Simple Sequence Repeat (SSR) profiling of cultivated Limau Madu (*Citrus reticulata* Blanco) in Malaysia

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Received 28 February 2011
Accepted 28 June 2011

Fruits, 2012, vol. 67, p. 67–76

DOI: 10.1051/fruits/2011070
www.fruits-journal.org

RESUMEN ESPAÑOL, *p. 76*

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Abstract — Introduction. In Malaysia, Limau Madu (*Citrus reticulata* Blanco; *Citrus suhuiensis* Hort. *ex* Tanaka) is commercially cultivated for local consumption. It is a loose-peel mandarin with fruits that are spherical in shape with shiny green or greenish yellow peel. In Malaysia, Limau Madu is vegetatively propagated, thus it is presumed to exhibit minimal variability. In our study, the genetic variability and genetic relatedness among cultivated samples collected from different states in Malaysia were assessed using Simple Sequence Repeat (SSR) primers. **Materials and methods**. Thirty pairs of SSR primers derived from *Citrus unshiu* were screened and 22 SSR primer pairs were utilized to assess genetic variability and relatedness among 118 cultivated samples. **Results and discussion**. The percentage of SSR transferability from *C. unshiu* to *C. reticulata* (Limau Madu) was 73.33%, which indicated that the primer sequences flanking simple sequence repeats were conserved among these *Citrus* species. Most SSR loci revealed a large excess of heterozygotes. In our study, low allelic diversity was shown, with an average of 5.227 alleles per locus. Polymorphic information content (PIC) ranged from 0.048 to 0.674. Based on UPGMA clustering analysis, four groups were identified from these citrus genotypes with a mean genetic distance of 0.170. Low genetic variability within species was probably due to vegetative propagation or inability to detect differences among samples using these primers.

Malaysia / *Citrus reticulata* / genetic resources / genetic variation / genetic markers / microsatellites

Profils de séquences répétées en tandem (SSR) pour la variété d'agrume Limau Madu (*Citrus reticulata* Blanco) cultivée en Malaisie.

Résumé — Introduction. En Malaisie, la variété d'agrume Limau Madu (*Citrus reticulata* Blanco ; *Citrus suhuiensis* Hort. *ex* Tanaka) est commercialement cultivée pour la consommation locale. C'est une mandarine facile à peler, avec un fruit de forme sphérique et une peau de couleur vert-brillant ou jaune-verdâtre. En Malaisie, la variété Limau Madu est propagée végétativement, de ce fait on présume qu'elle a une variabilité minimale. Dans notre étude, la variabilité génétique et la parenté génétique d'accessions de Limau Madu ont été évaluées parmi des échantillons cultivés et collectés dans différents états de la Malaisie, en utilisant des amorces de séquences répétées en tandem (SSR). **Matériel et méthodes**. Trente paires d'amorces de SSR dérivées de *Citrus unshiu* ont été examinées et 22 paires d'amorce de SSR ont été employées pour évaluer la variabilité génétique et la parenté parmi 118 échantillons de Limau Madu cultivés. **Résultats et discussion**. Le pourcentage de la transférabilité de SSR de *C. unshiu* vers *C. reticulata* (Limau Madu) a été de 73,33 %, ce qui indiquerait que les séquences d'amorce encadrant les séquences répétées en tandem ont été préservées parmi les espèces d'agrumes. La plupart des loci de SSR ont révélé un grand excès d'hétérozygotes. Dans notre étude, une faible diversité allélique a été enregistrée avec une moyenne de 5,227 allèles par locus. La valeur de l'information polymorphe a varié de 0,048 à 0,674. En se basant sur l'analyse des classes par la méthode UPGMA, quatre groupes ont été identifiés à partir de ces génotypes d'agrume avec une distance génétique moyenne de 0,170. La faible variabilité génétique au sein de l'espèce pourrait être attribuée soit à la multiplication végétative soit à l'incapacité de détecter des différences parmi des échantillons en utilisant nos amorces.

