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Molecular characterization and genetic diversity among Japanese acid citrus (*Citrus* spp.) based on RAPD markers

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SUMMARY
Randomly Amplified Polymorphic DNA (RAPD) markers were used to evaluate genetic similarity and inter-relationship among 31 acid citrus species and cultivars, including sour oranges (six accessions); 'Yuzu' (four accessions) and its relatives (21 accessions). Out of the 60 decamer primers screened, 27 were selected which produced 108 markers; 76 of which were polymorphic. Species or cultivar-specific RAPD markers were also found. A dendrogram based on genetic distance implied that sour oranges were very distinct from 'Yuzu' and its relatives. 'Yuzu' accessions were very closely linked to each other, however; for the other specimens genetic polymorphism could easily be detected by RAPDs and the genetic variation between accessions was quite high and revealed their different origins. In this study some RAPDs allowed the distinction of very close cultivars, for instance 'Kabosu' from 'Aka kabosu'.

The term "acid citrus" is conveniently used for *Citrus* species whose fruits are used for flavouring foods and for producing vinegar. In Japan, the cultivation of lemons and limes is very limited. The diversity of Japanese acid citrus mainly occurs in 'Yuzu' and its relatives, and sour oranges (Omura, 1996).

'Yuzu' (*Citrus junos* Sieb. ex Tanaka) originated in China and was introduced to Japan from China via Korea before the eighth century (Taninaka *et al.,* 1981; Omura, 1996). After introduction, through selfing or cross-pollination with other citrus forms, it has produced various progenies which form a special group of acid citrus in Japan (Rahman *et al.,* 2001). Many land clones also generated mutants which differ in crop yield, reduction of spines, variegation, ripening season and so on. Spontaneous seedless clones have also been identified (Omura, 1996).

'Yuzu' is utilized for making liqueur, juice, jam, marmalade, jelly and vinegar. Its rind is also used for cooking because it is less bitter than the pulp. 'Yuzu' is a cold tolerant citrus grown in the nothern part of Japan as well as in mountainous areas (Omura, 1996; Kimura and Taninaka, 1996). It is also cultivated as a rootstock because of its resistance to foot rot, the longevity of trees grafted on it and its ability to absorb nutrients more efficiently than trifoliate orange (Bitters, 1964).

'Sudachi' (*Citrus sudachi* Hort. ex Shirai) and 'Kabosu' (*Citrus sphaerocarpa* Hort. ex Tanaka), the 'Yuzu'-related species, originated spontaneously in Tokushima and Oita prefectures, respectively. Traditionally, their green-stage fruits have been used as acid citrus for flavouring in cooking and the demand has developed into a local citrus industry. In addition to these two, many other 'Yuzu' relatives and hybrids had been utilized for a long time (Omura, 1996).

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'Yuzu' and related acid citrus are characterized by their morphological traits (Taninaka *et al.,* 1981; Kimura and Taninaka, 1988, 1990, 1995) and recently using leaf isozyme analysis (Rahman *et al.,* 2001).

Introduction of DNA markers based on Polymerase Chain Reaction (PCR) technology has led to the development of several novel genetic assays, that can be used for many purposes in plant genetic analyses such as cultivar identification, gene mapping and so on.

Randomly amplified polymorphic DNA (RAPD) markers that result from the PCR amplification of genomic DNA fragments using short oligonucleotides (usually 10-mers) of arbitrary sequence as primers (Williams *et al.,* 1990) provide a fast and easy approach for taxonomic classification and cultivar-typing of fruit trees.

The RAPD assay has the advantages of being readily employed, requiring very small amounts of genomic DNA and eliminating the need for blotting and radioactive detection (Cipriani *et al.,* 1996). For these reasons many fruit crops have been successfully fingerprinted using RAPD markers e.g. olive (Sanz-Cortes *et al.,* 2001), feijoa (Dettori and Palombi, 2000), and kiwifruit (Cipriani *et al.,* 1996).

In *Citrus,* RAPD markers have been used for genetic mapping (Cristofani *et al.,* 1999), identification of cultivars (Coletta Filho *et al.,* 1998), hybrids (Elisiaro *et al.,* 1999), mutants (Deng *et al.,* 1995), chimeras (Sugawara *et al.,* 2002) and phylogenetic analyses (Nicolosi *et al.,* 2000).

