Fruit Characteristics, Chromosome and DNA Profiles of Four Mandarins (*Citrus reticulata* Blanco) Collected in West Sumatra, Indonesia

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Abstract

Fruit characteristics, chromosome and DNA profiles were analyzed in four mandarins (*Citrus reticulata* Blanco), 'Jeruk Brastagi', 'Jeruk Keprok', 'Jeruk Siem' and 'Limau Talang Babungo', collected in West Sumatra, Indonesia. In terms of fruit characteristics, all four mandarins possessed orange rind and flesh and green polyembryonic seed. The fruit diameter of 'Limau Talang Babungo' was the smallest. 'Jeruk Siem' possesed the thinnest rind. The brix of juice was high in 'Jeruk Brastagi' and 'Limau Talang Babungo'. The number of seeds per fruit of 'Jeruk Siem' was small. Fluorochrome staining with chromomycin A_3 (CMA) was used to characterize and compare the CMA banding patterns of chromosomes. The four mandarins of West Sumatra showed identical CMA banding patterns: two telomeric and one proximal positive bands were detected in one chromosome, one telomeric and one proximal positive bands were detected in one chromosome and one telomeric positive band was detected in eight chromosomes. In terms of results of sequence-related amplified polymorphism (SRAP) analysis using 18 primer pairs, there were no differences among the four mandarins of West Sumatra. A close relationship between mandarins of West Sumatra, Chinese mandarin Sunki and Japanese mandarin Shiikuwasha was revealed. On the other hand, the four mandarins of West Sumatra were distinct from Indian mandarin (Ponkan and Cleopatra) and Japanese mandarin Tachibana.

Key words: chromomycin, CMA, fluorochrome, genetic relationship, SRAP

Introduction

Citrus is one of the most important fruit crops worldwide. Although the taxonomy of citrus is complicated because of their wide cross-compatibility and polyembryony, recent DNA and chromosome analyses revealed various new findings (FEDERICI *et al.* 1998, GUERRA 1993, NICOLOSI *et al.* 2000, YAMAMOTO and TOMINAGA 2003, YAMAMOTO *et al.* 1993, 2007). This kind of study has been conducted mainly in citrus grown in temperate and subtropical zones. Recently, DNA analysis of tropical citrus has started to be conducted

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(AGISIMANTO *et al.* 2007, KARSINAH *et al.* 2002).

West Sumatra, Indonesia, is located in the tropical zone right on the equator. Many local citrus are cultivated in this area; however, their genetic characteristics have not been elucidated yet. Thus, in the present study, chromosome and DNA analyses were conducted to reveal the genetic profile of mandarins (*Citrus reticulata* Blanco) grown in West Sumatra. For chromosome analysis, we used guanine-cytosine (GC)-specific fluorochrome chromomycin A_3 (CMA) which has been useful for detecting variations of chromosome structure of citrus (GUERRA 1993, YAMAMOTO and TOMINAGA 2003, YAMAMOTO *et al.* 2005, 2007). Sequence-related amplified polymorphism (SRAP) analysis, which provides useful information on the genetic relationships of citrus (UZUN *et al.* 2009), was conducted. In addition, fruit characteristics of mandarins of West Sumatra were also investigated. Here, we report the results of these analyses.

Materials and Methods

Fruit characteristics

All fruit samples were collected at local markets with unclear origin of the trees in West Sumatra: 'Jeruk Brastagi', 'Jeruk Keprok' and 'Jeruk Siem' were collected at Padang and 'Limau Talang Babungo' was collected at Alahan Panjang. Just after collection, fruit characteristics were investigated at Andalas University. Five fruits were used as materials in each mandarin accession.

Chromosome analysis

Roots of young nucellar seedlings from the seed of the fruit collected from the markets with unclear origin of the trees of four mandarins were used as materials. Seeds were germinated in Petri dishes at 25 °C in the dark. Root tips about 1 cm long were excised, immersed in 2 mM 8-hydroxyquinoline at 10 °C for 4 h in the dark, fixed in methanol-acetic acid (3:1) and stored at -20 °C.

Enzymatic maceration and air-drying were performed as described by FUKUI (1996) with minor modifications. The root tips were washed in distilled water to remove the fixative and macerated in an enzyme mixture containing 1% Cellulase Onozuka RS, 0.75% Macerozyme R200 (Yakult, Japan), 0.15% Pectolyase Y-23 (Seishin Pharmaceutical Co., Ltd, Japan) and 1 mM EDTA, pH 4.2, at 37 °C for 55 min.