Malaisie / *Citrus reticulata* / ressource génétique / variation génétique / marqueur génétique / microsatellite

1. Introduction

Citrus belongs to the subfamily Aurantioideae [1] of the family Rutaceae, that comprises about 158 genera and 1900 species [2]. Citrus is well distributed worldwide but it is believed to be native to China, eastern India and Southeast Asia [3]. In Malaysia, the production of citrus fruits is mainly for the domestic market; they are grown in commercial orchards, backyard orchards and smallholdings in various parts of the country [4]. In this country, the general name for citrus is Limau. *Citrus* species commonly cultivated are *Citrus maxima* (Burm.) Merr. (Limau Bali), *Citrus reticulata* Blanco [Limau Madu, synonym *Citrus suhuiensis* Hort. ex Tanaka], *Citrus hystrix* DC. (Limau Purut), *Citrus medica* L. var. *sarcodactylis* (Noot.) Swingle (Limau Jari), and *Citrus aurantifolia* (Christm.) Swingle (Limau Nipis). The origin of *C. suhuiensis* is unknown. However, in China, it is known as Ssu-ui-kom (simhom) and it is one of the most popular citrus fruits in the tropics from the west of Canton to Hainan [1]. This species is also planted in Thailand, Indonesia and Vietnam, where its fruit is known as Somkeae Wan, Jeruk Siam and Duong, respectively. The fruit is spherical in shape with shiny green or greenish yellow peel, and its skin can be easily peeled [5]. It consists of nine to fifteen easily separated segments which are pale orange in color; the flesh is juicy and sweet. Fruits can be eaten raw, as squeezed fruit juice or used for flavoring. In Malaysia, a local variety, Limau Madu, is widely cultivated in the states of Pahang, Kedah, Perak, Terengganu, Johor and Sarawak. The fruits are cheaper than imported oranges and have high vitamin C content [5]. They normally have several seeds which are polyembryonic and recalcitrant [6].

Even though Limau Madu is considered as a cross-pollinated crop [6], it is expected to show minimal genetic variability as the plants are vegetatively propagated by the nurseries to ensure maintenance of elite genotypes with desirable traits [7]. The genetic variability observed within species might be due to spontaneous mutations, as most of the new or improved cultivars were obtained from careful selection of bud mutations [8]. Genetic variability studies on *Citrus* species such as *C. unshiu* and *C. sinensis* revealed that cultivated *Citrus* species cover a narrow genetic base [9, 10]. Genetic diversity and genetic relatedness among *Citrus* species have been conducted using morphological, biochemical and genetic markers [8, 11]. Compared with morphological and biochemical markers, molecular genetic markers were not influenced by environment or abundance in the genome and revealed a high level of polymorphism, which is an advantage in distinguishing between closely related individuals [12]. These genetic markers included Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Random Amplified Polymorphic DNA (RAPD), Inter-Simple Sequence Repeat (ISSR) and Simple Sequence Repeat (SSR).

Simple sequence repeats (SSRs), also known as microsatellites, consist of tandem repeats of short nucleotide motifs with a length of between one and six nucleotides [13]. They are abundant and distributed in nuclear, mitochondrial and chloroplast genomes [14]. SSRs have a high level of variation, which is believed to be due to DNA slippage during replication, unequal crossing over and genetic recombination [15]. The usage of SSRs in various studies has increased due to a few advantages: they are co-dominant, multiallelic and Mendelian-inherited; SSR genotyping can be semi-automated with the use of fluorescence-labeled primers; and they have high reproducibility and reliability [16, 17]. SSR primer pairs are transportable across species within a genus or between related genera, as sequences flanking microsatellite regions are highly conserved [18]. Previous studies reported that SSR primers synthesized from the citrus genomic library and citrus Expressed Sequence Tags (ESTs) were successfully amplified homologous loci in other *Citrus* species such as *C. maxima, C. medica*, *C. reticulata* and the related genera *Poncirus* [19, 20].

