

Genetic identification of ‘Limau Kacang’ (*Citrus* sp.), a local mandarin cultivated in West Sumatra by sequence-related amplified polymorphism (SRAP)

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Summary

Introduction – Mandarin ‘Limau Kacang’ (*Citrus* sp.) has been a specialty crop in the Solok region of West Sumatra, Indonesia. However, its genetic identification remains unknown. **Materials and methods** – Morphological characteristics were investigated using ‘Yoshida Ponkan’ (*C. reticulata* Blanco) as control. Sequence-related amplified polymorphism (SRAP) analysis of ‘Limau Kacang’ and several mandarins was conducted. **Results and discussion** – Morphological traits of ‘Limau Kacang’ and ‘Ponkan’ were almost the same. Two ‘Limau Kacang’ accessions showed identical or very similar banding patterns to those of ‘Ponkan’ (*Citrus reticulata* Blanco) in SRAP analysis. These results also showed some diversity among ‘Limau Kacang’ mandarines. **Conclusion** – Based on these results, it is clear that ‘Limau Kacang’ is a kind of ‘Ponkan’.

Keywords

Indonesia, citrus, mandarin, *Citrus reticulata*, DNA fingerprinting, genetic resources

Résumé

Identification génétique par polymorphisme séquentiel amplifié (SRAP) du ‘Limau Kacang’ (*Citrus* sp.), une mandarine locale cultivée à l’Ouest de Sumatra.

Introduction – La mandarine ‘Limau Kacang’ (*Citrus* sp.) est une ancienne spécialité fruitière de la région de Solok, dans l’Ouest de Sumatra, en Indonésie. Toutefois son identification génétique est inconnue. **Matériel et méthodes** – Les caractéristiques morphologiques des feuilles et des fruits ont été étudiées en utilisant ‘Yoshida Ponkan’ (*C. reticulata* Blanco) comme témoin. Une analyse de l’ADN de plusieurs accessions de mandarine a été effectuée par polymorphisme séquentiel amplifié (SRAP). **Résultats et discussion** – Les traits morphologiques de ‘Limau Kacang’ et de ‘Ponkan’ se sont révélés pratiquement identiques. Deux échantillons de ‘Limau Kacang’ ont montré des bandes identiques ou très similaires à celles de ‘Ponkan’ (*Citrus reticulata* Blanco) par analyse SRAP. Nos résultats ont également montré qu’une diversité existait parmi ‘Limau Kacang’. **Conclusion** – Sur la base de ces résultats, il est clair que ‘Limau Kacang’ est une sorte de ‘Ponkan’.

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Significance of this study

What is already known on this subject?

- Mandarin ‘Limau Kacang’ (*Citrus* sp.) has been a specialty crop in the Solok region of West Sumatra, Indonesia. However, its genetic identification remains unknown.

What are the new findings?

- Morphological traits of ‘Limau Kacang’ and ‘Ponkan’ (*Citrus reticulata* Blanco) were almost the same. Two ‘Limau Kacang’ accessions showed identical or very similar banding patterns to those of ‘Ponkan’ in DNA analysis. Based on these results, it is clear that ‘Limau Kacang’ is a kind of ‘Ponkan’.

What is the expected impact on horticulture?

- The results of genetic identification provide useful information for the project of rebuilding ‘Limau Kacang’ orchards in Solok region of West Sumatra.

Mots-clés

Indonésie, citrus, mandarine, *Citrus reticulata*, empreintes génétiques, ressources génétiques

Introduction

Mandarin ‘Limau Kacang’ (*Citrus* sp.) has been a specialty crop in the Solok region of West Sumatra, Indonesia. It was introduced from Garut of West Java to Solok in the 1930s. It became very popular in the 1970–80s because of its superior fruit quality. However, its production has rapidly decreased due to huanglongbing (HLB), a harmful disease of citrus (Bové, 2006) that invaded and spread in this region. There is a project to rebuild orchards of ‘Limau Kacang’ in Solok since it is the citrus representative of the region and still has popularity. For promoting the project, genetic identification of the cv. Limau Kacang is essential. It is an introduced cultivar and there is no similar or identical citrus in West Sumatra that could make its identification clear.

DNA analysis is a powerful tool for genetic identification (Fang and Roose, 1997). Among the various PCR-based DNA analysis methods, sequence-related amplified polymorphism (SRAP), which preferentially amplifies open reading frames (ORFs), is a simple and efficient marker system (Li and Quiros, 2001). In fruit trees including citrus, this technique has been

TABLE 1. Citrus materials used in this study and their geographic distribution.

