Communication

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Phylogenetic relationships among accessions in *Citrus* and related genera based on the insertion polymorphism of the *CIRE1* retrotransposon

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Abstract: A total of 145 accessions of the genus *Citrus* and related genera, maintained in the Conservation Garden for Citrus Germplasm at the Experimental Farm of Kindai University, Yuasa, Wakayama, Japan, were examined for their phylogenetic relationships. The present classification was conducted using an inter-retrotransposon amplified polymorphism (IRAP) method based on the insertion polymorphism of a retrotransposon, *CIRE1*, identified in *C. sinensis*. The objective of this study was to evaluate the applicability of the IRAP method for citrus classification. The constructed dendrogram showed that the 145 accessions and two outgroup species were successfully classified into five major clades. A large number of *C. sinensis* accessions were divided into three traditional groups, navel orange, sweet orange, and blood orange, almost corresponding to the sub-clades in the dendrogram. Several other accessions belonging to the same species, and also many hybrid cultivars from crossbreeding, were localized into the respective sub-clades or near positions in the dendrogram. Several unclassified accessions could also be located in the dendrogram, suggesting novel relationships with other accessions. It was concluded that the IRAP method based on *CIRE1* insertion polymorphism was suitable for the classification of citrus from a molecular point of view.

Keywords: *CIRE1*, citrus classification, farthest neighboring method, IRAP, Tyl-copia type

Abbreviations

| Abbreviation | Definition |
|--------------|------------|
| IRAP,        | inter-retrotransposon amplified polymorphism |
| LTR,         | long terminal repeat |
| NARO,        | National Agriculture and Food Research Organization |
| REMAP,       | retrotransposon-microsatellite amplified polymorphism |

1 Introduction

The classification system of the genus *Citrus* and related genera, including many important fruit trees all over the world, has been subject to some confusion. Two major systems have been proposed: one originating from Tanaka’s classification (Ikeda 2007) and the other from that by Swingle (Swingle 1943). The former identified more than 160 species, whereas the latter identified only 16 species. The confusion in citrus classification could derive from the definition policy of species, i.e., “species” in this case can traditionally include various cultivars, lines, and also hybrids between cultivars. This is particularly characteristic of Tanaka’s classification, in which species were defined as groups showing apparent and distinct morphological, physiological, and geographical characteristics compared with other groups.

Recent advances in molecular biology can demonstrate genome structure and function in numerous organisms, including citrus (Asins et al. 1999). Many trials based on molecular techniques have been conducted to classify the genus *Citrus* and related genera according to their genomic information. Nuclear genomic markers used in phylogenetic analyses include isozymes (Hirai et al. 1986), simple sequence repeats (Barkley et al. 2006), inter-simple sequence repeats (Fang et al. 1998; Biswas et al. 2010), restriction fragment length polymorphisms (Yamamoto et al. 1993), etc. Polymorphisms in chloroplast (Handa et al. 1986; Yamamoto et al. 1993; Li et al. 2007; Abkenar et al. 2008; Penjor et al. 2010;...
Penjor et al. 2013) and mitochondrial genomes (Yamamoto et al. 1993; Abkenar et al. 2008; Froelicher et al. 2011) were also examined mainly to evaluate maternal lineages.

Another useful and available genomic marker was derived from Class I transposable elements, retrotransposons, particularly those ubiquitously distributed in a wide range of related taxa (Kalender et al. 1999). In general, retrotransposons remain in their original positions after their transposition to other sites in the genome, due to their copy-and-paste mode (Kumar and Bennetzen 1999). This results in a consistent increment in their copy numbers during the evolutionary process. We can, therefore, elucidate the phylogenetic relationships of a wide range of taxa from the polymorphic patterns of a common retrotransposon shared by them and can determine the classification of target materials based on this system. For example, Shedlock and Okada (2000) re-examined the phylogeny of a wide range of vertebrates using a retrotransposon, Short Interspaced Nuclear Elements. Nishihara et al. (2006), thus, identified a novel clade, Pegasoferae in mammals.

