaka | *C. ichangensis* relative | Yuzu | Yamane | A 5403 |  
| 113 | *C. junos* Siebold ex Tanaka | *C. ichangensis* relative | Yuzu | Touchiki | A 5404 |  
| 113 | *C. junos* Siebold ex Tanaka | *C. ichangensis* relative | Yuzu | Tetraploid Yuzu | A 5402 |  
| 113 | *C. junos* Siebold ex Tanaka | *C. ichangensis* relative | Yuzu | Tadanishiki | B – |  
| 114 | *C. hanaju* Hort. ex Shirai | *C. ichangensis* relative | Hanaju | A 5500 |  
| 115 | *C. sudachi* Hort. ex Shirai | *C. ichangensis* relative | Sudachi | Mushu Yukaku | A 5511 |  
| 115 | *C. sudachi* Hort. ex Shirai | *C. ichangensis* relative | Sudachi | Sudachi | A 5501 |  
| 115 | *C. sudachi* Hort. ex Shirai | *C. ichangensis* relative | Sudachi | Yushi Mukaku | A 5512 |  
| 116 | *C. inflata* Hort. ex Tanaka | *C. ichangensis* relative | Mochiyu | A 5521 |  
| 117 | *C. yuko* Hort. ex Tanaka | *C. ichangensis* relative | Yuko | Yuko | A 5525 |  
| 117 | *C. yuko* Hort. ex Tanaka | *C. ichangensis* relative | Yuko | Mukaku Yuko | A 5526 |  
| 120 | *C. wilsonii* Tanaka | *C. ichangensis* relative | Ichang lemon | B 0611 |  
| 121 | *C. sphaerocarpa* Hort. ex Tanaka | *C. ichangensis* relative | Kabosu | A 5503 |  

Molecular Phylogeny of *Citrus* and Its Relatives
| No.  | Latin name                     | Common name | Accession | Source | Accession |
|------|-------------------------------|-------------|-----------|--------|-----------|
| 123  | C. nobilis Lour.               | C. reticulata Blanco | Kunenbo    | C      | –         |
| 123  | C. nobilis Lour.               | C. reticulata Blanco | King       | A      | 1522      |
| 124  | C. unshiu Markow.             | C. reticulata Blanco | Satsuma mandarin | A       | 1300      |
| 124  | C. unshiu Markow.             | C. reticulata Blanco | Satsuma mandarin | A       | 1401      |
| 124  | C. unshiu Markow.             | C. reticulata Blanco | Satsuma mandarin | A       | 1403      |
| 124  | C. unshiu Markow.             | C. reticulata Blanco | Satsuma mandarin | A       | 1415      |
| 124  | C. unshiu Markow.             | C. reticulata Blanco | Satsuma mandarin | A       | 1322      |
| 125  | C. yatsushiro hort. ex Tanaka | C. reticulata Blanco | Yatsushiro  | D      | JP117388  |
| 126  | C. keraji hort. ex Tanaka     | C. reticulata Blanco | Keraji     | A      | 1542      |
| 126  | C. keraji hort. ex Tanaka     | C. reticulata Blanco | Kabuchii   | A      | 1541      |
| 127  | C. oto hort. ex Y. Tanaka     | C. reticulata Blanco | Oto        | A      | 1540      |
| 128  | C. tarogayo hort. ex Y. Tanaka| C. reticulata Blanco | Tarogayo   | A      | 6000      |
| 128  | C. tarogayo hort. ex Y. Tanaka| C. reticulata Blanco | Unju      | C      | –         |
| 130  | C. reticulata Blanco          | C. reticulata Blanco | Ponkan     | Yoshida Ponkan | C      |
| 131  | C. deliciosa Ten.             | C. reticulata Blanco | Mediterranean mandarin | D      | JP117393  |
| 132  | C. genghukan hort. ex Tanaka  | C. reticulata Blanco | Genghukan  | C      | –         |
| 133  | C. tangerina hort. ex Tanaka  | C. reticulata Blanco | Dancy     | D      | JP117396  |
| 134  | C. clementina hort. ex Tanaka | C. reticulata Blanco | Clementine | C      | –         |
| 134  | C. clementina hort. ex Tanaka | C. reticulata Blanco | Clementine | Clementine de Nules | B      | 5653      |
| 137  | C. platymamma hort.ex Tanaka  | C. reticulata Blanco | Binkitsu   | B      | –         |
| 140  | C. suhuensis hort. ex Tanaka  | C. reticulata Blanco | Shikaikan  | A      | 1584      |
| 143  | C. tachibana (Makino) Tanaka  | C. reticulata Blanco | Tachibana  | C      | –         |
| 145  | C. kinokuni hort. ex Tanaka   | C. reticulata Blanco | Kinokuni   | Sakurajima Komikan | C      | –         |
| 145  | C. kinokuni hort. ex Tanaka   | C. reticulata Blanco | Kinokuni   | Hiraikshu | A      | 1518      |
| 145  | C. kinokuni hort. ex Tanaka   | C. reticulata Blanco | Kinokuni   | Mukaku Kishu | A      | 1519      |
| 145  | C. kinokuni hort. ex Tanaka   | C. reticulata Blanco | Soukitsu   | D      | JP117400  |
| 148  | C. sunki (Hayata) hort. ex Tanaka | C. reticulata Blanco | Sunki   | C      | –         |
| 149  | C. reshi hort. ex Tanaka      | C. reticulata Blanco | Cleopatra  | C      | –         |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Okitsu strain | C      | –         |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Kabishi     | A      | 1908      |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Mikangwu    | A      | 1916      |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Fusubuta    | A      | 1911      |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Kaachi      | A      | 1909      |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Ishikunibu  | A      | 1910      |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Shikunin     | C      | –         |
| 153  | C. depressa Hayata            | C. tachibana relative | Shikuwasha | Shikuribu    | C      | –         |
| 154  | C. leiocarpa hort. ex Tanaka  | C. reticulata Blanco | Koji       | C      | –         |
|      | C. hybrid cultivar             | C. reticulata Blanco | Shimamikan | Nagashima strain | A      | 559      |

