Investigation and DNA Analysis of Local *Citrus* Genetic Resources Grown on the Chuuk Islands of Micronesia

Masashi Yamamoto¹*, Yuta Natori¹ and Kei Kawai²

¹Faculty of Agriculture, Kagoshima University, Kagoshima 890-0065, Japan
²Research Center for the Pacific Islands, Kagoshima University, Kagoshima 890-8580, Japan

An investigation of local citrus genetic resources grown on the Chuuk Islands of the Federated States of Micronesia was conducted in August 2013. A total of 21 accessions were examined. According to the morphological traits, ten, four, four, two, and one accession resembled lime (local name “Nayimis”), sour orange (“Kurukur”), calamondin (“Tangerin” and “Gunkan”), sweet orange (“Kurukur” and “American Kurukur”), and pummelo (“Kurukur”), respectively. Next, cleaved amplified polymorphic sequence (CAPS) analysis was conducted. Then, precise accession identification was made based on the results of CAPS analysis in conjunction with morphological traits. “Nayimis” accessions were classified into at least two types. Genotypes of “Nayimis” type 1 were identical to those of Mexican lime. A genetic influence from mandarin was detected in “Nayimis” type 2. Although the morphological traits of all “Kurukur” sour orange types were similar, their diversity was revealed by CAPS analysis. The genotype of one “Kurukur” accession was identical to that of sour orange. Genotypes of the sweet orange-type “Kurukur” and “American Kurukur” were different. The types of “American Kurukur” and sweet orange were the same. Pummelo-type “Kurukur” was considered to be true pummelo based on the results of morphological and molecular markers. Morphologically, the calamondin-like accessions “Tangerin” and “Gunkan” showed the same genotype combinations on CAPS analysis. These genotypes were identical to those of calamondin used as a control. The combined results of morphological and molecular markers offer valuable information for the identification of citrus genetic resources on the Chuuk Islands.

Key Words: calamondin, CAPS, lime, pummelo, sour orange.

Introduction

The Chuuk Islands are part of the Federated States of Micronesia. They comprise more than 200 islands, are located between 0–11°S and 148–154°E, and have a tropical climate. The main crops are coconut, taro, and banana. Although citrus is not an important industrial crop, it is commonly utilized for cooking, fresh fruit, and juice (Yamamoto et al., 2015). On the Chuuk Islands, there are two well-known citrus accessions: “Nayimis” and “Kurukur”. Although “Nayimis” and “Kurukur” are considered to be lime and sour orange, respectively (Plant Threats to Pacific Ecosystems, Common names of PIER plants by language (Chuukese), http://www.hear.org/pier/commonnames/languages/Chuukese/index.html, January 9, 2018), their detailed characteristics have not yet been clarified. To elucidate the precise traits of each accession, investigations of morphological traits and DNA analysis have been very effective (Ueda and Higuchi, 2012). New findings regarding several local citrus genetic resources were also reported using both morphological and molecular markers (Nasir et al., 2017; Sharafi et al., 2016; Tshering Penjor et al., 2014a, b).

Among the various types of DNA analyses, the cleaved amplified polymorphic sequence (CAPS) is a simple and reliable method for DNA analysis (Konieczny and Ausubel, 1993). It is easy to assay, requires only a few nanograms of DNA for the PCR amplification, and does not require the use of a DNA sequencer. CAPS markers use amplified DNA fragments digested with a restriction endonuclease to reveal restriction-site polymorphisms. They have several advantages for cultivar identification because they are inherited in a codominant manner. Recently, Shimada
et al. (2014) developed 708 CAPS markers from sequenced-tagged-site (STS) primers with designs based on cDNA. Ninomiya et al. (2015) and Nonaka et al. (2017) reported the cultivar identification and parental analysis of new citrus cultivars in Japan using these markers. In addition, for chloroplast (cp) DNA analysis, CAPS uses universal primers and has proven to be a simple and reliable method for many genera (Cipriani and Morgante, 1993; Taberlet et al., 1991), including citrus (Asadi Abkenar et al., 2004; Fujii et al., 2016; Nicolosi et al., 2000; Yamamoto et al., 2013, 2017). These results indicate the usefulness of CAPS analysis in terms of both the nuclear genome and cpDNA for phylogenetic studies of citrus.