Chromosomes were stained with 2% Giemsa solution (Merck Co., Germany) in 1/30 M phosphate buffer (pH 6.8) for 15 min, rinsed with distilled water, air dried, and then mounted with xylene. After confirmation of each chromosome position on the slide glass, the chromosomes were de-stained with 70% methanol.

Chromosomes were also stained with 0.1 $g \cdot L^{-1}$ CMA according to HIZUME (1991) and observed under a fluorescence microscope (Nikon ECLIPSE 80i, Tokyo, Japan) with a BV filter cassette.

SRAP analysis

In the four mandarins of West Sumatra, three nucellar seedlings from the seed of the fruit collected from the markets with unclear origin of the trees of each accession were used as materials. Twelve mandarins, tangors and sweet oranges that originated in various regions were used as control accessions (Table 1). All control accessions grafted onto *Poncirus trifoliata* were preserved at the Faculty of Agriculture, Kagoshima University.

Total DNA was extracted from leaves using ISOPLANT II (Nippon Gene, Tokyo, Japan). All SRAP primer combinations used in this study were in accordance with UZUN *et al.* (2009) (Table 2, 3). PCR reactions were performed in a PC320 (Astec, Fukuoka, Japan) thermal cycler programmed as follows: initial heating at 95 °C for 10 min, then 5 cycles of denaturing at 94 °C for 1 min, annealing at 35 °C for 1 min and extension at 72 °C for 1 min. In the following 35 cycles, the annealing temperature was increased to 50 °C, with a final extension of 5 min at 72 °C. Amplified products were electrophoresed on 1.5% agarose gels and detected by staining with Mupid-Stain (Advance, Tokyo, Japan). The bands were recorded as 1 for present and as 0 for absent. Genetic distance was calculated between each pair of cultivars (NEI and LI 1979). For phylogenic analysis, a dendrogram was constructed with Molecular Evolutionary Genetic Analysis (MEGA, ver. 3.1) software (KUMAR *et al.* 2004) by applying the neighbor-joining (NJ) method.

Table 1. The materials used in SRAP analysis and their distribution.

No.	Common name	Latin name		Distribution
		Swingle system[1]	Tanaka system[2]	
1-3	Jeruk Brastagi	*Citrus reticulata* Blanco	*C.* sp.	West Sumatra, Indonesia
4-6	Jeruk Keprok	*C. reticulata* Blanco	*C.* sp.	West Sumatra, Indonesia
7-9	Jeruk Siem	*C. reticulata* Blanco	*C.* sp.	West Sumatra, Indonesia
10-12	Limau Talang Babungo	*C. reticulata* Blanco	*C.* sp.	West Sumatra, Indonesia
13	Ponkan 'Yoshida Ponkan'	*C. reticulata* Blanco	*C. reticulata* Blanco	India
14	Cleopatra	*C. reticulata* Blanco	*C. reshni* hort. ex Tanaka	India
15	Kunenbo	*C. reticulata* Blanco	*C. nobilis* Lour.	Vietnam
16	King	*C. reticulata* Blanco	*C. nobilis* Lour.	Vietnam
17	Sunki	*C. reticulata* Blanco	*C. sunki* (Hayata) hort. ex Tanaka	China
18	Kinokuni 'Sakurajima Komikan'	*C. reticulata* Blanco	*C. kinokuni* hort. ex Tanaka	China
19	Satsuma mandarin 'Unshiu Genboku'	*C. reticulata* Blanco	*C. unshiu* Marcow.	Japan
20	Koji	*C. reticulata* Blanco	*C. leiocarpa* hort. ex Tanaka	Japan
21	Tachibana	*C. tachibana* (Makino) Tanaka	*C. tachibana* (Makino) Tanaka	Japan
22	Shiikuwasha 'Shiikunin- Kara'	*C. tachibana* relative	*C. depressa* Hayata	Japan
23	Tankan 'Tarimizu 1 Gou'	*C. sinensis* hybrid	*C. tankan* Hayata	China
24	Sweet orange 'Hamlin'	*C. sinensis* (L.) Osbeck	*C. sinensis* (L.) Osbeck	China

1: Latin name by SWINGLE and REECE (1967).

2: Latin name by TANAKA system (1969, 1977).