Miscellaneous acid citrus

- C. sp. tenuissima hort. ex Tanaka C. hybrid cultivar Chosen Daidai | A | 2612
- C. sp. speciosa hort. ex Tanaka C. hybrid cultivar Zuishoyu | A | 5523
- C. yuzukichi hort. ex Y.Tanaka C. hybrid cultivar Yuzukichi | A | 5513
- C. kizu hort. ex Y. Tanaka C. hybrid cultivar Taninakakizuzu | A | 5515
- C. kizu hort. ex Y. Tanaka C. hybrid cultivar Kinosu | A | 5530
- C. jabara hort. ex Y. Tanaka C. hybrid cultivar Jabara | A | 5529
- C. takuma-sudachi hort. ex Tanaka C. hybrid cultivar Naoshichi | A | 5506
members of Clauseneae appeared to be an outgroup of Citraceae. This result did not support Swingle and Reece’s system of tribes. Thus, the matK data only partially supported Swingle and Reece’s system of tribes. The previous study using 9 cpDNA sequences [16] also showed that the members of the tribe Clauseneae did not belong to the same cluster.

Next, we focused on Swingle and Reece’s system of subtribes. The matK data did not completely support Swingle and Reece’s system of subtribes. For example, the Balsamocitrinae (Swinglea glutinosa, Aegle marmelos, Aegle paniculata, Feronia limonia, and Feroniella oblata) were not placed in 1 cluster. In contrast, Swingle and Reece [1] considered that the Balsamocitrinae paralleled the Citraceae and that both had evolved from a common ancestor. Among Balsamocitrinae, the Bael fruit group of Balsamocitrinae (Aegle marmelos and Aegle paniculata) is clustered together, which is consistent with previous reports [16,20,25]. Similarly, the “wood apple group” of Balsamocitrinae (Feronia limonia and Feroniella oblata) is clustered together, which is consistent with the previous reports by us [25] and Morton et al. [20].

Interestingly, Bayer et al. [16] concluded that Feroniella oblata is nested in Citrus species. The leaves of Feronia and Feroniella are odd-pinnate with paired opposite leaflets on a rachis. In addition, they are morphologically different from other genera of the orange subfamily; the core or axis of the ovary has disappeared and an odd-pinnate leaves (see Figure 4). Thus, they have some morphological similarities such as odd-pinnate leaves with broadly winged petioles and seeds with a hard testa. In addition, both are graft compatible with Citrus. However, Hesperethusa is native to Southeast Asia, whereas Citropsis is present only in Africa [1].