No.	Cultivar common names	Latin names		Geographic distribution	Sources ³
		Tanaka system ¹	Swingle system ²		
1	Limau Kacang #8	<i>Citrus reticulata</i> Blanco	<i>C. reticulata</i> Blanco	West Sumatra	WS
2	Limau Kacang #15	<i>C. reticulata</i> Blanco	<i>C. reticulata</i> Blanco	West Sumatra	WS
3	Ponkan (Yoshida Ponkan)	<i>C. reticulata</i> Blanco	<i>C. reticulata</i> Blanco	Japan	KG
4	Ponkan (Sasshu)	<i>C. reticulata</i> Blanco	<i>C. reticulata</i> Blanco	Japan	KG
5	Jeruk Siem	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Indonesia	KG
6	Jeruk Keprok	<i>C. sp.</i>	<i>C. reticulata</i> Blanco	Indonesia	KG
7	Satsuma mandarin (Original)	<i>C. unshiu</i> Marcow.	<i>C. reticulata</i> Blanco	Japan	KG
8	Clementine	<i>C. clementina</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Algeria	KG
9	Kinokuni (Sakurajima Komikan)	<i>C. kinokuni</i> hort. ex Tanaka	<i>C. reticulata</i> Blanco	Japan	KG
10	King	<i>C. nobilis</i> Lour.	<i>C. reticulata</i> Blanco	Indo-China	KG

¹ Latin name by Tanaka's system (Tanaka, 1969,1977).

² Latin name by Swingle and Reece (Swingle and Reece, 1967).

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used to analyze genetic diversity and for phylogenetic studies (Ahmad *et al.*, 2004; Guo and Luo, 2006; Uzun *et al.*, 2009; Guo *et al.*, 2012). The present study used SRAP analysis to genetically identify 'Limau Kacang' using several mandarins as control materials.

Materials and methods

Plant materials and morphological characteristics

Leaves and fruits of 'Limau Kacang #8' were collected from a small orchard located in Kacang-Solok, West Sumatra, Indonesia (S00.30003°, E100.35237°). Fruits of 'Limau Kacang #15' were purchased at a local market in Batu Sangkar, West Sumatra, Indonesia. Leaf and fruit characteristics of both 'Limau Kacang' were measured just after their collection. Nucellar seedlings from polyembryonic seeds were produced by 'Limau Kacang #15'.

'Yoshida 'Ponkan' (*C. reticulata* Blanco) was used as the control accession in the morphological characterization of fruits and leaves. Five leaves and fruits were sampled per accession. In the DNA analyses, eight accessions (number 3–10, Table 1) were used as control materials. Since 'Limau Kacang' expressed the morphological traits of a mandarin, several mandarins were chosen as control. Control accessions were as follows: *C. reticulata* 'Ponkan' (known as 'Suntara') is a major mandarin native to India. Both 'Jeruk Siem' and 'Jeruk Keprok' are important local mandarins in Sumatra, Indonesia. Satsuma mandarin (*C. unshiu* Marcow.) is native to Japan and has become a leading cultivar there. Kinokuni

(*C. kinokuni* hort. ex Tanaka) is one of the oldest mandarins in China. Clementine (*C. clementina* hort. ex Tanaka) was originated in Algeria and spread worldwide. 'King' (*C. nobilis* Lour.) is the most important mandarin accession in Vietnam. All the control materials were preserved at the Faculty of Agriculture of Kagoshima University, Japan.

DNA extraction and SRAP analysis

Total DNA was extracted from leaves using Isoplant II (Nippon Gene, Japan). SRAP analysis was conducted according to the study of Uzun *et al.* (2009). Fourteen primer combinations developed for citrus (Uzun *et al.*, 2009) were used in the present study (Tables 2 and 3). 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, Korea). PCR reactions were performed in a PC320 (Astec, Fukuoka, Japan) thermal cycler programmed as follows: initial heating at 95 °C for 10 min, the first five cycles of denaturation at 94 °C for 1 min, 35 °C for 1 min, and extension at 72 °C for 2 min, then the annealing temperature was raised to 50 °C for another 35 cycles, with final extension of 10 min at 72 °C. Amplified products were electrophoresed on 1.5% agarose gels (Seakem GTG Agarose; Takara Bio, Japan), and stained with GelRed (Biotium, USA). The bands were detected under UV light. Reproducibility of the patterns was tested by running some of the reactions in duplicate.