Table 2. List of forward and reverse SRAP primers and their sequences used in this study.

Forward primers	Reverse primers
Me1: 5'-TGAGTCCAAACCGGATA-3'	Em1: 5'-GACTGCGTACGAATTAAT-3'
Me2: 5'-TGAGTCCAAACCGGAGC-3'	Em2: 5'-GACTGCGTACGAATTTGC-3'
Me3: 5'-TGAGTCCAAACCGGAAT-3'	Em3: 5'-GACTGCGTACGAATTGAC-3'
Me4: 5'-TGAGTCCAAACCGGACC-3'	Em4: 5'-GACTGCGTACGAATTTGA-3'
Me5: 5'-TGAGTCCAAACCGGAAG-3'	Em5: 5'-GACTGCGTACGAATTAAC-3'
Me6: 5'-TGAGTCCAAACCGGACA-3'	Em6: 5'-GACTGCGTACGAATTGCA-3'
Me7: 5'-TGAGTCCAAACCGGACG-3'	Em7: 5'-GACTGCGTACGAATTCAA-3'
Me8: 5'-TGAGTCCAAACCGGACT-3'	Em9: 5'-GACTGCGTACGAATTCAG-3'
Me9: 5'-TGAGTCCAAACCGGAGG-3'	Em10: 5'-GACTGCGTACGAATTCAT-3'
Me11: 5'-TGAGTCCAAACCGGAAC-3'	Em11: 5'-GACTGCGTACGAATTCTA-3'
Me12: 5'-TGAGTCCAAACCGGAGA-3'	Em12: 5'-GACTGCGTACGAATTCTC-3'
	Em13: 5'-GACTGCGTACGAATTCTG-3'
	Em14: 5'-GACTGCGTACGAATTCTT-3'
	Em15: 5'-GACTGCGTACGAATTGAT-3'
	Em16: 5'-GACTGCGTACGAATTGTC-3'

Table 3. SRAP primer combinations that obtained polymorphic fragments in this study.

Em1/Me4	Em7/Me9
Em2/Me3	Em9/Me3
Em2/Me5	Em9/Me11
Em2/Me8	Em10/Me11
Em3/Me3	Em11/Me1
Em4/Me5	Em13/Me4
Em4/Me6	Em14/Me1
Em5/Me12	Em15/Me6
Em7/Me8	Em16/Me12

Results

All four citrus accessions collected in West Sumatra shared mandarin (*C. reticulata*) characteristics (Fig. 1, Table 4). All accessions possessed orange rind and flesh and green polyembryonic seed. The fruit diameter of 'Limau Talang Babungo' was smaller than that of the other three accessions. 'Jeruk Siem' possessed the thinnest rind. The brix of juice was high in 'Jeruk Brastagi' and 'Limau Talang Babungo' and the pH of juice was low in 'Limau Talang Babungo'. The number of seeds per fruit of 'Jeruk Siem' was the smallest in the four accessions investigated.

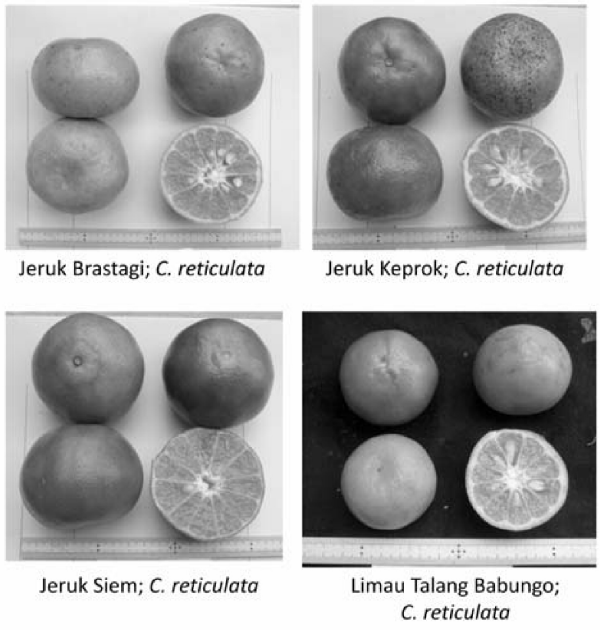

Fig. 1. Fruits of four mandarins collected in West Sumatra.

Table 4. Fruit characteristics of four mandarins collected in West Sumatra.