Since DNA fingerprinting using PCR-based markers are very important for breeding and taxonomy of *Citrus* and no DNA-based marker approaches to the systematic studies of Japanese acid citrus have been attempted, in this study we used RAPD markers to characterize Japanese acid citrus. The objectives were to achieve a better understanding of genetic variation and to investigate their inter-relationships.

MATERIALS AND METHODS

Plant material

A total of 31 species and cultivars belonging to sections *Osmocitrus* and *Aurantium* after Tanaka's (1954) systematic study of *Citrus* were investigated in this study (Table I). The plant materials were maintained in the citrus germplasm collection at Saga University, Japan.

DNA isolation

Total DNA was isolated from fully expanded fresh leaves using the CTAB (hexadecyltrimethylammonium-bromide) method (Murray and Thompson, 1980) with few modifications. Briefly, 2 g of leaves which were cleaned with moist paper towels were ground to a fine powder in liquid nitrogen. The powder was added to 6 ml of extraction buffer {Tris-HCl (100 mM, pH 8.0), EDTA (20 mM), NaCl (1.4 M), CTAB [2% (wv-1)], 2-mercaptoethanol [2% (vv^{-1})], and PVP [polyvinylpyrollidone, Mol.Wt. 40,000; 1% (wv^{-1})]} and incubated at 65°C for 30 min. DNA was extracted with chlorform-octanol (24:1). DNA was washed with 70% ethanol and dissolved in 100–400 μl of TE {Tris-HCl (10 mM, pH 8.0), and EDTA (1 mM)} containing RNase (ribonuclease A, 0.2 mg ml^{-1}). DNA concentration was determined by spectrophotometry at 260 nm using different concentrations of phage lambda DNA (Toyobo Co., Ltd.) as standards. A portion of the DNA was diluted to 20 ng μl^{-1} for use, and both the stock and diluted portions were stored at –20°C.

PCR procedure

RAPD primers were purchased from Operon Technologies, Alameda, CA, USA. A total of 60 decamer oligonucleotides, of arbitrary sequence (kits OP-A, OP-B and OP-E) were tested for PCR amplification. The basic protocol reported by Williams *et al.* (1990) for PCR was followed with slight modifications. PCR was performed in a total volume of 12.5 μl, containing 25 ng of template DNA, 0.4 μM of a single primer, 0.6 U Taq DNA polymerase (Takara Shuzo Co. Ltd.), 200 μm each dNTP (Takara Shuzo Co. Ltd.), 1.5 mM $MgCl_2$, 10 mM Tris-HCl, 50 mM KC1, pH 8.3. Each reaction mixture was overlaid with one drop of mineral oil (Sigma-Aldrich).

DNA amplification was carried out in a Progene thermal cycler (Techne Ltd., UK) and followed the thermal cycling adopted by Williams *et al.* (1990). An initial denaturation cycle of 1 min 30 s at 94°C was followed by 45 cycles comprising 1 min at 94°C, 1 min at 36°C and 2 min at 72°C. An additional cycle of 7 min at 72°C was used for final extension. Amplification products were separated by electrophoresis (8.3 V cm^{-1}) in 1.5% agarose gels and stained in ethidium bromide. A photographic record was taken for each PCR run. The DNA size reference standard was the 200 base pairs (bp) ladder (Takara Shuzo Co., Ltd.). Reproducibility of the patterns was tested by running part of the reactions in duplicate.