Our matK data showed that Severinia buxifolia and 5 Atalantia species (Atalantia bilocularis, Atalantia ceylanica, Atalantia monophylla, Atalantia roxburghiana, and Atalantia spinosa) belong to a monophyletic clade. Our previous rbcL data did not lead to the same conclusions, probably because of low discrimination power [25]. Araújo et al. [15] reported that Severinia buxifolia is clustered with Atalantia monophylla, and Bayer et al. [16] reported that Severinia buxifolia is clustered with 3 Atalantia species. (Atalantia ceylanica, Atalantia citroides, and Atalantia monophylla). Our analysis showed that Severinia buxifolia is most closely related to Atalantia bilocularis. Severinia species were considered to be species of Atalantia for many years, but Swingle segregated them out in 1938 [6].

Our matK data showed that Murraya paniculata and Merrillia caloxylon are clustered together, which is consistent with the previous reports by us [25] and Bayer et al. [16]. However, Murraya koenigii belongs to an independent cluster, which is consistent with our previous report [25]. Although the flowers of Merrillia caloxylon, which are very long (55–60 mm long) and trumpet shaped, are unique to the orange subfamily, this species resembles Murraya paniculata in its general growth habit, leaf shape, and wood texture. These 2 species grown in the wild are sometimes confused by natives of Malay Peninsula. As shown by the results from a series of studies [1], Swingle considered that Merrillia had probably evolved from a Murraya-like ancestral form. On the other hand, the leaf shapes of Murraya paniculata and Murraya koenigii are different. The leaf number of Murraya koenigii is larger than that of Murraya paniculata, although both species have odd-pinnate leaves (see Figure 4).

Our matK data showed that Clausena amissata, Clausena harmandiana, and Clausena laurisamum formed a monophyletic group, which is consistent with our rbcL data [25]. Bayer et al. [16] reported that Clausena harmandiana is clustered with Clausena escatana. Furthermore, our matK data showed that Glycosmis citrifolia and Glycosmis pentaphylla formed a monophyletic group, which is consistent with our rbcL data [25]. Bayer et al. [16] reported that Glycosmis pentaphylla is clustered with Glycosmis trichanthera and Glycosmis naruriana. Thus, each of Clausena and Glycosmis forms a monophyletic group.

### Table 2. Cont.

| No.* | Latin name | Swingle and Reece* | Common name | Accession | Source* | Accession |
|------|------------|--------------------|-------------|-----------|---------|-----------|
| Tanaka* | Matsuura Sudachi | A | 5510 | |
| – | C. acidoglboosa hort ex Tanaka | C. hybrid cultivar | Matsuda Sudachi | A | 5519 |
| – | C. nanseimensis hort. ex Tanaka | C. hybrid cultivar | Zanbo | A | 5516 |
| – | C. hybrid cultivar | C. hybrid cultivar | Kozu | A | 1904 |
| – | C. hybrid cultivar | C. hybrid cultivar | Nagata Kozu | A | |
| – | C. hybrid cultivar | C. hybrid cultivar | Hedzukia Daidai | C | – |
| – | C. hybrid cultivar | C. hybrid cultivar | Shibahara Sour | B | – |
| – | C. hybrid cultivar | C. hybrid cultivar | Hebezu | B | – |
| – | C. hybrid cultivar | C. hybrid cultivar | Genko | B | – |
| – | C. hybrid cultivar | C. hybrid cultivar | Tosu | B | – |

*Classification number and Latin names using Tanaka’s system [53,54].

*Latin name using Single’s system [1].

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Molecular Phylogeny of Citrus and Its Relatives

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Both phylogenetic trees produced a well-supported (BS, 99%) clade that contained all members of the “true citrus fruit trees,” thus supporting their monophyletic origin (Figures 1 and 2). All the genera belonging to “true citrus fruit trees” were incorporated into *Citrus* in accordance with the results reported by Mabberley [37,38], Zhang et al. [39], Bayer et al.
Our results support their concept. These “true citrus fruit trees” contain the genus *Citrus* having high economic and nutritional value. Therefore, we phylogenetically analyzed “true citrus fruit trees” by creating ML and NJ trees with *Triphasia trifolia* as an outgroup (Figures 5 and 6, respectively). The topologies of both trees were essentially identical and could be classified into 2 clusters. One large cluster included...
Citrus, Fortunella, and Poncirus. The other cluster included Microcitrus and Eremocitrus. Clymenia was isolated from these 2 clusters. No differences were observed among 7 Fortunella and 2 Poncirus accessions. The origins of these genera are as follows: Citrus, India to China; Fortunella, China; Poncirus, China, Clymenia, New Guinea; Microcitrus, Australia to New Guinea; and Eremocitrus, Australia. Some divergence of the matK sequence probably occurred between the genera originating in Southeast Asia and other places.