Accession	Collected place[1]	Collected Date	Fruit diameter (mm)	Fruit shape index[2]	Rind color	Flesh color	Rind thick-ness (mm)	Peeling	Brix	pH	No. of seeds per fruit	Embryo		Notes
												Color	Poly or Mono	
Jeruk Brastagi	Padang	Jan. 6, 2010	68	127	Orange	Orange	3.7	Easy	11.5	4.5	10.0	Green	Poly	Fresh fruit use
Jeruk Keprok	Padang	Jan. 6, 2010	68	123	Orange	Orange	3.7	Easy	9.8	3.8	13.8	Green	Poly	Fresh fruit use
Jeruk Siem	Padang	Jan. 6, 2010	70	118	Orange	Orange	2.3	Easy	8.0	4.7	5.0	Green	Poly	Fresh fruit use
Limau Talang Babungo	Alahan Panjang	Jan. 9, 2010	50	118	Orange	Orange	2.8	Easy	11.2	2.8	11.2	Green	Poly	Fresh fruit use

1: All samples were collected at local markets.

2: (Diameter/length) × 100.

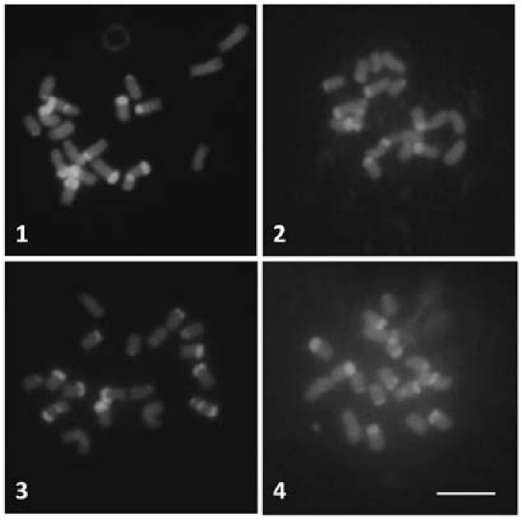

Fig. 2. CMA staining of somatic chromosomes in mandarins of West Sumatra. 1: Jeruk Brastagi, 2: Jeruk Keprok, 3: Jeruk Siem, 4: Limau Talang Babungo. Bar in 4 represents 5μm for all figures.

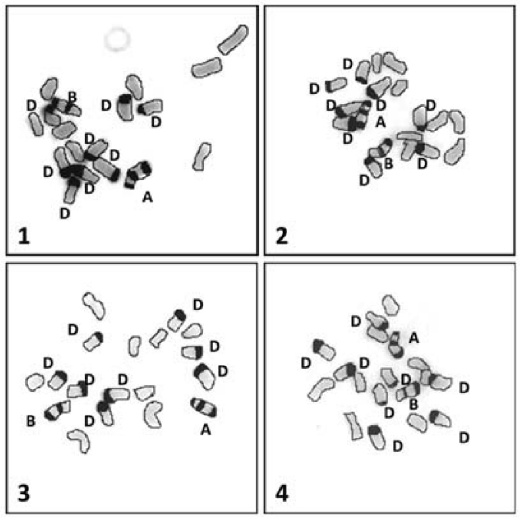

Fig. 3. Schmatic representation of CMA staining of somatic chromosomes in mandarins of West Sumatra. 1: Jeruk Brastagi, 2: Jeruk Keprok, 3: Jeruk Siem, 4: Limau Talang Babungo. A, B and D: See Table 5. The black regions indicate CMA-positive bands.

All accessions had 2n=18 chromosomes and no variation in CMA banding patterns was found within the seedlings. Moreover, the four mandarins of West Sumatra showed identical CMA banding patterns. Two telomeric and one proximal positive bands were detected in one chromosome, one telomeric and one proximal positive bands were detected in one chromosome and one telomeric positive band was detected in eight chromosomes. No CMA-positive band was detected in the remaining eight chromosomes (Fig. 2, 3, Table 5).

Table 5. CMA banding patterns of somatic chromosomes of mandarins of West Sumatra and their control accessions.