Table I
Acid citrus species and cultivars used in this study

No.	Accession	Scientific name
1	'Daidai'	*C. aurantium* L.
2	'Myrtle-leaf orange'	*C. myrtifolia* Rafinesque
3	'Kaiseito'	*C. aurantium* L.
4	'Nanshou daidai'	*C. taiwanica* Tan. & Shimada
5	'Kiku daidai'	*C. canaliculata* Y. Tanaka
6	'China daidai'	*C. aurantium* L.
7	'Yuzu' (tetraploid)	*C. junos* Sieb. ex Tanaka
8	'Tochikei yuzu'	*C. junos* Sieb. ex Tanaka
9	'Yamanekei yuzu'	*C. junos* Sieb. ex Tanaka
10	'Seedless yuzu'	*C. junos* Sieb. ex Tanaka
11	'Mushi yuukaku sudachi'	*C. sudachi* Hort. ex Shirai
12	'Yuukaku yushi sudachi'	*C. sudachi* Hort. ex Shirai
13	'Yushi mukaku sudachi'	*C. sudachi* Hort. ex Shirai
14	'Sudachi'	*C. sudachi* Hort. ex Shirai
15	'Seedless sudachi'	*C. sudachi* Hort. ex Shirai
16	'Kabosu'	*C. sphaerocarpa* Hort. ex Tanaka
17	'Aka kabosu'	*C. sphaerocarpa* Hort. ex Tanaka
18	'Taninaka kizu'	*C. kizu* Hort. ex Y. Tanaka
19	'Kinosu'	*C. kizu* Hort. ex Y. Tanaka
20	'Kizu'	*C. kizu* Hort. ex Y. Tanaka
21	'Matsuda sudachi'	*C. acidoglobosa* Hort. ex Tanaka
22	'Zanbo'	*C. nanseiensis* Hort. ex Tanaka
23	'Yuzukichi'	*C. yuzukichi* Hort. ex Y. Tanaka
24	'Mochiyu'	*C. inflata* Hort. ex Tanaka
25	'Zuishouyu'	*C. speciosa* Hort. ex Tanaka
26	'Jabara'	*C. jabara* Hort. ex Y. Tanaka
27	'Kinkouyu'	*C. parvifructa* Hort. ex Tanaka
28	'Naoshichi'	*C. takuma-sudachi* Hort. ex Tanaka
29	'Hanayu'	*C. hanaju* Sieb. ex Shirai
30	'Yukou'	*C. yuko* Hort. ex Tanaka
31	'Kiyooka daidai'	*Citrus* sp.

Data analysis

Only clear and repeatable amplification products were scored as 1 for present bands and 0 for absent ones. The specific bands useful for identifying species and cultivar were named with primer number followed by the approximate size of the amplified fragment in base pairs. Amplified products wree analysed by pairwise comparisons of the genotypes based on the percentage of common fragments, and a similarity matrix was calculated (Nei and Li, 1979). A dendrogram was constructed based on the genetic distance matrix by applying an unweighted pair group method with arithmetic averages (UPGMA) cluster analysis using the MEGA (Molecular Evolutionary Genetics Analysis) version 2.0 (Kumar *et al.,* 2001).

RESULTS

Polymorphism and identity

After screening 60 primers, 27 primers produced polymorphic and repeatable products (Table II). Between one and nine clear and repeatable bands were obtained for each of these primers and of a total 108 scored bands, 76 were polymorphic (RAPDs), with an average of 2.8 markers per primer. The number of polymorphic fragments per primer ranged from one to eight and fragment sizes ranged from 400 bp (OPA-18) to 3200 bp (OPA-01). The amplification products obtained by primer OPB-12 are illustrated in Figure 1, which exemplifies the typical RAPD banding patterns observed.

The gels were also screened for primers revealing RAPDs unique to particular species or cultivar. In such a way among the polymorphic RAPDs those present only in one genotype were considered to be unique fragments (Table III). For seven of 31 accessions, it was possible to find at least one such fragment. Some fragments also allowed the distinction of close cultivars, for instance, A17 (2200), A17 (1100) and E20 (675) were present in

TABLE II
List of the primers that produced RAPD polymorphisms among 31 Japanese acid citrus species and cultivars