The bands were recorded as 1 for present and as 0 for absent. The genetic distance was calculated between each

TABLE 2. Forward and reverse SRAP primers used in the present study.

Forward primers	Reverse primers
Me1: TGAGTCCAAACCGGATA	Em1: GACTGCGTACGAATTAAT
Me3: TGAGTCCAAACCGGAAT	Em2: GACTGCGTACGAATTTGC
Me4: TGAGTCCAAACCGGACC	Em3: GACTGCGTACGAATTGAC
Me5: TGAGTCCAAACCGGAAG	Em4: GACTGCGTACGAATTTGA
Me6: TGAGTCCAAACCGGACA	Em7: GACTGCGTACGAATTCAA
Me8: TGAGTCCAAACCGGACT	Em9: GACTGCGTACGAATTCAG
Me9: TGAGTCCAAACCGGAGG	Em10: GACTGCGTACGAATTCAT
Me10: TGAGTCCAAACCGGAAA	Em14: GACTGCGTACGAATTCTT
Me11: TGAGTCCAAACCGGAAC	Em15: GACTGCGTACGAATTGAT
Me12: TGAGTCCAAACCGGAGA	Em16: GACTGCGTACGAATTGTC

TABLE 3. SRAP primer combinations used in the present study.

Primer combinations	
Em1/Me4	Em7/Me9
Em2/Me5	Em9/Me11
Em2/Me8	Em10/Me11
Em3/Me3	Em14/Me1
Em4/Me5	Em15/Me6
Em4/Me6	Em15/Me10
Em7/Me8	Em16/Me12

pair of accessions (Dice, 1945). For the phylogenetic analysis, cluster analysis was conducted using Molecular Evolutionary Genetic Analysis (MEGA, ver. 4.1) software (Tamura *et al.*, 2007) by the neighbor-joining (NJ) method.

Results and discussion

Leaf and fruit characteristics of 'Limau Kacang' and 'Yoshida Ponkan' are shown in Figure 1 and Table 4. The wing leaf was small in both accessions. Characteristics of fruit, fruit surface, flesh color, degree of puffing, granulation and peeling, embryo color, and polyembryony in both accessions were quite similar. In particular, there was no difference in flavor between the accessions.

All the 14 primer combinations showed a clear polymorphic fragment in the SRAP analysis. There were 32 polymorphic fragments in total and 2.3 polymorphic fragments per primer on average. The number of polymorphic fragments produced by each primer combination ranged from one by Em1/Me4, Em2/Me5, Em4/Me6, Em7/Me8, Em9/Me11, and Em15/Me10 to six by Em15/Me6.

A dendrogram was constructed from SRAP data (Figure 2). All the accessions except 'Limau Kacang #8', 'Yoshida Ponkan', and 'Sasshu' could be distinguished from each other. These three accessions always showed identical fragment patterns. The results of 'Limau Kacang #15' and these three accessions were very much the same except in Em4/Me5. Ten accessions were classified into three types: "A", "B", and "C". "A" included 'Limau Kacang #8', 'Limau Kacang #15', 'Yoshida Ponkan', and 'Sasshu', "B" included 'Jeruk Siem' and 'Jeruk Keprok', and "C" included 'Clementine', 'Sakurajima Komikan', 'Sastuma mandarin', and 'King'.