The aim of our work was to evaluate the genetic variability and assess the genetic relationships among samples of Limau Madu using SSR primer pairs synthesized from the genomic library of *C. unshiu* [21].

Potential varieties that are associated with agronomic traits such as fruit size, fruit color and taste can be identified based on the unique allelic profiles obtained. By studying the genetic variability within species, this would greatly assist the breeders in managing the germplasm that is available for cultivar improvement programs.

2. Materials and methods

2.1. Plant materials

A total of 118 cultivated samples were collected from different states in Malaysia. The sources of these samples included the Malaysian Agricultural Research and Development Institute (MARDI) citrus germplasm collection, the germplasm collection of the Universiti Kebangsaan Malaysia (UKM) and private orchards *(figure 1)*. The locations of the source trees were determined using a global positioning system (GPS) (*table I*).

Figure 1.
Location of sites where samples were collected for studying genetic variability in the Limau Madu (*Citrus reticulata* Blanco) variety in Peninsular Malaysia.

2.2. DNA extraction

Genomic DNA was extracted from fresh leaves based on Doyle and Doyle's method [22] with minor modifications according to Puchooa [23]. The purity and the concentration of DNA were estimated using the Nanodrop ND-100 (Dupont Agricultural Genomics Laboratory). The final concentration was adjusted to 10 ng·uL^{-1} for the amplification step.

Table I.
List of locations and number of samples collected for studying genetic variability in the Limau Madu (*Citrus reticulata* Blanco) variety in Malaysia.

Geographical description	Latitude (N)	Longitude (E)	Number of samples
Germplasm collection at Universiti Kebangsaan Malaysia, Bandar Baru Bangi	2°55'18"	101°47'10"	19
Citrus germplasm collection at MARDI Kluang Station, Johor	1°56'53"	103°21'19"	20
Orchard in Kuala Terla, Cameron Highlands, Pahang	4°32'4"	101°27'42"	20
Orchard in Felda Ciku 3 Gua Musang, Kelantan	4°57'55"	102°12'9"	10
Orchard in Chalok Setiu, Terengganu	5°28'50"	102°47'49"	16
Orchard in Beaufort, Sabah	5°16'1"	115°39'45"	2
Orchard in Kampung Tanjung Parang, Kota Samarahan, Sarawak	1°27'8"	110°31'16"	11
Orchard in Kampung Lian Seng, Kuala Kurau, Perak	4°59'3"	100°29'57"	20

2.3. PCR amplification

PCR amplification was performed with a three-primer system which included a sequence-specific forward primer with a universal M13 (-21) attached at its 5' end, a sequence-specific reverse primer and a M13 (-21) universal primer labeled with fluorescent dye [24]. A total of 30 SSR primer pairs synthesized from the genomic library of *C. unshiu* were supplied by the National Academy of Agricultural Science, RDA, Republic of Korea [21]. These 30 SSR primer pairs were screened and only primers that produced easily scored, clear and visible bands were selected to assess the genetic diversity of Limau Madu (*table II*). Amplification reactions were carried out in a total volume of 25 µL, containing 20 ng template DNA, 1X PCR buffer, 0.2 mM of each dNTP, 1 U Hotstart Taq DNA polymerase, 5 pmol of each forward and the fluorescent-labeled M13 (-21) primer and 10 pmol of the reverse primer. The conditions of the PCR amplification were as follows: 95 °C (15 min) then 3 cycles each at 95 °C (20 sec), 52 °C (40 sec) and 72 °C (1 min), followed by 14 cycles of 95 °C (30 sec), 53 °C (45 sec) and 72 °C (1 min) and a final extension at 72 °C for 15 min. PCR products were separated by the ABI Prism 3130 automated sequencer (Applied Biosystems) and sized precisely based on the GeneScan ROX 500 internal size standard using GENOTYPER ver. 3.7. Electropherograms were analyzed using GENESCAN ver. 3.7.