Common name	CMA banding pattern1	Reference
Jeruk Brastagi	1A+1B+8D+8E	
Jeruk Keprok	1A+1B+8D+8E	
Jeruk Siem	1A+1B+8D+8E	
Limau Talang Babungo	1A+1B+8D+8E	
Ponkan 'Yoshida Ponkan'	1B+1C+10D+6E	YAMAMOTO and TOMINAGA (2003)
Cleopatra	15D+3E	YAMAMOTO and TOMINAGA (2003)
Kunenbo	1A+1B+2C+5D+9E	YAMAMOTO and TOMINAGA (2003)
King	1A+1B+1C+8D+7E	YAMAMOTO and TOMINAGA (2003)
Sunki	12D+6E	YAMAMOTO and TOMINAGA (2003)
Kinokuni 'Sakurajima Komikan'	1C+8D+9E	YAMAMOTO and TOMINAGA (2003)
Satsuma mandarin 'Okitsu Wase'	1A+1C+8D+8E	YAMAMOTO and TOMINAGA (2003)
Koji	2B+1C+6D+9E	YAMAMOTO and TOMINAGA (2003)
Tachibana	1C+10D+5E+2F	YAMAMOTO and TOMINAGA (2003)
Shiikuwasha	1C+10D+6E+1F	YAMAMOTO and TOMINAGA (2003)
Tankan 'Tarimizu 1 Gou'	1A+1B+1C+8D+7E	YAMAMOTO *et al.* (2005)
Sweet orange 'Comuna'	2B+2C+7D+7E	YAMAMOTO *et al.* (2007)

1: A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without band, F: one proximal band.

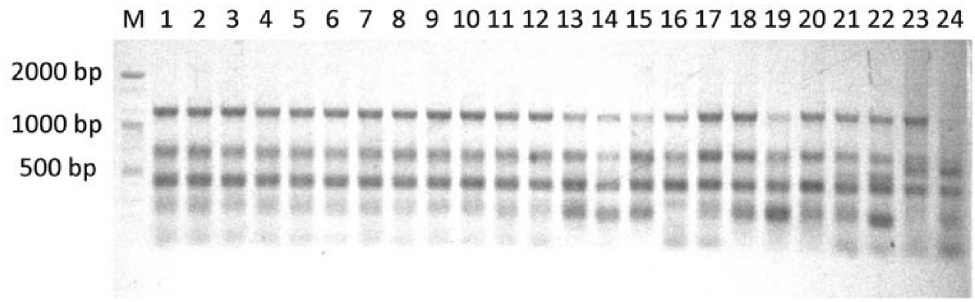

Fig. 4. DNA amplifications of mandarins of West Sumata and their controls using SRAP primers Em1/Me4. 1-3: Jeruk Brastagi, 4-6: Jeruk Keprok, 7-9: Jeruk Siem, 10-12: Limau Talang Babungo, 13: Yoshida Ponkan, 14: Cleopatra, 15: Kunenbo, 16: King, 17: Sunki, 18: Sakurajima Komikan, 19: Unshiu Genboku, 20: Koji, 21: Tachibana, 22: Shiikunin-Kara, 23: Tarumizu 1 Gou, 24: Hamlin, M: Molecular markers.

In SRAP analysis, every seedling that arose from the same accession always showed identical results in each mandarin of West Sumatra. Moreover, there were no differences in results of SRAP analysis using 18 kinds of primer pairs in the four mandarins of West Sumatra. One example of the results of agarose gel electrophoresis is shown in Fig. 4.

