Introduction

Only two species, *Citrus depressa* Hayata (Shiikuwasha) and *C. tachibana* (Makino) Tanaka (Tachibana), are indigenous to Japan, although various accessions of citrus are cultivated there. The former is native to the subtropical Ryukyu Islands (Tanaka, 1926), while the latter mainly grows on the temperate Pacific side of the southwest of Japan’s main islands, but some populations are also found on the Ryukyu Islands (Tanaka, 1924; 1926).

Kagoshima Prefecture, Japan, is located between 27° and 32° N (Fig. 1). Various local citrus, including Shiikuwasha, are grown there and we have investigated them (Yamamoto et al., 2006; 2021). The origins and/or phylogenetic relationships of many of them have been already reported (Yamamoto et al., 2011; 2017; 2022a; 2022b). However, there are some small mandarin accessions whose genetic background is unknown. Although their fruits resemble those of Shiikuwasha or Tachibana, phylogenetic relationships among them remain unclear.

DNA analysis is essential for the study of accession identification and phylogenetic relationships. Among the various types of DNA analyses, the cleaved amplified polymorphic sequence (CAPS) is a simple and reliable method (Konieczny and Ausubel, 1993). It is a PCR-based assay and does not require the use of a DNA sequencer. It has several advantages for such a phylogenetic study because inheritance occurs in a codominant manner. Shimada et al. (2014) developed 708 CAPS markers for citrus, which are useful for phylogenetic studies in citrus genetic resources (Yamamoto et al., 2022a; 2022b).

Thus, in the present study, we performed CAPS analysis of the local small mandarin, grown in several places in Kagoshima. Based on the results of molecular markers, their genetic characteristics were examined, and as a result, new findings, regarding the distribution of Shiikuwasha, were obtained.

Materials and Methods

Six local citrus accessions, grown in Kagoshima Prefecture, were investigated: ‘Yamato #8’ on Amami Oshima, ‘Kozu’ (A) and ‘Kozu’ (B) on Yakushima, ‘Kozumikan’ on Kuroshima, ‘Yamatate’ on Koshikijima, and ‘Yamamikan’ on Bonotsu (Figs. 1 and 2). They were collected and preserved in the Toso Orchard of the Experimental Farm, Faculty of Agriculture, Kagoshima University (Kagoshima, Japan, ca. N 31.34°, E 130.32°, and 65 m high).

The six local citrus and 16 control accessions, preserved there were used as materials (Table 1). Total DNA was extracted from fresh leaves using Isoplant II (Nippon Gene, Tokyo, Japan), according to the manufacturer’s instructions.

A CAPS analysis of the nuclear genome was carried out, using 16 of the citrus CAPS markers (Table 2), developed by Shimada et al. (2014). A PCR reaction mixture of 12.5 μL consisted of 10 ng of template DNA, 10 pmol of each primer, 1 × reaction buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, and 0.5 units of Prime Taq DNA polymerase (GeNet Bio, Daejeon, Korea). These reactions were performed in a Veriti 200 (Applied Biosystems, ThermoFisher Scientific, Waltham, MA, USA) thermal cycler that was programmed as follows: initial heating at 94°C for 3 min, then two cycles each 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 a final extension for 7 min at 72°C.

The PCR products were digested with restriction enzymes (Takara Bio, Shiga, Japan) under the following
conditions: Each of the 4.0 μL of the PCR products 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 products 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 results of the banding pattern of the gel electrophoresis, each genotype was designated as “aa”, “ab”, or “bb” according to fragment size. The genetic distance was calculated between each pair of accessions according to Dice (1945). To examine genetic relationships, cluster analysis was conducted using a Molecular Evolutionary Genetic Analysis (MEGA, ver. 4.1) software (Tamura et al., 2007) and the Neighbor-Joining (NJ) Method.
Table 2. Characteristics of the CAPS markers used in this study.

<table>
<thead>
<tr>
<th>Marker name</th>
<th>Restriction enzyme</th>
<th>Approximate size of polymorphic allele (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cp1143</td>
<td>Hha I</td>
<td>800</td>
</tr>
<tr>
<td>Gn0029</td>
<td>Hinf I</td>
<td>450</td>
</tr>
<tr>
<td>T0001</td>
<td>Msp I</td>
<td>650</td>
</tr>
<tr>
<td>T0042</td>
<td>Hha I</td>
<td>300</td>
</tr>
<tr>
<td>T0154</td>
<td>Hae III</td>
<td>1100</td>
</tr>
<tr>
<td>T0168</td>
<td>Ra I</td>
<td>1200</td>
</tr>
<tr>
<td>T0235</td>
<td>Hae I</td>
<td>700</td>
</tr>
<tr>
<td>T0300</td>
<td>Bam III</td>
<td>700</td>
</tr>
<tr>
<td>T0315</td>
<td>Hinf I</td>
<td>300</td>
</tr>
<tr>
<td>T0350</td>
<td>Dra I</td>
<td>900</td>
</tr>
<tr>
<td>T0364</td>
<td>Hinf I</td>
<td>800</td>
</tr>
<tr>
<td>T0368</td>
<td>Ra I</td>
<td>350</td>
</tr>
<tr>
<td>T0386</td>
<td>Msp I</td>
<td>550</td>
</tr>
<tr>
<td>T0396</td>
<td>Eco RV</td>
<td>600</td>
</tr>
<tr>
<td>T0419</td>
<td>Pvu II</td>
<td>700</td>
</tr>
<tr>
<td>T0420</td>
<td>Hae III</td>
<td>400</td>
</tr>
</tbody>
</table>

Shimada et al. (2014).