The region from northeastern India (e.g., Assam) to southwestern China (e.g., Yunnan) is probably where some Citrus species originated (Gmitter and Hu 1990; Tanaka, 1959). Since the Chuuk Islands are located far from these regions, citrus growing there seems likely to have been associated with human activity. Subsequently, characteristic local citrus, introduced types and those originating as chance seedlings, has grown there. There is a possibility that these are important citrus resources that have adapted to a tropical climate. Actually, a hardy new type of lime was found in an investigation of local citrus in Bhutan (Tshering Penjor et al., 2014b, 2016). However, there is no study of the distribution and tree/fruit traits on the Chuuk Islands. Information on these citrus resources is considered useful for use and cultivation of citrus fruits on the Chuuk Islands. In addition, elucidation of the genetic characteristics of these citrus resources will contribute to an understanding of the distribution and differentiation of citrus genetic resources globally. Thus, in the present study, we first investigated the morphological characteristics of local citrus genetic resources grown on the Chuuk Islands. Then, CAPS analysis was carried out. Based on the combined results of morphological and molecular markers, the genetic characteristics of these accessions were elucidated.

Materials and Methods

Plant materials and morphological characteristics

Samples of local citrus genetic resources grown on the Weno, Fefen, Eot, Romanum, and Tonoas Islands in the Chuuk Islands (Fig. 1) were gathered in August 2013. All samples were collected from trees grown in home gardens. The location of the tree was determined via a Global Positioning System (GPS) (Poke Navi Mini EX; Enpex, Tokyo, Japan). Tree, leaf, and fruit characteristics of each accession were recorded on site just after collection. These characteristics were analyzed and are shown in Tables 1 and 2. Mexican lime (Citrus aurantifolia (Cristm.) Swingle), sour orange (C. aurantium L.), and calamondin (C. madurensis Lour.) cultivated at the Faculty of Agriculture of Kagoshima University, Japan, were used as the control accessions in the morphological characterization. Since Mexican lime did not bear any fruit, its fruit characteristics could not be measured. Five leaves and fruits were sampled per accession.

In DNA analysis, the 13 accessions shown in Table 3 were used as control materials. All the control materials except rangpur lime were preserved at the Faculty of Agriculture of Kagoshima University. The rangpur lime was kindly provided by the NARO Institute of Fruit Tree and Tea Science.

DNA extraction and CAPS analysis

Dried and fresh leaves from local accessions and controls were used as plant materials, respectively. Total DNA was extracted from them using Isoplant II (Nippon Gene, Tokyo, Japan) following the manufacturer's instructions.