Kalender et al. (1999) proposed new techniques for retrotransposon fingerprinting, inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-microsatellite amplified polymorphism (REMAP). The IRAP relies on PCR amplification products between two identical primers against two retrotransposons’ long terminal repeats (LTRs). These products can be amplified when two retrotransposons are in the vicinity in a genome. REMAP uses the products between simple sequence repeats and retrotransposons’ LTRs. For example, both techniques were found to be suitable for the fingerprinting of barley genotypes using a retrotransposon, BARE-1 (Kalender et al. 1999). Taheri et al. (2018) also examined the relationships among Triticum urartu and T. boeoticum populations from Iran using the IRAP and REMAP.

In citrus, a Class I, Ty1-copia-type element retrotransposon, CIRE1, was identified in the genome of C. sinensis, which consists of approximately 5 kbp with typical LTRs and other intrinsic elements (Rico-Cabanas and Martínez-Izquierdo 2007). The copy number of CIRE1 per genome was estimated to be approximately 2,200 in C. sinensis, occupying about 2.9% of the genome of this species. In the C. sinensis genome, there are seven families, CIRE1 to CIRE7, which share more than 85% nucleotide identity. Particularly for CIRE1, this element showed root-specific expression. In addition, the expression levels in leaf tissues could be enhanced by wounding or the application of methyl jasmonate and auxins.

This study conducted an examination of the phylogenetic relationships among the genus Citrus and related genera using the IRAP method based on CIRE1 insertion polymorphism. Biswas et al. (2010) also used the IRAP method based on the insertion of a Ty1-copia-like-type retrotransposon in Citrus species (GenBank accession number AF506028.1), which was apparently different from CIRE1. The present target population consisted of 145 accessions maintained in the Conservation Garden for Citrus Germplasm at the Experimental Farm of Kindai University. They consisted of various cultivars, hybrids, landraces, etc., not only from Japan but also from other countries. They also included accessions that have not been classified according to traditional morphological evaluation. The aim of this study was to evaluate the applicability of the IRAP using CIRE1 as a probe and to confirm the phylogenetic relationships among citrus accessions, particularly for the unclassified accessions, in this garden.

2 Materials and methods

A total of 147 accessions belonging to Citrus, Fortunella, Poncirus, and other genera were used as materials (Figures 4–8). Their species names basically followed the classification by Tanaka. Of these, 145 accessions were cultivated and maintained in the Conservation Garden for Citrus Germplasm at the Experimental Farm of Kindai University, located at Yuasa, Wakayama, Japan (34°, 2′ N, 135°, 12′ E, 27 m above sea level). Two others, orange jasmine (Murraya paniculata (L.) Jack) and Japanese pepper (Zanthoxylum piperitum (L.) DC), were used as outgroup species in the present classification of citrus. From every accession, several fresh leaves were sampled from one plant on November 24, 2015, and stored at −80°C until use.

A piece of each leaf, weighing about 100 mg, was frozen in liquid nitrogen and crushed in a 2-ml tube with a micro-pestle, and total DNA was extracted using a DNeasy Plant Mini Kit (QIAGEN GmbH, Hilden, Germany) in accordance with the manufacturer’s instructions and used as a template for PCR.