Results and Discussion

Among the six local citrus accessions, the genotype combinations of 16 CAPS markers were identical to one another in ‘Kozu’ (A), ‘Yamatate’, and ‘Yamamikan’ (Fig. 3). The three remaining local citrus demonstrated characteristic genotypes. Two Kozu accessions showed different genotype combinations. On the other hand, four Shiikuwasha accessions were closely related to each other and distinct from otheraccessions in the control materials. In addition, small mandarin-type accessions clustered together (Fig. 3). All the CAPS genotypes of ‘Kozu’ (B) were identical to those of ‘Shiikunin’ (Ama). The genotypes of ‘Kozumikan’ were also identical to those of ‘Shiikuribu’. ‘Kozu’ (A), ‘Yamatate’, and ‘Yamamikan’ were closely related to the Japanese mandarin Tachibana. The estimated genetic distance between ‘Yamato #8’ and the Chinese mandarin Sunki was closer than that to the Japanese mandarins Shiikuwasha and Tachibana (Fig. 3).

The phylogenetic relationships of control accessions were consistent with those in previous reports (Curk et al., 2015; Yamamoto et al., 2017; Wu et al., 2018; Yu et al., 2018). The Citrus species were largely classified into citron, pummelo, and mandarin. Small mandarin was distinct from large ones. Shiikuwasha could be differentiated from others. Hence, the results of the present study are considered to be appropriate for the elucidation of phylogenetic relationships of citrus and strongly suggested that ‘Kozu’ (B) and ‘Kozumikan’ are kinds of Shiikuwasha. The former and latter were identical to ‘Shiikunin’ (Ama) on Tokunoshima and ‘Shiikuribu’ on Okinorabujima, respectively.

Shiikuwasha is distributed on the Ryukyu Islands of Japan and Taiwan. It has been considered that its northern limit is Amami Oshima (ca. N 28°) (Tanaka, 1926; Tanaka, 1948). Although some Shiikuwasha plants are found on Takarajima (ca. N 29°) on Tokara Islands, there is a possibility that they were artificially introduced. (Shiuchi and Hotta, 2015). ‘Kozu’ (B) and ‘Kozumikan’ are distributed on Yakushima and Kuroshima, respectively. Both islands are located at approximately N 30°. Thus, they are the northernmost areas where Shiikuwasha is grown as local citrus that we know of. However, an important issue remains. DNA analysis could not reveal their history regarding whether they are indigenous or were artificially introduced. Neither is cultivated in large-scale orchards for commercial use; being mainly grown in backyards, at roadsides, or in small-scale orchards. On the islands of Kagoshima Prefecture, some introduced citrus are considered to be local, for instance, Kunenbo (C. nobilis Lour.), introduced from the Indochina Peninsula before the sixteenth century (Tanaka, 1948), is a representative local citrus of the Amami Islands (Yamamoto et al., 2006). Thus, it is difficult to elucidate its history, based on the current growing conditions.

‘Kozu’ (A), ‘Yamatate’, and ‘Yamamikan’ were closer to Tachibana than Shiikuwasha. Since their distribution areas overlap those of Tachibana (Tanaka, 1924), these results could be obtained. However, they were not identical to Tachibana used in the present study. It is
well known that there is some diversity in this citrus and natural hybrids are derived from it (Hirai et al., 1990; Shimizu et al., 2016). Comparisons of these three accessions and the plural type of Tachibana accessions are necessary to clarify their phylogenetic relationships accurately. In addition, the parent-offspring relationships with these three could not be denied in Tachibana, based on the results of the CAPS genotype combinations. The three accessions, showing the same genotypes, were distributed in different places. As in ‘Kozu’ (B) and ‘Kozumikan’, the reason for the wide distribution, either natural or artificial, could not be elucidated. However, since these accessions are polyembryony (data not shown), nucellar seedlings have been considered to play an important role in their distribution. In addition, ‘Kozu’ (A) and ‘Kozu’ (B) on Yakushima were different types of citrus although their name are the same. This result indicates the presence of homonym in local citrus.

Unlike the five above-mentioned accessions, ‘Yamaoto #8’ was closely related to the Chinese mandarin Sunki. It was also related to the Indian mandarin Cleopatra and Chinese mandarin Kishumikan. It was closer to Chinese and Indian mandarins than Japanese mandarins, Tachibana and Shiikuwasha. A previous study on isozyme analysis reported that the genotypes of ‘Yamaoto #8’ were identical to those of the Chinese mandarin (Yamamoto et al., 2011). Therefore, these results suggest that ‘Yamaoto #8’ may be a Chinese mandarin from the Asian Continent or a close relative of this mandarin.

In conclusion, genetic characteristics of some small local mandarins, grown in Kagoshima Prefecture, were discovered based on the results of CAPS analysis. In particular, we demonstrated that ‘Kozu’ (B) and ‘Kozumikan’ distributed on Yakushima and Kuroshima, respectively, might be kinds of Shiikuwasha. However, there are still unresolved issues. Thus, we plan to conduct the following research in the future. A field survey over a wider area and a literature research are necessary to investigate the distribution and history, indigenous or artificially introduced, of small mandarin accessions. Morphological traits are also important for genetic characterization. Since the phylogenetic relationships of citrus were revealed, based on them (Handa and Oogaki, 1985), investigating detailed morphological traits, is necessary. In the present study, 16 CAPS markers were used. Although they were useful for the identification of the accessions, DNA analysis, using more markers, are needed to increase the accuracy of determining their genetic characteristics.

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References