Primer	Sequence	Number of bands		
		Monomorphic	Polymorphic	Total
A01	CAGGCCCTTC	2	5	7
A04	AATCGGGCTG	–	5	5
A05	AGGGGTCTTG	–	5	5
A07	GAAACGGGTG	1	1	2
A11	CAATCGCCGT	1	1	2
A12	TCGGCGATAG	1	8	9
A17	GACCGCTTGT	2	3	5
A18	AGGTGACCGT	1	5	6
A19	CAAACGTCGG	–	2	2
A20	GTTGCGATCC	–	4	4
B01	GTTTCGCTCC	2	2	4
B04	GGACTGGAGGT	2	3	5
B05	TGCGCCCTTC	–	2	2
B07	GGTGACGCAG	–	1	1
B08	GTCCACACGG	2	3	5
B10	CTGCTGGGAC	–	2	2
B11	GTAGACCCGT	–	3	3
B12	CCTTGACGCA	–	7	7
E01	CCCAAGGTCC	2	2	4
E05	TCAGGGAGGT	4	1	5
E06	AAGACCCCTC	1	2	3
E09	CTTCACCCGA	2	1	3
E13	CCCGATTCGG	2	1	3
E15	ACGCACAACC	4	1	5
E16	GGTGACTGTG	2	2	4
E17	CTACTGCCGT	–	2	2
E20	AACGGTGACC	1	2	3
Total		32	76	108

'Kabosu' and absent in 'Aka kabosu'; A20 (1400), B05 (990) and E16 (1000) were present in 'Aka kabosu' and absent in 'Kabosu'; A01 (750), A05 (1200), A07 (1980), A17 (750), B01 (1900), E06 (1950) and E15 (500) were present in 'Taninaka kizu' and absent in 'Kinosu'; A01 (1200), A05 (1180), A12 (2750), A17 (1100), A18 (1600), A18 (400), A19 (930), A20 (1400), B01 (940), B11 (520), B12 (1500) and E17 (750) were present in 'Kinosu' and absent in 'Taninaka kizu'.

Relationships between acid citrus accessions

Based on the pairwise analysis of the amplification products obtained with the 27 selected primers, relationships between the 31 acid citrus species and cultivars were analysed. The similarity values based on 76 RAPDs ranged from 0.20 for 'Daidai' and 'Hanayu' to 1.00 for 'Yuzu' (tetraploid accession) and 'Tochikei yuzu' (data not shown). The dendrogram generated, based on the matrix obtained by genetic distance (Figure

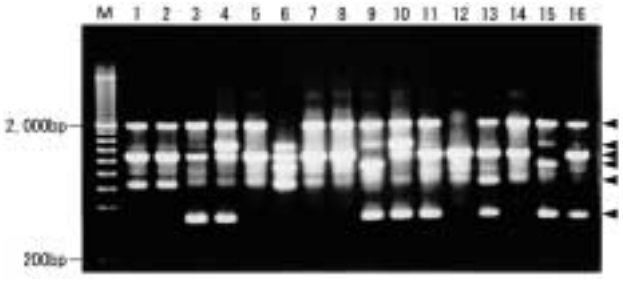

FIG. 1
Amplification products from primer OPB-12. M: 200 bp DNA Ladder; Lane 1; 'Kabosu', 2; 'Aka kabosu', 3; 'Taninaka kizu', 4; 'Kinosu', 5; 'Matsuda sudachi', 6; 'Zanbo', 7; 'Yuzukichi', 8; 'Mochiyu', 9; 'Zuishouyu', 10; 'Kizu', 11; 'Jabara', 12; 'Kinkouyu', 13; 'Naoshichi', 14; 'Hanayu', 15; 'Yukou', 16; 'Kiyouka daidai'. Arrowheads at the right indicate the position of the polymorphic bands.

TABLE III
Unique RAPD fragments among acid citrus species and cultivars

Accession	Primers revealing unique RAPDs (No. of base pairs of a band)
'Myrtle-leaf orange'	A12 (950), A18 (700), A20 (1700) B08 (1120), B11 (730), E01 (930)
'China daidai'	A12 (820)
'Yushi mukaku sudachi'	A12 (1900)
'Seedless sudachi'	A12 (900)
'Zanbo'	A04 (530), B08 (1500)
'Zuishouyu'	A12 (1200)
'Yukou'	A12 (1250)