In the phylogenetic trees, Eremocitrus glauca was placed within the Microcitrus group (Microcitrus australasica, Microcitrus australis, Microcitrus inodora, Microcitrus papuana, and Microcitrus warburgiana). This result is consistent with Barrett and Rhodes's results, which show a very close relationship between Microcitrus and Eremocitrus [3]. The analysis of the rbcL sequence [25] and 9 cpDNA sequences [16] also showed a close relationship between both genera. It is generally believed that the species of Microcitrus and Eremocitrus arose over millions of years of slow evolution in geographically isolated land masses (Australia and New Guinea), separate from the other genera of the true citrus group in Southeast Asia and other places.

Our matK data showed that Fortunella species formed a monophyletic group, which is consistent with previous reports [16,23,25]. Swingle and Reece [1] placed Fortunella at the genus level only because it has 2 collateral ovules near the top of each locale, whereas Citrus has 4–12. However, Fortunella was placed within the Citrus group in the present study. Fortunella has also been classified within the Citrus group in previous studies on DNA analysis [10–14,22,23,25,40,41]. It is considered to be difficult to distinguish Fortunella from Citrus at the DNA level, based on these results.

Poncirus was also placed within the Citrus group in the present study. However, Swingle and Reece [1] placed Poncirus at the genus level mainly because it is deciduous, its flowering period differs from Citrus, it has trifoliolate leaves, and its geographical distribution differs from that of Citrus. Several DNA analysis studies on citrus phylogeny [10,12,40] have revealed that Poncirus is distant from Citrus. However, some studies involving cpDNA analysis have suggested a close relationship between Poncirus and Citrus [15,16,20,22,23,25]. These studies, along with the present study, strongly suggest that Poncirus is closely related to Citrus at the DNA level.
Next, we focused on the members of the genus Citrus that are economically and nutritionally important fruit trees. Our current study is the most extensive phylogenetic study of Citrus species among other studies (e.g., [16,22,23,25]) because we studied 93 accessions of the Citrus species. The phylogenetic tree showed that Citrus can be classified into 3 clusters: the citron cluster, the pummelo cluster, and the mandarin cluster (Figures 5 and 6). This finding is consistent with the results of previous studies using not only cpDNA but also nuclear and mitochondrial DNA (mtDNA) [10–16,23,42]. We found that Poncirus and Fortunella belonged to the parent cluster containing the members of the genus Citrus, but did not belong to these 3 clusters.

The mandarin cluster can be divided into 1 major cluster and some minor subclusters. The major subcluster includes, amongst others, C. reticulata, C. unshiu, C. clementina, C. kinokuni, C. deliciosa, C. reshni, and C. sunki. One of the minor subclusters includes C. tachibana and C. ichangensis.

Our matK analysis showed that most mandarin accessions belonged to the mandarin cluster. However, our extensive analysis showed that there are some exceptions. The mandarin accessions, Citrus keraji, Citrus oto, Citrus tarogayo, Citrus platymamma, and Citrus yatsushiro belonged to the pummelo cluster, and not to the mandarin cluster. Interestingly, Citrus nobilis (King) belonged to the mandarin cluster, and Citrus nobilis (Kunenbo) belonged to the pummelo cluster. Similarly, the previous study [22] showed that most mandarin landraces formed a monophyletic clade and some exceptions were similar to pummelo, although their tested accessions were different from ours.

The major subcluster of the mandarin cluster includes edible mandarins such as C. reticulata, C. unshiu, and C. clementina. In the present study, it was difficult to further classify members of this subcluster. Similarly, the previous researchers who conducted analyses based on cpDNA [12,22,43,44] and mtDNA [14,41] did not subdivide these members. Thus, our analysis confirmed the similarities of these mandarins with respect to organellar DNA. The major subcluster of the mandarin also includes C. reshni, C. sunki, and C. depressa, which are small-fruited mandarins mainly used as rootstocks. In contrast, the previous study using 3 cpDNA sequences [23] separated Citrus sunki from Citrus reticulata. One of the minor subclusters of the mandarin consists of C. reshni, C. sunki, and C. depressa, which are native to Japan. Hence, C. tachibana and some C. depressa accessions are different from other members of the mandarin cluster.