The CAPS analysis of the nuclear genome was carried out using four of the citrus CAPS markers (Table 4) developed by Shimada et al. (2014) that showed efficiency for cultivar identification in a previous study (Ninomiya et al., 2015). In cpDNA analysis, two regions were amplified with universal primers (Cipriani and Morgante, 1993; Taberlet et al., 1991) (Table 4). The PCR reaction mixture of 12.5 μL consisted of 10 ng of template DNA, 10 pmol of each primer, 10 × reaction buffer, and 0.5 units of Prime taq DNA polymerase (GeNet Bio, Daegjeon, Korea). PCR reactions were per-
| No. | Date (year/month/day) | Accession Type | Place | Altitude (m) | Latitude | Longitude | Tree Leaf | Island | Place name | Height (cm) | Width (cm) | Vigor | Habit | Spine* | Spinex | Length (mm) | Width (mm) | Wing | Leaf blade Width (mm) | Length (mm) | Width (mm) | Height (mm) | Width (mm) |
|-----|----------------------|----------------|-------|--------------|-----------|-----------|----------|--------|------------|-------------|------------|-------|-------|-------|--------|-----------|------------|------|----------------------|------------|------------|-------------|-----------|
| 2   | 2013/8/5             | Nayimis (Type 1) | Lime  | 31           | N7.27521  | E151.52609| Weno     | Peniesene village | 5            | 8          | Medium | Strong | Spreading | None   | 7.79       | 4.19       | 7       | 3.0       |
| 7   | 2013/8/6             | Nayimis (Type 1) | Lime  | 18           | N7.26984  | E151.49195| Fefen    | Onongoch village  | 6            | 5          | Slightly weak | Medium | Spreading | None   | 96.0      | 42.6       | 6.4      | 7.6       |
| 12  | 2013/8/7             | Nayimis (Type 1) | Lime  | 36           | N7.23138  | E151.44442| Eot      | None           | —            | —          | —       | Medium | Spreading | None   | 98.0       | 45.0       | 10.5    |
| 21  | 2013/8/9             | Nayimis (Type 1) | Lime  | 36           | N7.23207  | E151.55806| Tonoas   | Kuchua  | 50          | 4          | Medium | Slightly weak | Medium | Spreading | None   | 101.5     | 53.4       | 10.5     |
| 5   | 2013/8/5             | Nayimis (Type 2) | Lime  | 31           | N7.27521  | E151.52609| Weno     | Peniesene village | 6            | 5          | Slightly weak | Medium | None       | None   | 99.4       | 49.0       | 5.9      | 7.4       |
| 9   | 2013/8/6             | Nayimis (Type 2) | Lime  | 33           | N7.21177  | E151.49355| Fefen    | Onongoch village  | 3            | 5          | Weak    | Spreading | None       | None   | 93.4       | 42.5       | 6.6      |
| 15  | 2013/8/7             | Nayimis (Type 2) | Lime  | 49           | N7.27252  | E151.57905| Fefen    | None           | 4            | 5          | Weak    | Spreading | None       | None   | 85.4       | 41.5       | 5.6      |
| 3   | 2013/8/5             | Nayimis (Type 2) | Lime  | 31           | N7.27521  | E151.52609| Weno     | Peniesene village | 5            | 8          | Medium | Spreading | None       | None   | 81.9       | 44.9       | 6.7      |
| 10  | 2013/8/6             | Nayimis (Type 2) | Lime  | 49           | N7.26984  | E151.55806| Fefen    | Onongoch village  | 6            | 8          | Slightly weak | Medium | Spreading | None   | 99.4       | 49.0       | 5.9      | 7.4       |
| 13  | 2013/8/7             | Nayimis (Type 2) | Lime  | 33           | N7.21177  | E151.49355| Fefen    | None           | —            | —          | —       | Medium | Spreading | None   | 91.5       | 42.6       | 6.6      |
| 14  | 2013/8/7             | Nayimis (Type 2) | Lime  | 49           | N7.26984  | E151.55806| Fefen    | Onongoch village  | 6            | 8          | Slightly weak | Medium | Spreading | None   | 99.4       | 49.0       | 5.9      | 7.4       |
| 21  | 2013/8/9             | Nayimis (Type 2) | Lime  | 36           | N7.23138  | E151.44442| Eot      | None           | —            | —          | —       | Medium | Spreading | None   | 98.0       | 45.0       | 10.5     |
| 10  | 2013/8/6             | Kurukur (Type 1) | Sour orange | 35           | N7.23207  | E151.55806| Romanum | None           | 5            | 8          | Medium | Medium | Spreading | None   | 81.5       | 43.5       | 6.7      |
| 16  | 2013/8/7             | Kurukur (Type 2) | Sour orange | 36           | N7.23138  | E151.44442| Eot      | None           | —            | —          | —       | Medium | Spreading | None   | 98.0       | 45.0       | 10.5     |
| 8   | 2013/8/6             | Kurukur (Type 3) | Sour orange | 36           | N7.23138  | E151.44442| Eot      | None           | —            | —          | —       | Medium | Spreading | None   | 98.0       | 45.0       | 10.5     |
| 5   | 2013/8/5             | Tangerin (Type 1) | Calamondin | 18           | N7.26984  | E151.55806| Fefen    | Onongoch village  | 6            | 8          | Slightly weak | Medium | Spreading | None   | 99.4       | 49.0       | 5.9      | 7.4       |
| 17  | 2014/1/9             | Mexican lime (Citrus aurantifolia (Cristm.) Swingle) |  | 65           | N31.34062 | E130.31930| —        | —        | 4.5         | 3          | Medium | Upright | None   | 60.5       | 32.5       | 2.5      | 5.9       |
| 18  | 2013/8/8             | Sour orange ‘Kabusu’ (C. aurantium L.) |  | 60           | N31.34074 | E130.31374| —        | —        | 8           | 6          | Medium | Spreading | None   | 81.4       | 41.2       | 7.3      | 5.9       |
| 19  | 2013/8/8             | American Kurukur (C. aurantium L.) |  | 64           | N31.34077 | E130.31384| —        | —        | 121.1       | 82.5      | Medium | Medium | Slightly strong | None   | 131.1      | 85.2       | 14.2    |

The standards for each characteristic are as follows:

- Vigor: weak, slightly weak, medium, slightly strong, strong.
- Habit: spreading, slightly spreading, medium, slightly upright, upright.
- Spine: none, short, medium, long.
| No. | Accession      | Skin color       | Fruit surface | Flesh color | Flavor   | Peeling | Bitterness | Sweetness | Sourness | Diamter (mm) | Height (mm) | D/H index | Brix of juice | pH of juice | Embryo color | Embryony | Number |
|-----|---------------|------------------|---------------|------------|----------|---------|------------|-----------|----------|--------------|-------------|-----------|---------------|------------|--------------|----------|--------|
| 2   | Nayimis (Type 1) | Yellowish green  | Smooth        | Pale green | Lime     | Difficult | None        | Low       | High     | 43.8         | 45.5        | 97.0      | 7.7           | 2.2        | Cream, Pale green | Poly    | 3.5     |
| 7   | Nayimis (Type 1) | Yellowish green  | Smooth        | Pale green | Lime     | Difficult | None        | Low       | High     | 42.8         | 46.0        | 93.2      | 7.2           | 2.1        | Cream          | Poly    | 3.8     |
| 12  | Nayimis (Type 1) | Yellowish green  | Smooth        | Yellow     | Lime     | Difficult | None        | Low       | High     | 33.9         | 32.0        | 105.6     | —             | —          | Cream          | Poly    | —       |
| 14  | Nayimis (Type 1) | Yellowish green  | Smooth        | Pale green | Lime     | Difficult | None        | Low       | High     | 49.2         | 53.4        | 92.1      | 9.0           | 2.3        | Pale green     | Mono?   | 6.0     |
| 21  | Nayimis (Type 1) | Green            | Smooth        | Pale green | Lime     | Difficult | None        | Low       | High     | 41.1         | 43.8        | 93.9      | 8.2           | 2.2        | —              | Poly    | 4.4     |
| 3   | Nayimis (Type 2) | Green            | Slightly smooth| Orange    | Lime     | Difficult | None        | Low       | High     | 42.2         | 44.0        | 95.8      | 7.2           | 2.3        | Cream          | Poly    | 11.4    |
| 9   | Nayimis (Type 2) | Green            | Slightly smooth| Yellow    | Lime     | Difficult | None        | Low       | High     | 35.8         | 38.4        | 93.2      | —             | —          | Pale green     | Poly    | 7.4     |
| 5   | Nayimis (Type 3) | Green            | Smooth        | Pale green | Lime     | Difficult | None        | Low       | High     | 46.5         | 47.7        | 97.4      | 8.3           | 2.3        | Pale green     | Poly    | 8.6     |
| 22  | Nayimis (Type 3) | Green            | Coarse        | Yellowish orange | Unknown | Difficult | None        | Low       | High     | 54.2         | 60.1        | 90.2      | 8.0           | 2.3        | Cream          | Poly    | 10.6    |
| 8   | Kurukur        | Green            | Medium        | Yellow    | Sour orange | Difficult | Medium     | Medium    | Medium     | 71.9         | 66.6        | 107.8     | 8.4           | 3.1        | Cream          | Poly    | 68.0    |
| 11  | Kurukur        | Green            | Coarse        | Yellowish orange | Sour orange | Difficult | None  | Medium     | Low        | 66.9        | 60.9        | 109.9     | —           | —          | Cream          | Poly    | 24.7    |
| 16  | Kurukur        | Green, Yellow    | Coarse        | Sour orange | Difficult | Medium     | Medium    | Medium     | 77.7        | 71.5        | 108.7     | —           | —          | Cream          | Poly    | 20.8    |
| 23  | Kurukur        | Green, Yellow    | Coarse        | Sour orange | Difficult | Medium     | Medium    | Low        | 73.5        | 70.9        | 103.5     | 9.4          | 3.2        | Cream          | Poly    | 29.5    |
| 1   | Tangerin       | Yellow           | Slightly smooth| Yellow    | Calamondin | Slightly easy | None  | Low       | High     | 28.7        | 27.7        | 103.4     | 7.7          | 2.4        | Pale green     | Poly    | 7.2     |
| 6   | Tangerin       | Green            | Smooth        | Orange    | Calamondin | Medium     | None       | Low       | High     | 28.5        | 27.4        | 104.0     | 6.6          | 2.3        | Green, Pale green | Poly    | 6.5     |
| 13  | Tangerin       | —                | —             | —         | Calamondin | —         | —         | —         | —         | —            | —           | —          | —             | —          | —           | —       | —       |
| 18  | Gunkan         | Orange           | Smooth        | Orange    | Calamondin | Medium     | None       | Low       | High     | 32.9         | 31.2        | 105.2     | 7.4           | 2.3        | Green, Pale green | Poly    | 6.6     |
| 4   | Kurukur        | Green            | Medium        | Yellowish orange | Sour orange | Difficult | None  | Medium     | Low        | 91.6        | 93.0        | 99.2      | 7.2          | 3.0        | Cream          | Poly    | 12.5    |
| 19  | American Kurukur | Green           | Coarse        | Yellowish orange | Sour orange | Difficult | None  | Medium     | Medium    | 94.7        | 90.6        | 104.5     | 8.6          | 3.2        | Cream          | Poly    | 2.5     |
| 17  | Kurukur        | Yellowish green  | Smooth        | Cream     | Pummelo    | Difficult | Medium     | —         | —         | 122.2        | 133.8       | 92.1      | —           | —          | —              | —       | 0.0     |