2) revealed that 'Myrtle-leaf orange' formed a distinct cluster. Sour oranges, including 'Daidai', 'Kaiseito', and 'China daidai' clustered apart from other accessions. 'Zanbo', with an obscure origin (Taninaka *et al.,* 1981) and 'Nanshou daidai' were placed close to sour oranges. Accessions of 'Sudachi' were placed close to different accessions of 'Yuzu'. Other relatives of 'Yuzu' were clustered separately and distributed across the dendrogram; for example 'Taninaka kizu' was placed close to 'Sudachi' group and apart from 'Kinosu' and 'Kizu'. In the present work 'Kiku daidai', 'Zuishouyu' and 'Yukou' clustered distant from other 'Yuzu' relatives. The genetic diversities among 'Yuzu' and 'Sudachi' accessions are 0.02 and 0.08, respectively, which are quite narrow for genetic variability.

DISCUSSION

Classification of Japanese acid citrus, due to the vast number of selections; has long been complicated and problematical. Nearly all the accessions analysed here include uncertain origins (Kimura and Taninaka, 1988, 1990, 1995). These accessions have originated in a variety of ways including selection from open pollinated populations, chance seedlings that could be apomictic or zygotic, and mutations that occur in buds or branches of existing cultivars. Here we discuss the variations and inter-relationship among the accessions based on the results of our RAPDs data.

The distance of 'Myrtle-leaf orange' from other specimens, revealed by RAPDs in the present work; was expected from its unique morphological characters. These characters have been described in detail by Hodgson (1967). It also expressed the highest number of unique fragments.

Unique character and affinity of 'Nanshou daidai' to sour orange have been suggested by Tanaka (1954); however, the affinity was not demonstrated by Hirai *et al.* (1986), who studied phylogenetic relationships of citrus by isozyme analysis. In the present study, in agreement with Tanaka (1954); 'Nanshou daidai' was clustered with sour oranges.

'Taninaka kizu' was separated clearly from 'Kizu' and 'Kinosu', therefore it is possible that it did not originate from mutation of 'Kizu' or 'Kinosu', although they are assigned to the same species. Its isozyme phenotype at GOT-2 was also different from those of 'Kizu' or 'Kinosu' (Rahman *et al.,* 2001). It was placed within the 'Sudachi' group in the present study.

Hirai *et al.* (1986) considered 'Kiku daidai' to be a hybrid between a Japanese mandarin and pummelo. The