2.4. Statistical analysis

The number of alleles (N_a) and observed heterozygosity (H_o) in each SSR locus were estimated using GenAIEx 6 [25]. As these samples collected did not constitute a natural population, expected heterozygosity was not estimated in this study. Polymorphic Information Content (PIC) for each SSR primer pair was calculated using Power Marker ver. 3.25 [26]. The Probability of Identity among siblings (PID-*sib*) [27] was used to estimate the probability of two siblings having identical genotypes drawn randomly from a given population using GIMLET ver. 1.3.2 [28]. Genetic relatedness among samples was estimated based on Nei [29] using Power Marker ver. 3.25. The samples were grouped using the unweighted pair-group method using the arithmetic average (UPGMA) clustering method and dendrograms were built using MEGA ver. 4.0 [30]. The genetic relatedness among samples was also evaluated using principal component analysis (PCA) which was available in MultiVariate Statistical Package 3.0 (MVSP) [31].

3. Results

3.1. Genetic diversity of Limau Madu

Out of the thirty primer pairs that were screened in our study, 22 SSR primer pairs produced alleles that could be scored. The transferability rate was 73.33%. The size of the alleles amplified by these 22 SSR loci ranged from 158 bp to 342 bp. All the SSR primers were polymorphic, with the number of alleles at each locus ranging from 2 (GB-CU-077 and GB-CU-176) to 10 (GB-CU-096). An average of 5.227 alleles per locus was detected. The observed heterozygosity (H_o) obtained ranged from 0.051 to 1.000 with a mean H_o of 0.511. Polymorphic Information Content (PIC) ranged from 0.048 to 0.674 with an average of 0.345 (*table II*).

For individual loci, the PID-*sib* ranged from 0.420 to 0.951 and the overall probability of identity among siblings was $5.76E^{-5}$ (*table II*). In order to minimize the time and cost, a smaller number of highly polymorphic loci would be preferred for distinguishing between individuals in citrus. Thus, PID-*sib* served as an upper limit to provide the most conservative number of loci required for distinguishing Limau Madu samples. Overall, PID-*sib* became close to zero after nine selected SSR loci (GB-CU-133, GB-CU-104, GB-CU-182, GB-CU-098, GB-CU-031, GB-CU-096, GB-CU-198, GB-CU-125 and GB-CU-038) with the lowest PID-*sib* were applied (*figure 2*).

The combination of genotypes obtained across 22 SSR loci was taken as the SSR

Table II.
List of the 22 SSR primers, primer sequence, repeat motifs, allele size and diversity of Limau Madu (*Citrus reticulata* Blanco) variety samples collected in Malaysia for studying their genetic variability.