Control

- Sour orange ‘Kabusu’
- Orange
- Slightly smooth
- Yellowish orange
- Sour orange
- Difficult
- Medium
- Low
- High
- 86.4
- 74.1
- 116.6
- 11.5
- 2.3
- Cream
- Poly
- 11.2
- Calamondin
- Orange
- Smooth
- Orange
- Calamondin
- Medium
- Low
- High
- 32.4
- 27.7
- 117.0
- 9.5
- 2.5
- Cream
- Poly
- 3.0

The standards for each characteristic are as follows;

A Skin color: green, yellowish green, yellow, orange, pink, red.
B Fruit surface: smooth, slightly smooth, medium, slightly coarse, coarse.
C Flesh color: pale green, cream, yellow, yellowish orange, orange, pink, red.
D Peeling: easy, slightly easy, medium, slightly difficult, difficult.
E Bitterness: none, low, medium, high.
F Sweetness: low, medium, high.
G Sourness: low, medium, high.
H Embryo color: cream, pale green, green.
I Embryony: mono, poly.
formed in a Veriti 200 (Applied Biosystems, Thermo-
Fisher Scientific, Waltham, MA, USA) thermal cycler
that was programmed as follows: initial heating at 94°C
for 3 min, next two cycles of denaturation at 94°C for
30 s, at 62, 60, 58, and 56°C for 30 s, extension at 72°C
for 1 min, followed by 35 cycles for 30 s at 94°C, 30 s
at 54°C, and 2 min at 72°C, and final extension for
7 min at 72°C.

The PCR products were digested with restriction en-
zymes (Takara Bio, Shiga, Japan) under the following
conditions: 4 μL of each PCR product was mixed with
1.0 μL of the reaction buffer and 2 to 3 units of the
restriction enzyme, and then the final volume was
adjusted to a total of 10 μL with sterile water. After
digestion at 37°C for more than 4 h, the digested prod-
cuts were electrophoresed on 1.5% agarose gels
(Seakem GTG Agarose; Takara Bio), and stained with
GelRed (Biotium, Hayward, CA, USA). The resulting
bands were detected under UV light. Based on the re-
sults of the banding pattern of the gel electrophoresis,
each genotype was designated as “aa”, “ab”, or “bb” ac-
cording to the fragment size in the nuclear genome
(Table 4). In cpDNA analysis, the type of each acces-
sion was confirmed according to the fragment size that
appeared (Table 4).

## Results

**Local citrus genetic resources on the Chuuk Islands and
their morphological traits**

The results of our investigation on the Chuuk Islands
are shown in Figures 1 and 2 and Tables 1 and 2. Five,
four, four, five, and three accessions were investigated
on Weno, Fefen, Eot, Romanum, and Tonoas Island,
respectively. The total number of accessions investigated
was 21. Morphologically, ten, four, four, two, and one
accession resembled lime (local name “Nayimis”), sour
orange (“Kurukur”), calamondin (“Tangerin” and
“Gunkan”), sweet orange (“Kurukur” and “American
Kurukur”), and pummelo (“Kurukur”), respectively.
Lime, sour orange, and calamondin-type accessions
were grown on at least four of the islands investigated.

All “Nayimis” accessions except #5 and 22 pos-
sessed characteristic lime features (e.g., round fruit and
a highly acidic juice with a distinctive aroma, described
in Table 2). They were separated into three types based
on their characteristics: “Nayimis type 1”, “Nayimis

| No. | Latin name | Common name                      |
|-----|------------|----------------------------------|
| C1  | C. aurantifolia (Cristm.) Swingle | Mexican lime |
| C2  | C. medica L. ‘Marubushukan’ | citron |
| C3  | C. limon (L.) Burm. f. ‘Allen Eureka’ | lemon |
| C4  | C. limonia Osbeck | rangpur lime |
| C5  | C. maxima (Burm.) Merr. ‘Chandler’ | pummelo |
| C6  | C. maxima (Burm.) Merr. ‘Banpeiyu’ | pummelo |
| C7  | C. aurantium L. ‘Kaiseito’ | sour orange |
| C8  | C. aurantium L. ‘Kabusu’ | sour orange |
| C9  | C. sinensis (L.) Osbeck ‘Hamlin’ | sweet orange |
| C10 | C. reticulata Blanco ‘Yoshida Ponkan’ | mandarin (ponkan) |
| C11 | C. reshni hort. ex Tanaka | mandarin (cleopatra) |
| C12 | C. depressa Hayata | mandarin (shiikuwasha) |
| C13 | C. madurensis Lour. | calamondin |