To confirm the existence of CIRE1 in the two outgroup species, their DNA samples and those from several accessions were amplified using a primer pair designed near to both LTRs of CIRE1. The sequence of CIRE1 (CIRE1.1) was referred to the GenBank/NCBI database as the accession number AM040263 (Rico-Cabanas and Martínez-Izquierdo 2007). The sequences of
the forward and reverse primers for the detection of 

*CIRE1* itself were 5'-CGATTGTCCTGGTGTCTGACT-

CAC-3' and 5'-GACCCCATCAACTGAACTCACTGC-3', re-

spectively. The template DNA (diluted at about 5 ng μL⁻¹) 

was added to a reaction mixture involving 0.2 μL⁻¹ 

KOD PLUS Neo polymerase, 1x KOD PLUS Neo buffer, 

1.5 mM MgSO₄, 0.2 mM dNTPs (TOYOBO Co., Ltd, Osaka, 

Japan), and 0.6 μM of each primer pair, to make a total of 

10 μL. This reaction mixture was amplified using the 

following PCR conditions: 94°C for 2 min, and 35 cycles 

of 98°C for 10 s, 58°C for 30 s, and 68°C for 2.5 min, then 

68°C for 1 min. Amplified fragments were resolved using 

0.7% agarose gel in 1x Tris/Acetic acid/EDTA buffer and 

visualized by ethidium bromide staining. From this PCR, 

a fragment of 4,279 bp was expected to be amplified 

(Rico-Cabanas and Martinez-Izquierdo 2007).

To apply the IRAP method on the 147 accessions 

with *CIRE1*, each DNA sample from these accessions 

was added to the reaction mixture. A single primer was 

used in the IRAP (Kalendar et al. 1999). Its sequence was 5’-

TGAGGGATCACTGAATCACTGCAGG-3’, designed to be 

flanked by the LTR of *CIRE1*, and oriented in the 

outward direction of *CIRE1*. The 10 μL reaction mixture 

included 0.2 U μL⁻¹ KOD PLUS Neo polymerase, 1x KOD 

PLUS Neo buffer, 1.5 mM MgSO₄, 0.2 mM dNTPs 

(TOYOBO Co., Ltd), 0.6 μM primer, and template DNA. 

This reaction mixture was amplified using the following 

PCR procedure: 94°C for 2 min, and 35 cycles of 98°C for 

10 s and 68°C for 6 min, then 68°C for 7 min. Amplified 

fragments were resolved using 1% agarose gel in 1x Tris/

Borate/EDTA buffer and visualized by ethidium bromide 

staining. The fragments were expected to be generated 

from neighboring *CIRE1* pairs at available distances to be 

amplified, each of which was oriented in a reverse 

direction to each other.

Amplified fragments, which were apparently distin-

guishable and longer than about 1 kbp, were estimated 

for their sizes using regression analysis against standard 

fragments in the size marker (O’RangeRuler™ 1 kbp DNA 

Ladder; Thermo Fisher Scientific KK, Yokohama, Japan).

These fragments were classified into one of the groups, 

each of which showed the same fragment size. The 147 

accessions were evaluated for the presence (scored as 

“1”) or absence (as “0”) of every fragment-size group, 

thereby constructing a matrix representing accessions 

and fragments to apply a cluster analysis. An add-in 

software of Excel, “MULCEL” (Yanai 2005) was used in 

this cluster analysis. From this matrix, a dissimilarity 

matrix was calculated, in which each element (corre-

sponding to squared Euclid distance in categorical data) 

was the number of fragments for their presence or 

absence between a given pair of accessions (Yanai 2005). 

Clusters according to a farthest neighboring method 

were constructed and visualized as a dendrogram. 

Although several methods to form clusters had been 

tried, the most plausible dendrogram was obtained from 

the present farthest neighboring method.

3 Results and discussion

Figure 1 shows that all of the *Citrus*, *Fortunella*, and 

*Poncirus* species demonstrated a common amplified 

fragment from *CIRE1* corresponding to an expected size 

of 4,729 bp, although several other non-specific frag-

ments were also observed. Moreover, the two outgroup 

species in this study, *M. paniculata* (L.) Jack and 

*Z. piperitum* (L.) DC., showed the same fragment as 

other *Citrus* species, though at a weaker intensity. For 

*M. paniculata* (L.) Jack, the fragment was slightly 

shorter, probably due to the inclusion of structural 

variation. This indicated that these two species had the
CIRE1 as other species, and they could be used as outgroup species in the present classification.