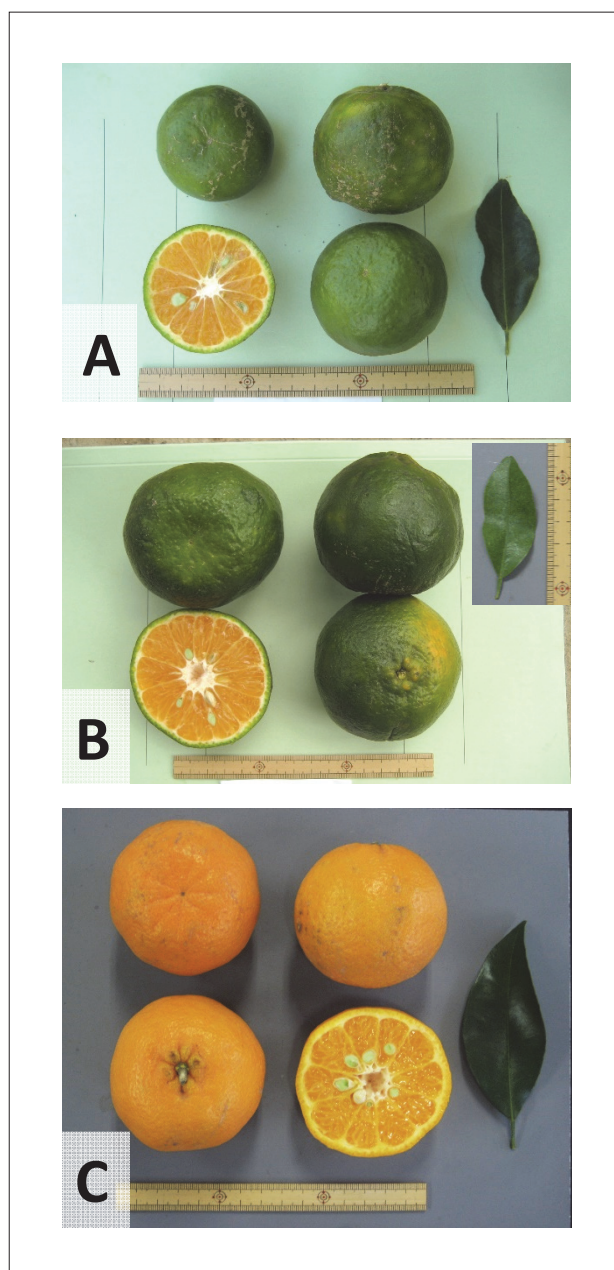


FIGURE 1. Fruit and leaves of 'Limau Kacang #8' (A), 'Limau Kacang #15' (B), and 'Yoshida Ponkan' (C). Horizontal ruler length = 15 cm.

FIGURE 2. Genetic relationships of 'Limau Kacang' and some mandarins by neighbor-joining method cluster analysis of SRAP data.

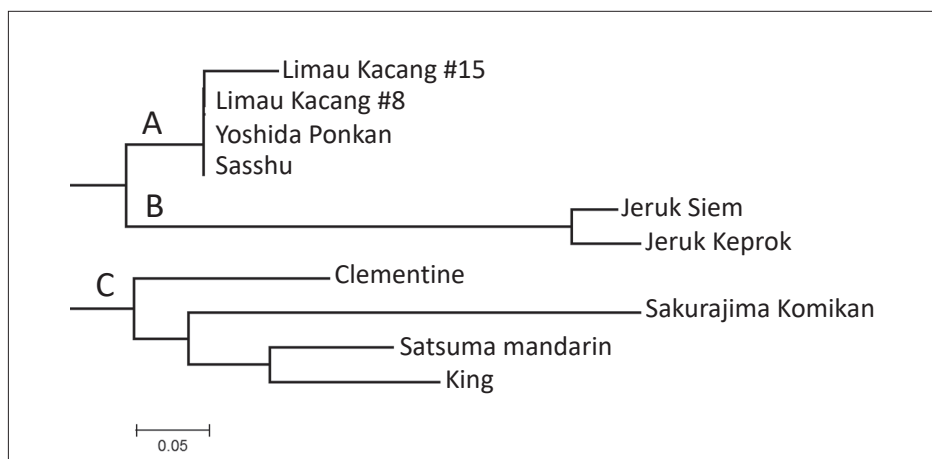


TABLE 4. Leaf and fruit characteristics of 'Limau Kacang' and 'Yoshida Ponkan' (*Citrus reticulata*).

No.	Common names	Leaf				Fruit					Earliness
		Leaf blade		Wing		Juice		Seeds			
		Length (mm)	Width (mm)	Length (mm)	Width (mm)	°Brix	pH	Embryo color	Poly-embryony	Number	
1	Limau Kacang #8	76.1 ± 1.2	37.5 ± 1.9	6.6 ± 0.6	2.3 ± 0.4	9.3	2.7	Pale green	Poly	20.7 ± 1.2	Pre-mature
2	Limau Kacang #15	–	–	–	–	9.8	3.7	Pale green, Green	Poly	10.0 ± 3.8	Fully mature
3	Yoshida Ponkan	93.2 ± 3.7	40.3 ± 1.7	9.4 ± 0.7	2.4 ± 0.1	10.4	3.6	Pale green, Green	Poly	4.6 ± 1.5	Fully mature