| Marker name | PCR product size (bp) | Restriction enzyme | Polymorphic allele size (bp) | a | b |
|-------------|----------------------|--------------------|-----------------------------|---|---|
| Nuclear genome |  |  |  |  |  |
| Tf0235 | 700 | HaeIII | 700 | 450 |
| Tf0420 | 400 | HaeIII | 400 | 200 |
| Tf0419 | 700 | PvuII | 700 | 400 |
| Gn0029 | 450 | HinfI | 450 | 250 |
| Chloroplast DNA |  |  |  |  |  |
| trnL-trnF | 450 | Sau3AI | 450 | 250 |
| rbcL | 1100 | HhaI | 850 | 450 |

* Shimada et al. (2014).
† Taberlet et al. (1991).
‡ Cipriani and Morgante (1993).
type 2”, and “Nayimis type 3”. Accessions #2, 7, 12, 14, and 21 belonged to type 1. The characteristics of the foliage and fruit of “Nayimis” type 1 were very similar to those of Mexican lime (C. aurantifolia (Cristm.) Swingle). The fruit surface was smooth and the petiole wing was relatively large (Table 1). In contrast, the morphological traits of “Nayimis” type 2 (#3, 9, and 15) were slightly different from those of type 1 and Mexican lime. The fruit surface was slightly smooth and they had a smaller and thinner petiole wing compared with type 1 and Mexican lime. In addition, type 1 had fewer seeds than type 2. “Nayimis” type 3 (#5 and 22) did not show typical lime characteristics. The flesh color of #5 was orange and #22 did not have a lime flavor.

“Kurukur” and “American Kurukur” accessions were separated into three types: a sour orange type (#8, 11, 16, and 23), a sweet orange type (#4 and 19), and a pummelo type (#17) (Tables 1 and 2). Local accessions that possessed sour orange (C. aurantium L.) characteristics are known as “Kurukur”. The morphological traits (e.g., a relatively large petiole wing, external appearance of fruit, and cream polyembryonic seeds), including flavor, of all accessions of this “Kurukur” type (#8, 11, 16, and 23) were similar to those of ordinary sour orange except for the sourness of the juice. Although highly acidic juice is common in sour orange, the taste of these “Kurukur” cultivars was not sour. Their juice pH values were 3.1 and 3.2, which were higher than that of the acid citrus “Nayimis”.

“Kurukur” #4 was similar to sweet orange (C. sinensis (L.) Osbeck) in terms of morphological traits. Although the flavor of “American Kurukur” resembled that of sweet orange, its rind was thick and the rind of the fruit surface was coarse compared with sweet orange. According to an interview, “American Kurukur” was introduced from the USA.

“Kurukur” #17 showed typical pummelo (C. maxima (Burm.) Merr.) characteristics: a large petiole wing, large fruit, hard skin, and characteristic aroma. It was seedless.

Morphological traits of three “Tangerin” and one “Gunkan” were similar to those of calamondin (C. madurensis Lour.). All accessions had a small petiole wing, small, round, seedy fruits with a distinctive flavor and a low juice pH. There were no clear differences between “Tangerin” and “Gunkan”. In general, tangerin means a reddish mandarin. However, the “Tangerin” on Chuuk was difficult to class as a mandarin.

**CAPS analysis of the local citrus genetic resources on the Chuuk Islands**

Polymorphisms were observed in all primer and restriction enzyme combinations on CAPS analysis of both the nuclear genome and cpDNA (Tables 4 and 5; Fig. 3). First, we classified the accessions based on the results of genotype combinations of the nuclear genome. Control species, C. aurantifolia, C. medica, and C. madurensis, could not be distinguished from each other in the present study. Each accession analyzed was divided into eight types (Table 5). “Nayimis” was divided into two types based on the genotype combinations: one was type 1 and the other included types 2 and 3. The genotypes of type 1 were identical to those of Mexican lime. Types 2 and 3, rangpur lime, and cleopatra belonged to same type. “Tangerin”, “Gunkan”, and calamondin showed identical genotypes. Each of the three “Kurukur” sour orange-types showed characteristic genotype combinations. Genotypes of “Kurukur” #8 were identical to those of sour orange. Sweet orange-type “Kurukur” #4 and “American Kurukur” #19 could be discriminated from each other. “American Kurukur” #19 and sweet orange showed the same genotype. Pummelo-type “Kurukur” #17 and pummelo showed identical genotype combinations.