Figure 5. Maximum likelihood tree of the matK genes from accessions belonged to “true citrus fruit trees.” Numbers at the nodes indicate bootstrap values (% over 1,000 replicates). Numbers in parenthesis indicate the number of accessions. Citrus depressa contains 6 accessions (Kaachi, Mikangawa, Shiikunin, Shiikuribu, Ishikunibu, and Okitsu strains). Citrus depressa contains 2 accessions (Fusubuta and Kabishii).

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members of the mandarin cluster. Thus, our analysis confirms that
*Citrus tachibana* differs from other members of the mandarin cluster.

Members of *Citrus depressa* are placed in both the major and the minor
subclusters. Consistent with our analysis, the analyses by Urasaki
et al. [44] and Yamamoto et al. [45] showed the genetic diversity
in cpDNA among the members of *Citrus depressa*. These results suggest
that different *Citrus depressa* accessions have different origins.

The pummelo cluster contained, amongst others, *Citrus maxima*,
*papeda*, *Citrus sinensis*, *Citrus limon*, and *Citrus aurantifolia*. As described above,
this cluster also contained some mandarin accessions such as
*Citrus nobilis* (Kunenbo) and *Citrus keraji*. This cluster can be divided into 7
subclusters, which are represented by the species *Citrus sinensis*,
*Citrus maxima*, *Citrus latipes*, *Citrus aurantifolia* (plus papeda),
*Citrus aurantium*, and *Citrus limon* and the Citrus hybrid cultivar 'Tosu'. A number of mandarins
and Japanese acid citrus belonged to the *Citrus sinensis* subcluster.

Similarly, Bayer et al. [16] reported that *Citrus sinensis*, *Citrus maxima*,
*Citrus aurantifolia*, papeda, *Citrus aurantium*, and *Citrus limon* were clustered
together. However, because the numbers of tested accessions are
larger in our study, we addressed the classification of the pummelo
cluster extensively, as discussed below.

Some of the mandarins are clustered exclusively with pummelo
accessions. This result strongly suggests that their maternal origins
are members of the pummelo cluster. Previous studies showed that
*Citrus nobilis* (Kunenbo) has organellar DNA derived from a member
of the pummelo cluster [14,46]. Its maternal predecessor is probably a sweet orange with cytoplasmic DNA originally derived
from a member of the pummelo cluster. *Citrus nobilis* (Kunenbo) is
thought to be the maternal ancestor of *Citrus keraji* (Keraji and
Kabuchii) and *Citrus oto* [46,47]. Consistent with that hypothesis, the results of the current study show that the *matK*
sequences from *Citrus keraji* (Keraji and Kabuchii), *Citrus oto*,
and *Citrus nobilis* (Kunenbo) are identical. In agreement with the results of a previous morpholog-
ical characterization [48], the results of our analysis suggest that *Citrus nobilis*
(Kunenbo) is also the maternal origin of *Citrus tarogayo*.

We also studied the nucleotide variation in the *matK* genes from
accessions that were grouped in the pummelo cluster, although the
diversity is low. By using SSR analysis, Deng et al. [43] showed
that cpDNAs of pummelos and their relatives have many types of
nucleotide polymorphisms. Except for pummelo (*Citrus maxima*)
and papeda, all members of the pummelo cluster originate from
hybrids. Therefore, the nucleotide variation among the maternal
ancestors can contribute to the variation in the pummelo cluster.

Our results show that the *matK* sequences from *Citrus aurantium*, *Citrus sinensis*,
*Citrus limon*, and *Citrus limettioides* are derived from members of the
pummelo cluster, and this finding is consistent with the results of
previous studies using cpDNA analysis [12,15,16,40,47,49]. *Citrus wilsonii*
and *Citrus latifolia* also have pummelo-type cpDNA sequences,
and to our knowledge, this is the first study to highlight this point.
The *matK* sequences from *Citrus aurantium* and *Citrus limon* in the pummelo
cluster differ by single-base pair mismatches. Previous analyses of
cytoplasmic DNA suggested that *Citrus aurantium* is the maternal
ancestor of *Citrus limon* [14,50]. Thus, further work is required to
confirm this result.