Locus	Forward sequence	Reverse sequence	Repeat motifs	Allele size range (bp)	Number of alleles	Ho	PIC	PID-*sib*
GB-CU-133	TGCAGCTCGTGTGTGTTC	TGACACAACTTGACCTTGTCAC	(TG)2(T)(TG)6, (TG)5(GC)(TG)3	158–195	6	0.839	0.674	0.420
GB-CU-098	TCTTCTCCCAGTTGGGGT	CCGGACCCAGAATCAGTC	(AC)8	256–290	9	0.966	0.485	0.530
GB-CU-038	AACGACGGCATACCTGC	GAGTCCTCCTCCCTGTCC	(AG)2(GA)(AG)4	310–342	8	0.525	0.450	0.564
GB-CU-085	TGGCAAACGTACCAAACC	CTCCCAAACGGACCCTAC	(GCG)4	271–295	5	0.556	0.357	0.596
GB-CU-125	TGACCAATTATGTGGCTCC	CTTCTCCCAGTTGGGGTC	(TG)3TT(TG)9	216–226	5	0.983	0.435	0.559
GB-CU-052	GTCAATACGATCCACGGG	TTGAGCCAAAGAACGGTG	(TC)5CC(TC)5	231–259	6	0.568	0.408	0.586
GB-CU-182	GCTGATGCAAAATCGGAG	GCCATTTCCTTTTCCACC	(TC)11, (TG)5GG(TG)2	220–223	7	1.000	0.514	0.513
GB-CU-096	GCTCTCAGTCCCTTCGGA	GCACTCGCGGTGTAACTC	(GGAGGC)4	259–298	10	0.873	0.462	0.543
GB-CU-014	TCTAGGCTGTCCGTGTGC	TCCGGCTTCTTTGTCAGA	(GAC)8	209–217	3	0.898	0.392	0.638
GB-CU-198	TGTTGGCTGATAGGACGG	GCTAACACAAGTTAACACACGC	(TG)5	187–229	3	0.983	0.462	0.544
GB-CU-017	GACCATGGGATTACCCAAA	ATCACTCCAGGCTCAGCA	(CTG)10	198–218	5	0.085	0.081	0.920
GB-CU-104	ACGGATGCGACTTCAATG	GCCACCATTTACAAGGCA	(TTC)13	252–278	6	0.983	0.643	0.438
GB-CU-173	GAGATGCAGACGGCTCAC	CTGCACAGTCACTCGCAA	(GT)9	253–281	6	0.483	0.430	0.585
GB-CU-120	CCAGTAGGGGTAGTGGGC	GGGAAGAACAGACAATGAAATC	(TTGGTG)7	256–281	4	0.052	0.050	0.951
GB-CU-031	AGATTGCAGACTGGCGAA	ACACAAATCACACTCGCAGA	(TG)2(T)2(TG)4	204–268	6	0.051	0.466	0.542
GB-CU-040	TTAGCCCAACAGTGCCC	GGAAGCGCTTGAACCTTT	(TGC)5	280–300	5	0.090	0.391	0.579
GB-CU-097	AAATAGAGCACGGGCCAT	GCATCGCTATTGCCGTTA	(ACC)3(GCT)(ACC)3	278–312	6	0.222	0.211	0.795
GB-CU-176	CCACGTGCTTTCAACCAT	AGGGAAGGGAGTGCAATG	(CCG)4	171–176	2	0.051	0.048	0.951
GB-CU-022	ATCTAGGGTTTTGCCGGA	ATCCGTACACGCTGCACT	(CAG)5	218–228	4	0.085	0.095	0.906
GB-CU-110	ATAAAATGAGGGCGCCAG	GCATTTTCACAGTCTCGCA	(CT)8(G)(CT)6(TG)8	203–207	3	0.838	0.417	0.573
GB-CU-033	TTTGCAAAGTTGGGAGGA	TAAAAATCCCGTCACCGC	(CAG)4	268–282	4	0.068	0.072	0.928
GB-CU-077	CAGCTGCTGAAGAACAACAA	GTTGCTGAACTTGTCCGC	(AGC)6	214–218	2	0.051	0.049	0.950

H_o: Observed heterozygosity.

PIC: Polymorphic Information Content.

PID-*sib*: Probability of identity among siblings.