In cpDNA analysis, local accessions were classified into two types (Table 5). “Nayimis” types 2 and 3 belonged to type 2 cpDNA. On the other hand, all remaining accessions had type 1 cpDNA. In control species, rangpur lime, shiikuwasha, and cleopatra were type 2, and the remaining species except ponkan had type 1 cpDNA.

**Discussion**

In the present study, we identified local citrus accessions grown on the Chuuk Islands using morphological and molecular markers. The combined results offer...
valuable information for the identification of citrus genetic resources on the Chuuk Islands. In addition to the classification based on the morphological traits, many local citrus accessions showed characteristic genotypes on CAPS analysis.

There are at least two groups of “Nayimis”. Morphologically, “Nayimis” type 1 was identical or similar to Mexican lime. Its petiole wing was larger and the fruit surface was smoother than those of “Nayimis” type 2. Moreover, “Nayimis” types 1 and 2 could be clearly distinguished by DNA analysis. Both the nuclear genome and cpDNA of types 1 and 2 were different. There were no polymorphisms within accessions of the same types. The morphological traits of “Nayimis” type 1 were similar to those of Mexican lime. In addition, their types of nuclear and cpDNA were the same. Thus, “Nayimis” type 1 is considered to be the same or a close relative of Mexican lime. Although the nuclear genome and cpDNA of type 2 were identical to those of rangpur lime and cleopatra, it is difficult to classify it as a mandarin because of morphological traits. Morpho-

| No. | Accession          | Nuclear genome | Chloroplast DNA | Type |
|-----|--------------------|----------------|-----------------|------|
| 2   | Nayimis (Type1)    | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 7   | Nayimis (Type1)    | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 14  | Nayimis (Type1)    | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 21  | Nayimis (Type1)    | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 3   | Nayimis (Type2)    | aa ab bb aa 2  | trn-trnL Sauc3Al | b b 2|
| 9   | Nayimis (Type2)    | aa ab bb aa 2  | trn-trnL Sauc3Al | b b 2|
| 15  | Nayimis (Type2)    | aa ab bb aa 2  | trn-trnL Sauc3Al | b b 2|
| 5   | Nayimis (Type3)    | aa ab bb aa 2  | trn-trnL Sauc3Al | b b 2|
| 22  | Nayimis (Type3)    | aa ab bb aa 2  | trn-trnL Sauc3Al | b b 2|
| 8   | Kurukur            | ab aa bb ab 3  | trn-trnL Sauc3Al | a b 1|
| 16  | Kurukur            | ab aa bb aa 4  | trn-trnL Sauc3Al | a b 1|
| 23  | Kurukur            | ab aa bb bb 5  | trn-trnL Sauc3Al | a b 1|
| 1   | Tangerin           | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 6   | Tangerin           | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 18  | Gunkan             | aa aa bb aa 1  | trn-trnL Sauc3Al | a b 1|
| 4   | Kurukur            | ab ab ab aa 6  | trn-trnL Sauc3Al | a b 1|
| 19  | American Kurukur   | ab ab ab ab 7  | trn-trnL Sauc3Al | a b 1|
| 17  | Kurukur            | ab ab ab bb 8  | trn-trnL Sauc3Al | a b 1|

Control

C1  C. aurantifolia (Mexican lime)  aa aa bb aa 1  a b 1
C2  C. medica (citron)             aa aa bb aa 1  a b 1
C3  C. limon (lemon)              ab aa bb aa 4  a b 1
C4  C. limonia (rangpur lime)      aa ab bb aa 2  b b 2
C5  C. maxima (pummelo)           bb aa bb bb 8  a b 1
C6  C. maxima (pummelo)           bb aa bb bb 8  a b 1
C7  C. aurantium (sour orange)    ab aa bb ab 3  a b 1
C8  C. aurantium (sour orange)    ab aa bb ab 3  a b 1
C9  C. sinensis (sweet orange)    ab ab ab ab 7  a b 1
C10 C. reticulata (mandarin (ponkan)) aa ab ab ab 9  b a 3
C11 C. reshni (mandarin (cleopatra)) aa ab bb aa 2  b b 2
C12 C. depressa (mandarin (shiikuwasha)) aa ab ab bb 10 b b 2
C13 C. madurensis (calamondin)    aa aa bb aa 1  a b 1