Figure 2 shows a typical electrophoresis profile resulting from the IRAP method using CIRE1 as a probe. A total of 97 polymorphic amplified fragments were generated in the 147 accessions. Biswas et al. (2010) also detected 79 polymorphic fragments by IRAP, and also REMAP, in 48 accessions. In the present study, a fragment of 2.1 kbp was the most prevalent one shared by 81 accessions. On average, there were 9.83 fragments per accession ranging from 4 to 17. Of these 147 accessions, only one pair, Pineapple sweet orange (C. sinensis) and Calabrese orange (C. sinensis), showed completely identical fragment patterns. From the dissimilarity matrix as
Figure 4: Details of major clade A in the whole dendrogram. Underlined accessions are unclassified ones without species names.

Figure 5: Details of major clade B in the whole dendrogram. Underlined accessions are unclassified ones without species names.
above, a dendrogram was constructed (Figure 3). This dendrogram consisted of major five clades divided at a squared Euclid distance of about 23, tentatively designated from A to E (Figures 4–8).

For clade A (Figure 4), various species were included, and *C. paradisi* (grapefruit group), *C. reticulata* (Ponkan group), *C. maxima* (pummelo group), *C. kinokuni*, *C. unshiu* (mandarin group), *C. tankan* (tangor group), etc. tended to be identified as the respective sub-clades in this major clade. Two *Fortunella* species (kumquat group) belonged to the same sub-clade near one of the sub-clades of *C. reticulata*. The four unclassified accessions, Hisagokomikan, Kouda mandarin, Hozodaka mandarin, and USSR tangelo, were positioned near Halligan (*C. sinensis × C. reticulata*), Miyagawawase mandarin (*C. unshiu*), round-shaped Kinokuni mandarin (*C. kinokuni*), and Kotokan (*C. kotokan*), respectively. Notably, one accession of the species *C. maxima* Merr. (Hirado buntan pummelo) was the most dissimilar accession in this clade A.

One of the sub-clades of the clade B consisted exclusively of *C. sinensis* (L.) Osbeck var. brasiliensis Tanaka (navel orange group) with close similarity, except for one *C. sinensis* (L.) Osbeck (Fukumoto navel orange), which was not classified as var. brasiliensis (Figure 5). The other characteristics of clade B were that most of the hybrid species (cultivars) ("Okitsu" group in Figure 5) developed by crossbreeding in the Institute of Fruit Tree and Tea Science, National Agriculture and Food Research Organization (NARO), Japan, formed several sub-clades. Only two accessions in the “Okitsu” group, Okitsu 24 (*C. funadoko* hort. ex Tanaka) and

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**Figure 6**: Details of major clade C in the whole dendrogram. Underlined accessions are unclassified ones without species names.

**Figure 7**: Details of major clade D in the whole dendrogram. Underlined accessions are unclassified ones without species names.
Okitsu 12 (C. unshiu × C. sinensis), were assigned to other major clades (clade C and clade D, respectively).

Two of the sub-clades in clade C were dominated by the C. sinensis (L.) Osbeck, except for five accessions, Yuzu (C. junos Siebold ex Tanaka), sweet lime (C. limettioides Tanaka), Kara mandarin (C. unshiu × C. nobilis), Kurenbo (C. nobilis Lour.), and Okitsu 24 (C. junadoko hort. ex Tanaka) (Figure 6). Sweet orange group and blood orange group in the same C. sinensis (L.) Osbeck were separately located in the two different sub-clades. An unclassified accession, Shina-mikan, may be classified as C. sinensis (L.) Osbeck, because of its position in clade C. One more unclassified accession, Hayayu, was most similar to Andoukan (C. maxima Merr. [hybrid]).