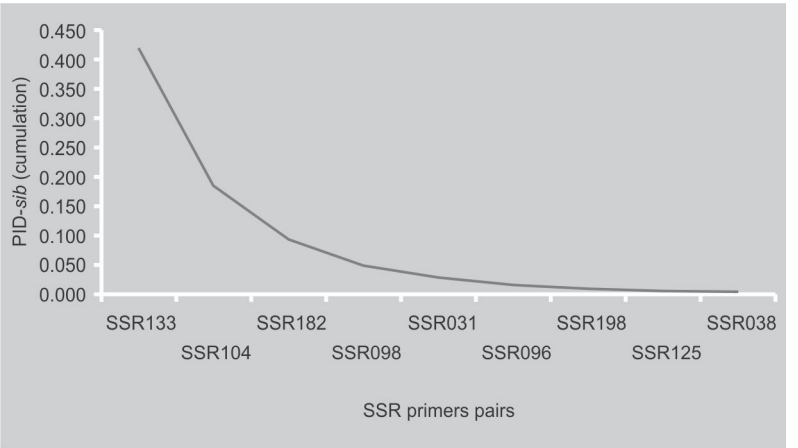

Figure 2.
Probabilities of identity among siblings (PID-*sib*) obtained from 118 samples of the Limau Madu (*Citrus reticulata* Blanco) variety collected in Peninsular Malaysia.

profile. Based on their SSR profiles, samples that were fully matched across 22 loci were considered as clones. A total of 44 SSR profiles were observed. Out of the 118 samples that were analyzed, 85 samples were identified as clones.

3.2. Genetic relationships among samples

The average genetic distance among individuals in Limau Madu was 0.170. The dendrogram (*figure 3*) shows the samples were separated into four groups. The first group comprised samples cs32, cs24 and cs27. Group two comprised samples cs40, cs36, cs37, cs38, cs39, cs31, cs23, cs28, cs30, cs21, cs25, cs26 and cs29. The third group comprised samples cs22, cs33 and cs34. The final group comprised samples collected from orchards, which was further divided into two subgroups. The samples within the subgroups had short branch lengths, with most of them less than 0.01 (or zero) since they had identical SSR profiles. Calculations showed that the first three principal components in total accounted for 94.04% of changes in Limau Madu. The first principal component (PC1) accounted for 51.65% of total variance. The second (PC2) and third component (PC3) alone accounted for 36.31% and 6.44%, respectively. Eigenvalues for the first three principal components were 60.49, 42.85 and 7.60, respectively. From the scatter plot, samples collected from MARDI and samples collected from orchards were clearly separated and two groups were observed within orchard samples (*figure 4*), which is consistent with the dendrogram obtained.

4. Discussion

The high transferability rate of the SSR primer pairs across *Citrus* species and related genera, with some that exceeded 50%, has been reported in previous studies [32, 33]. The present study revealed that the successful transferability rate of *C. unshiu* microsatellite loci to be utilized in *C. reticulata* (Limau Madu) was 73.33%. These 30 SSR primer pairs were previously utilized by Dixit [21] to assess the genetic diversity of 40 *Citrus* accessions representing different *Citrus* species and some varieties of *C. unshiu*. All microsatellite loci were amplified and produced between two and ten alleles [21]. The high rate of conservation of these SSR loci between *Citrus* species is an advantage as it allows a great use of these SSR markers in *Citrus* genetic research and may be shared among different laboratories.

The degree of informativeness in different sets of SSR primers and the diversity of studied species would influence the results obtained. In our study, the level of heterozygosity observed in Limau Madu was intermediate, with mean observed heterozygosity (H_o) of 0.511. An intermediate level of heterozygosity was also reported by Cristofani-Yaly *et al.* [34] as well as in Luro *et al.* [35] in different *Citrus* spp. and related genera, which ranged between 8% and 58%. One of the reasons for an intermediate to high level of heterozygosity can be attributed to wide cross-compatibility among species and related genera, and spontaneous mutations [36]. The maintenance of heterozygosity was also due to selection of desirable clones which were propagated vegetatively [37]. In this study, most of the SSR loci revealed a large excess of heterozygotes, which indicated that the selection had favored heterozygous genotypes. As reviewed by Sonnante *et al.* [38] in domesticated plants, human selection has favored the maintenance of high levels of heterozygosity since

the plants may display hybrid vigor derived from outcrossing and those plants were then vegetatively propagated to ensure that the favorable genotypes were passed on to the next generation. The mean number of alleles detected in our study was lower compared with other *Citrus* species reported: *C. unshiu* (5) [9], *C. limon* (6.14) [32] and *C. maxima* (9.85) [39]. In addition, the Polymorphic Information Content (PIC) obtained in the present study was lower compared with other *Citrus* species reported [9, 39]. The low allelic diversity and PIC obtained in our study can be attributed to the fact that the samples studied covered a narrow genetic base and the SSR loci used in this study were less informative.