Fig. 3. Restriction pattern obtained after digestion of Tf0235 amplified products with Hae III. 1–23: Accession numbers of local citrus on the Chuuk Islands evaluated in this study (see Table 1). M: molecular marker. The genotype in this figure corresponds to the genotype in Table 5.
logical traits of type 2 and rangpur lime were somewhat different; the fruit of rangpur lime is similar to that of mandarin and it does not have a lime flavor (Hodgson, 1967; Tanaka, 1946). However, since wide variation exists in fruit characteristics of rangpur lime (Hodgson, 1967), the genetic relationships between type 2 and rangpur lime should be elucidated using more DNA markers. Moreover, recently a new type of lime grown in Bhutan was reported (Tshering Penjor et al., 2014a, 2016). Its morphological traits were similar to those of “Nayimis” type 2: a relatively small and thin petiole wing and a slightly smooth rind texture. This type of lime was found to be genetically intermediate between mandarin and citron, whereas Mexican lime is considered to be a hybrid of papeda and citron (Tshering Penjor et al., 2016). The cpDNA of “Nayimis” type 2 was identical to that of mandarin (cleopatra). These results suggest a close relationship between “Nayimis” type 2 and the new lime found in Bhutan, although Bhutan is far from Chuuk. Therefore, it is an urgent issue to elucidate their genetic relationships and distribution. We could not define “Nayimis” type 3. Morphologically, it is different from types 1 and 2, but its genotype based on CAPS analysis was identical to type 2 and rangpur lime. It is considered that there are some genetic relationships between types 2 and 3 and rangpur lime. Further study is needed to precisely identify “Nayimis” accessions.

Sour orange, sweet orange, and pummelo are known as “Kurukur”. This means that “Kurukur” refers to not only sour orange, but also other large citrus fruits for fresh use on the Chuuk Islands.

All accessions of the “Kurukur” sour orange type were similar to those of ordinary sour orange, except for the sourness of the juice, in terms of morphological traits. Although highly acidic juice is common in sour orange, no “Kurukur” had a sour taste. The reason for this was not clear; the tropical environmental conditions may affect juice acidity, or accessions with a low juice acidity may have been selected. Genetic diversity was found among the “Kurukur” accessions on CAPS analysis. The genotype combinations of #8 were identical to those of sour orange. The CAPS genotypes of #16 and 23 may have arisen from self-fertilized seedlings of sour orange. This is because sour orange is self-compatible (Yamamoto et al., 2006) and seedling propagation has been common there.

Genotypes of the sweet orange-type “Kurukur” and “American Kurukur” were different, while those of “American Kurukur” and sweet orange were the same. Their cpDNA was identical. The CAPS genotypes of “Kurukur” #4 could not have arisen from self-fertilized seedlings of sweet orange. Thus, genetic influences from other species should be investigated. “American Kurukur” is probably different from sweet orange because of its morphological traits although they showed the same genotype on CAPS analysis.

Pummelo-type “Kurukur” #17 was considered to be a true pummelo based on the results of morphological and molecular markers. It is considered to be an important genetic resource because of its seedless fruit.

Morphologically, the calamondin–like accessions “Tangerin” and “Gunkan” showed the same genotype combinations on CAPS analysis. These genotypes were identical to those of calamondin used as a control. “Tangerin” and “Gunkan” seem to be true calamondin based on the results of morphological traits and CAPS analysis.

The accessions morphologically similar to some lime and sour orange grown on the Chuuk Islands were genetically dissimilar to standard lime (C. aurantifolia), and sour orange (C. aurantium), respectively. Morphologically similar, but genetically dissimilar, local citrus accessions also existed in Bhutan and Ambon Island in Indonesia (Raharjo et al., unpublished; Tshering Penjor et al., 2016). Hence, in citrus species morphological similarities are not always predictive of genetic similarities. This indicates the importance of investigation of local citrus accessions to understand the distribution and differentiation of citrus genetic resources. Local accesses showing characteristic genotypes could have been selected because they adapted to the local environment. The local citrus grown on the Chuuk Islands is considered to be adapted to a tropical rain forest climate. It is necessary to investigate tree and fruit characteristics of these local accessions and compare them with the standard ones in detail. However, it is very difficult to conduct this research since it must be carried out on the Chuuk Islands where no citrus researchers are based.

In the present study, valuable new genetic information was revealed based on the results of CAPS analysis, although we used only four and two primer/restriction enzyme combinations for nuclear genome and cpDNA, respectively. A number of CAPS markers are available in citrus (Shimada et al., 2014). In conclusion, CAPS analysis using more markers of local citrus grown on the Chuuk Islands is necessary to identify each accession and clarify phylogenetic relationships.

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