No.	Common names	Fruit									
		Skin color	Fruit surface	Flavor	Flesh color	Puffing	Granulation	Peeling	Diameter (mm)	Height (mm)	D/H index
1	Limau Kacang #8	Green	Smooth	Ponkan	Orange	None	None	Slightly easy	54.4 ± 2.5	46.6 ± 2.0	116.6 ± 0.5
2	Limau Kacang #15	Yellowish green	Slightly smooth	Ponkan	Orange	None	None	Easy	79.1 ± 1.7	65.6 ± 1.9	120.9 ± 4.7
3	Yoshida Ponkan	Orange	Smooth	Ponkan	Orange	None	None	Easy	67.8 ± 0.6	61.9 ± 0.7	109.6 ± 1.3

Based on the results of morphological and molecular markers, it is clear that 'Limau Kacang' is a kind of 'Ponkan' (*C. reticulata*). Recent investigations on morphological traits combined with DNA analysis have been conducted on studies about fruit genetic resources (Tshering Penjor *et al.*, 2014a, 2014b; Ueda and Higuchi, 2012; Sharafi *et al.*, 2016). These studies reported new findings to elucidate their diversity and/or genetic evaluation and identification. Our results are consistent with the results of those studies; the genetic identification of 'Limau Kacang' seems accurate and reliable. In addition, we could demonstrate the efficacy of DNA analysis for the genetic identification of local citrus germplasm. Thus, DNA analysis is considered to be essential for further study on the genetic resource management of fruit trees.

Although 'Ponkan' is a major mandarin cultivar ranging from India to China, Taiwan, and Japan, it is not popular in Southeast Asia. Since there is probably no 'Ponkan' germplasm in West Sumatra, no one recognized it as a 'Ponkan'. 'Ponkan' did not originate from West Java where original trees of 'Limau Kacang' were cultivated; these trees however, also considered to have been introduced from other areas.

The results of SRAP analysis also revealed some diversity among 'Limau Kacang'. 'Limau Kacang #8' and 'Limau Kacang #15' did not show identical banding patterns. Since 'Limau Kacang #15' was collected at a local market, the site of the orchard is unknown. Thus, it is necessary to investigate the diversity in 'Limau Kacang' using a number of trees cultivated in various orchards. In addition, 'Limau Kacang #15' was slightly different from the major Japanese 'Ponkan', 'Yoshida Ponkan' and 'Sasshu' in the SRAP analysis. Qualitative fruit characteristics of 'Limau Kacang #15' were almost the same as those of 'Yoshida Ponkan' except skin color. Quantitative fruit characteristics of both accessions were slightly different. However, conditions of cultivation of both cultivars were quite different; tropical Indonesia and temperate Japan.

Thus, it is necessary to investigate fruit of 'Limau Kacang' and to compare it with the other 'Ponkan' accessions cultivated in the same condition.

'Limau Kacang' was not closely related to 'Jeruk Siem' or 'Jeruk Keprok', both mandarins cultivated in Sumatra. 'Jeruk Siem' and 'Jeruk Keprok' were considered to be Indonesian mandarins because they are not related to major mandarins originating from overseas (Yamamoto *et al.*, 2012). 'Limau Kacang' has been propagated vegetatively since its introduction to the Solok region. No genetic influence from Indonesian citrus was found.

The results obtained in the present study contribute to the development of citrus industry in Indonesia. Moreover, it is considered to offer important information for horticulturists throughout the world. 'Ponkan' (*C. reticulata*) is the most widely grown mandarin in the world, being common throughout South China and Southern Japan and India. It is also the most popular mandarin in Brazil (Saunt, 2000). Various improved cultivars adapted the growing environmental conditions have been selected in each region (Iwamasa, 1976; Chinese Society for Horticulture, 2008). Since its main production area is the subtropics, most cultivars seem to adapt to subtropical climate. In contrast, Indonesia is located at the tropics. Thus, 'Limau Kacang' possibly adapts to tropical climate. It is necessary to investigate tree and fruit characteristics of 'Limau Kacang' and compare it with the other 'Ponkan' cultivars in detail.

Conclusion

In the present study, we succeeded in the genetic identification of 'Limau Kacang', a specialty crop in Solok of West Sumatra, Indonesia. It is clear that this cultivar is a kind of 'Ponkan' (*C. reticulata*). The results were obtained from morphological and molecular markers. SRAP analysis offers reliable results but does not require special analysis instruments such as a DNA sequencer (Li and Quiros, 2001). The results of the present study also show the usefulness

of SRAP for genetic identification. In addition, the results of genetic identification provide useful information for the project of rebuilding 'Limau Kacang' orchards in Solok of West Sumatra.

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