Clade D included only five species: C. sinensis × Poncirus trifoliata (Rusk citrange), C. jabara hort. ex Tanaka (Jabara), C. hanaju Siebold ex Shirai (Hanayu), C. sphaerocarpa Tanaka (Kabosu), and C. wilsonii Tanaka (Ichang lemon [Shangyuan]) (Figure 7). These five species appeared not to be closely related to each other within this major clade from their squared Euclid distances.

The last clade E included the two outgroup species in this experiment as an isolated sub-clade, as expected (Figure 8). This sub-clade also included C. tachibana (Makino) Tanaka (Tachibana), suggesting that this species was the most distantly related to other citrus species, but it was close to the outgroup species among the present accessions. One of the other two sub-clades of clade E was characterized by the prevalence of C. limon (L.) Burm. f. (lemon group). Poncirus trifoliata (L.) Raf. (Trifoliolate orange) was included in the last sub-clade. The unclassified accessions in this major clade, Parauan kumquat and leyasu-kishu mandarin, were suggested to be close to Kobeni mandarin (C. erythrosa hort. ex Tanaka).

The present results of the phylogenetic relationships among 145 accessions of Citrus and related genera basically corresponded to those of the classification system by Tanaka (Ikeda 2007). For example, C. sinensis was divided into three major groups, i.e., in clade B and two distinct sub-clades in clade C. One of these three groups of C. sinensis in clade B included predominantly the navel orange group, which was classified as var. brasiliensis (which means “Brazilian”) by Tanaka. The other two groups as two sub-clades in clade C corresponded mainly with the sweet orange group and blood orange group. This result strongly suggested that accessions in C. sinensis (L.) Osbeck were differentiated into three groups: navel orange (brasiliensis) (clade B), sweet orange (clade C), and blood orange (clade C). Of these, the sweet orange group showed the highest similarity within a group from lower squared Euclid distances, compared with the other two groups (Figure 3). C. paradisi (grapefruit group) (clade A), C. reticulata (Ponkan group) (clade A), Fortunella species (clade A), and C. limon (lemon group) (clade E) also showed lower squared Euclid distances within each species group and occupied similar positions in the dendrogram.

On the other hand, we can find that accessions belonging to the same species were not always clustered at similar positions in the dendrogram. C. maxima (Hirado buntan, Suishou buntan pummelo, Andoukan, and Kuchinotu 2) and C. unshiu (Kikumikan, Miyagawawase-unshiu mandarin, Hayashi-unshiu mandarin, Beni-unshiu mandarin, and Variegated unshiu mandarin) were
examples of these. Handa and Oogaki (1985) detected a wide morphological variation within the mandarin group using numerical taxonomy. Fuji et al. (2016) reported that Satsuma mandarin (C. unshiu) appeared to be generated from the occasional cross between Kuishu-mikan-type mandarin and Kunenbo-type mandarin using SNP markers. This result suggests that a citrus group showing similar morphological characteristics does not always have a similar CIRE1 fingerprinting.

Some accessions of "Okitsu" group, which were developed by crossbreeding from the Institute of Fruit Tree and Tea Science, NARO, Japan, tended to be located mainly in one of the sub-clades of clade B, although several "Okitsu" members were scattered in different clades. This similarity in phylogeny among "Okitsu" group may be attributable to the relatively narrow range of their parents, C. hassaku, C. maxima, C. tanumiana, C. reticulata, etc. In other words, the IRAP method could detect this similarity.

In conclusion, the present IRAP method based on CIRE1 was highly suitable for the classification of many accessions of Citrus and related genera. Particularly for closely related accessions such as navel orange group, sweet orange group, and blood orange group of C. sinensis, this method can detect polymorphisms among these three groups and describe their relationship successfully. In addition, the unclassified accessions were located in adequate positions in the dendrogram. This IRAP method with CIRE1, or other retrotransposons in Citrus species, may also be applied successfully to other groups of unclassified accessions, providing available information to the future breeding of citrus.

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