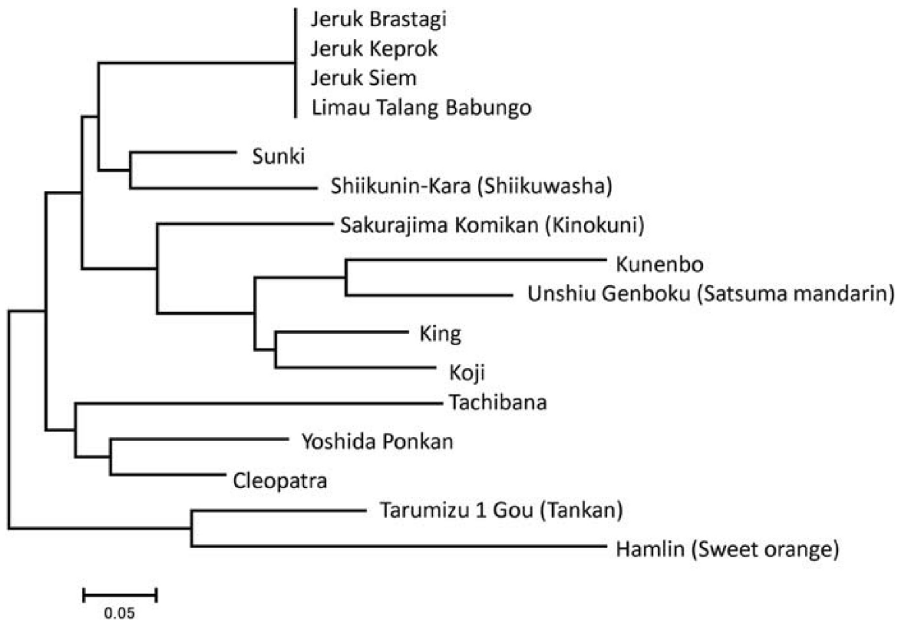

Fig. 5. Dendrogram of mandarins of West Sumata and their controls generated by neighbor-joining method cluster analysis of SRAP data.

On the basis of the SRAP data, a dendrogram was constructed using NJ cluster analysis (Fig. 5). From this dendrogram, Sweet orange and Tankan (tangor) were distinguished from all mandarins. Mandarins could be classified into two major clusters. The four mandarins of West Sumatra belonged to the same cluster as Japanese mandarins (Shiikuwasha, Satsuma mandarin and Koji), Chinese mandarin (Sunki and Kinokuni) and Vietnamese mandarin (Kunenbo and King). In particular, there was a close relationship between mandarins of West Sumatra and Sunki and Shiikuwasha. On the other hand, Indian mandarin (Ponkan and Cleopatra) and Japanese mandarin Tachibana were included in another cluster.

Discussion

We could detect no difference of results from chromosome and DNA analyses among four mandarins of West Sumatra investigated in the present study although some differences were observed in fruit morphological traits. In citrus, almost all accessions possessed characteristic CMA banding patterns (YAMAMOTO 2007) although CMA banding patterns were uniform in all Sweet orange cultivars that arose from mutation (PEDROSA *et al.* 2000). Moreover, all citrus accessions were distinguished from each other in SRAP analysis except for two Sweet orange cultivars (UZUN *et al.* 2009). Therefore, it can be considered that

differentiation of mandarins of West Sumatra has occurred by mutation. There is a possibility that 'Jeruk Brastagi' and 'Jeruk Keprok' are synonyms because their fruit characteristics are very similar.

CMA banding patterns of various citrus have been reported (YAMAMOTO 2007). Chromosomes could be classified into six types based on the number and position of CMA-positive bands: A: two telomeric and one proximal band, B: one telomeric and one proximal band, C: two telomeric bands, D: one telomeric band, E: without bands and F: one proximal band. According to this classification, the CMA banding pattern of four mandarins of West Sumatra was 1A+1B+8D+8E. GUERRA (2009) postulated that true mandarins possessed type C, D and E chromosomes and mandarins have type A or B chromosomes that arose from hybridization with other citrus. As shown in Table 5, true mandarin, Cleopatra, Sunki and Kinokuni, does not possess type A and B chromosomes whereas hybrid origin of Kunenbo and King possess those chromosomes. Type F is characteristic chromosome of Japanese mandarin (YAMAMOTO and TOMINAGA 2003). The mandarins of West Sumatra used in the present study seem to be hybrids between mandarin and other citrus because all have type A and B chromosomes.

However, the hybrid origin of mandarins of West Sumatra was not clarified in this SRAP analysis. There is a close relationship between mandarins of West Sumatra and both Sunki and Shiikuwasha, which are considered to be true mandarins (GUERRA 2009, TANAKA 1948). Genetic relationships between mandarins of West Sumatra and King, Kunenbo and Satsuma mandarin, which possess genetic features from Sweet orange, were not strong. Sweet orange was distinguished from mandarins of West Sumatra clearly. Moreover, genetic relationships between mandarins of West Sumatra and Indian mandarins were weak.

In conclusion, we investigated the genetic profile of four mandarins collected in West Sumatra, Indonesia. Since there are many local citrus accessions in this area, their genetic characteristics should be analyzed. Fruits purchased in local markets were used as materials in the present study. Environmental conditions probably affect the fruit characteristics in each accession. In addition, it has been well known that there is a wide variation in a given accessions; several types of 'Jeruk Keprok' and 'Jeruk Siem' are distributed in Indonesia. Thus, it is necessary to use standard accessions of each area as materials and investigate fruits produced under the same conditions.

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