Swingle and Reece [1] divided the genus *Citrus* into the subgenera *Citrus* and *Papeda*. However, in the present study,
papeda belonged to the pummelo cluster, and it is difficult to

![Figure 6. Neighbor-joining tree of the *matK* genes from accessions belonged to "true citrus fruit trees." Numbers at the nodes indicate bootstrap values (% over 1,000 replicates). Numbers in parenthesis indicates the number of accessions. *Citrus depressa* contains 6 accessions (Kaachi, Mikangwua, Shiikunin, Shiikuribu, Ishikunibu, and Oikitsu strains). *Citrus depressa* contains 2 accessions (Fusubuta and Kabishi).](https://doi.org/10.1371/journal.pone.0062574.g006)
discern the subgenera Citrus and *Papaya*. Thus, our analysis does not support the classification proposed by Swingle and Reece [1]. Similar results were reported based on the cpDNA analyses [12,16]. The *matK* sequences from *C. hystrix*, *C. micrantha*, and *C. macroptera* are identical, which is consistent with the results of previous analyses of cpDNA by Nicolosi et al. [12]. They suggested that *C. micrantha* was the maternal ancestor of *C. aurantifolia*. Our present results show that the *matK* sequence from *C. aurantifolia* is identical to that from *C. hystrix*, *C. micrantha*, and *C. macroptera*, which confirms the results of the study by Nicolosi et al. [12]. However, the *rbcL* genes and chloroplast SSRs of *C. micrantha* and *C. aurantifolia* differ [25,43]. Therefore, their maternal relationship is unclear.

Most of miscellaneous acid citrus species grown in Japan belonged to the mandarin major cluster, the *C. pannos* cluster, or the *C. sinensis* cluster. Although this result almost agrees with that of Asadi Abkenar et al. [51] who analyzed cp and mtDNA using PCR-RFLP, our analysis showed that *matK* sequences of some accessions (C. *sp. tenusissima* and *C. nanseensis*) were identical with that of *C. maxima*. As above mentioned, *matK* sequence of *C. sinensis* was similar to that of *C. maxima* but not identical with it. Our result was more informative than the previous study [51] and it is considered to be useful information for estimation of genetic background of Japanese acid citrus. These data obtained from cytoplasmic DNA analysis were not coincident with the RAPD results of nuclear and cytoplasmic genomes; further studies are required to elucidate their exact origin.

Swingle and Reece [1] have classified *C. ichangensis* into the subgenera *Papaya*. However, our analysis shows that *C. ichangensis* belongs to the mandarin cluster. There are conflicting reports regarding the cytoplasmic relationships between *C. ichangensis* and other Citrus species. Consistent with our current result, based on the analyses of cpDNAs, Asadi Abkenar et al. [40], Bayer et al. [16], Deng et al. [43], and Nicolosi et al. [12] showed that *C. ichangensis* is closely related to the mandarins. However, according to the phylogeny of *rbcL* [25], *C. ichangensis* is most closely related to the *Poncirus* species. On the other hand, cpSSR analysis [49] failed to show a relationship to other species. A comparison of mtDNA sequences [41] suggested that *C. ichangensis* was not related to the mandarins and was identical to *C. hystrix* and *C. aurantifolia*. A complete understanding of the cytoplasmic relationships of *C. ichangensis* with the Citrus species and its relatives including the *Poncirus* species requires further analyses.

Our analysis clearly separated citron from pummelo and mandarin. Considerable variation between citron and the other Citrus accessions has been reported previously based on F1 protein [32], mtDNA [14] and cpDNA [12,15,16,22,23,42].

### Conclusions

Based on the chlorophyll *matK* sequences, the present study provides novel information that resolves the genetic relationships among members of the Aurantioideae, especially of the genus *Citrus*, and confirms previous observations. Because the *matK* gene has a relatively fast rate of nucleotide substitutions, our study provides more information on interspecific relationships within the genus *Citrus* than the analysis of the *rbcL* gene [25]. Our extensive classification of 135 accessions from 22 genera of the Aurantoideae could be useful for the breeding of these trees.

### Author Contributions

Conceived and designed the experiments: TP MY YN. Performed the experiments: TP MU MI NM YN. Analyzed the data: TP MY RM YN. Wrote the paper: TP MY YN.

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