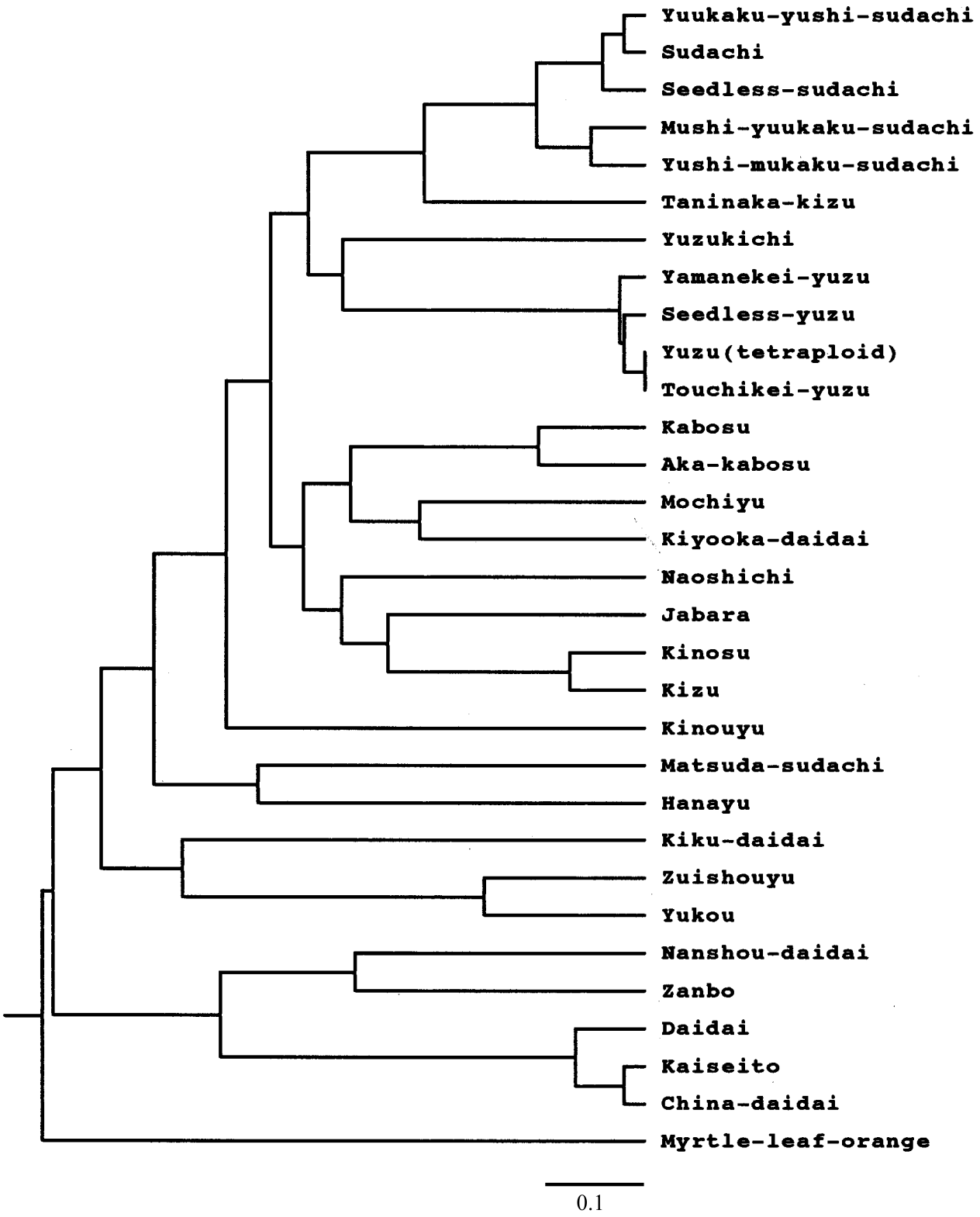

Fig. 2
Dendrogram obtained by UPGMA cluster analysis of the RAPD data. Scale bar shows distance value.

dendrogram obtained revealed that 'Kiku daidai' had no close relationship with any other specimen.

Rahman *et al.* (2001) concluded that 'Aka kabosu' originated from a mutation of 'Kabosu'; the only difference between these two cultivars is the pulp colour. The pulp of 'Aka kabosu' is red while that of 'Kabosu' is white. The RAPD assay generated accession-specific markers for these two cultivars and they could be discriminated from each other.

In 'Sudachi' group, 'Mushi yuukaku sudachi' and 'Yushi mukaku sudachi' which might be mutants of 'Sudachi' (Rahman *et al.,* 2001); expressed some accession-specific bands that differentiated them from other accessions of this group.

Four cultivars of 'Yuzu'; 'Yuzu' (tetraploid), 'Tochikei yuzu', 'Yamanekei yuzu' and 'Seedless yuzu' were included in the present study. All yielded very similar patterns, suggesting either that they originated from a common cultivar, or that the technique was not able to detect cultivar variation. The RAPD technique is rarely able to discriminate between very closely related genotypes, such as sports and clonal variants (Dettori and Palombi, 2000), and this could be the case for these four cultivars. These findings agree with those of Rahman *et al.* (2001) who also observed similar isozyme patterns in 'Yuzu' cultivars.

Taninaka *et al.* (1981) concluded that 'Sudachi', 'Kabosu' and 'Kizu' originated as chance seedlings and the origin of 'Mochiyu' is uncertain. Because other kinds of citrus like mandarins, citrons and pummelos were not included in the present study our results were not sufficient to support further discussion on the origin of most species and cultivars analyzed here.

In this study we confirmed that RAPD markers, as a fast and simple technique, can detect enough polymorphism to differentiate between Japanese acid citrus species and cultivars and to understand their interrelationships. However, if a finer molecular analysis of 'Yuzu' or 'Sudachi' accessions is required, the use of other molecular marker techniques, e.g. amplification fragment length polymorphisms (AFLPs) (Vos *et al.,* 1995) or inter-simple sequence repeat markers (ISSRs) (Fang and Roose, 1997) should be considered.

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