Low genetic polymorphism within species was observed in our study, with a mean genetic distance of 0.170. The present study revealed that most samples have identical SSR profiles even though they were collected from different places. Our study indicated that the low genetic variability was probably due to propagation of a small number of cultivars which were used commercially as scions in order to obtain a genetically uniform crop, ensure early yield and overcome citrus juvenility [12]. Additionally, the improvement for most of the cultivars in sweet oranges, satsumas, clementines, grapefruits and lemons came from the carefully selected mutated buds or branches, which could not be distinguished by molecular markers [40].

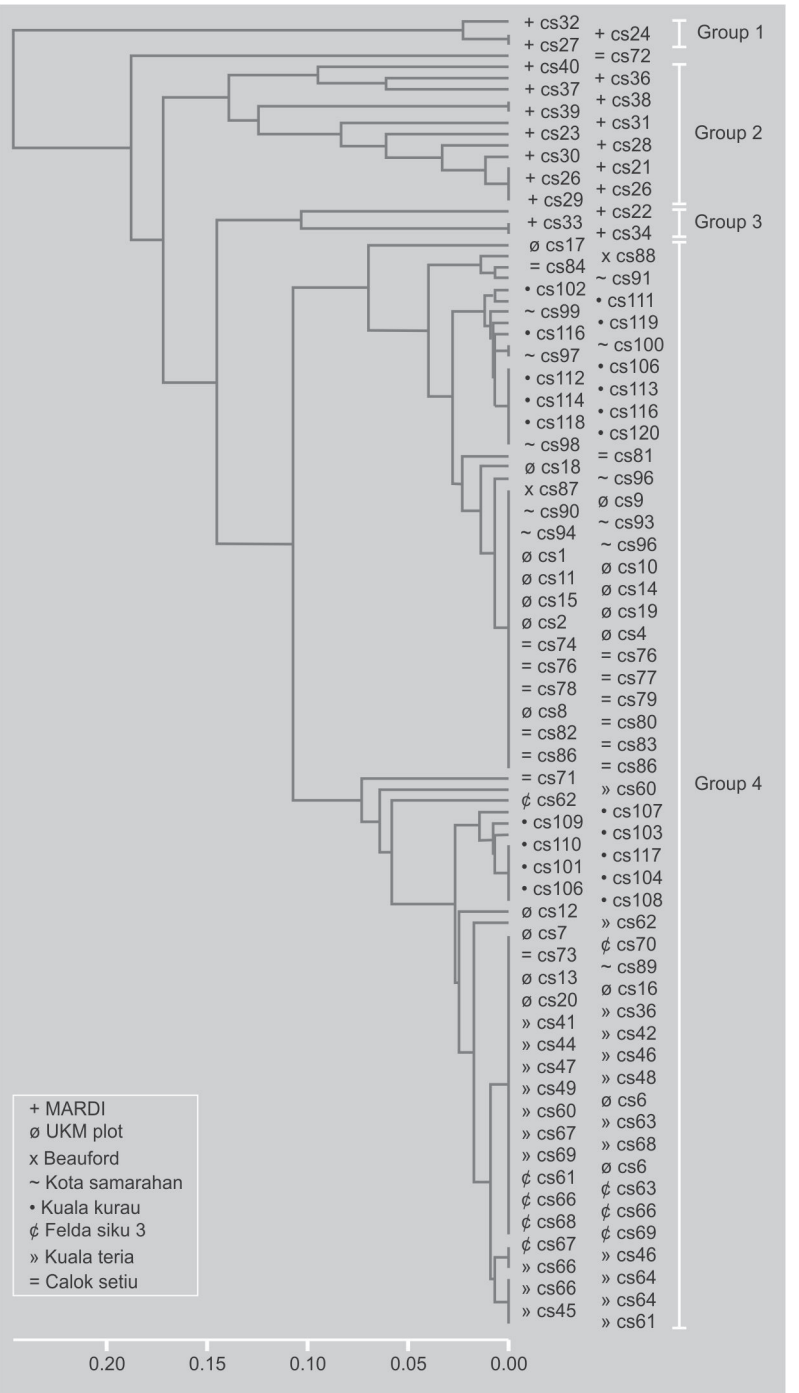

Figure 3.
Dendrogram of 118 Limau Madu (*Citrus reticulata* Blanco) variety samples, obtained from the unweighted pair group with arithmetic mean (UPGMA) method using Nei's genetic distance after amplification with 22 SSR primer pairs.

Acknowledgements

We are grateful to Dr. Jung Wook Chung and Dr. Wei Guo Zhao for their support, guidance, helpful comments and technical information in completing this study. We are also very grateful to the National Academy of Agricultural Science (RDA) for providing primers, facilities and financial support during the research attachment of the first author. We would like to thank the Malaysian Agricultural Research and Development Institute (MARDI) for providing the samples. This research was supported by a Bioversity International – Korean Biogreen 21 grant.

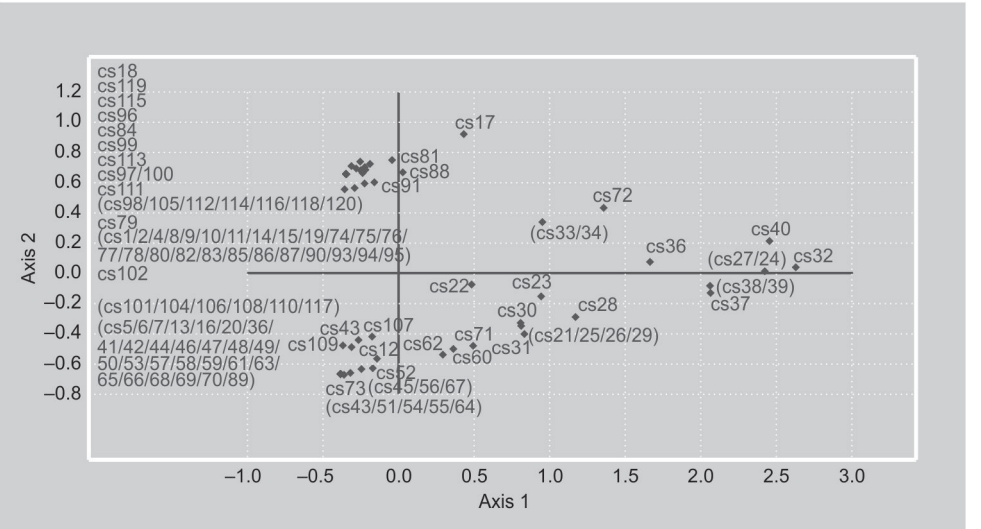

Figure 4.
Plot of the first two components (axis 1 = PC1; axis 2 = PC2) of principal component analysis on the similarity matrix for 118 Limau Madu (*Citrus reticulata* Blanco) variety samples studied from their Simple Sequence Repeat (SSR) profiles.

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Perfiles de repetición de secuencia única (SSR) para la variedad cítrica Limau Madu (*Citrus reticulata* Blanco) cultivada en Malasia.

Resumen — Introducción. En Malasia, la variedad cítrica Limau Madu (*Citrus reticulata* Blanco; *Citrus suhuiensis* Hort. *ex* Tanaka) se cultiva con fines comerciales para el consumo local. Se trata de una mandarina fácil de pelar, con un fruto en forma de esfera y una piel de color verde-brillante o amarillo-verdoso. En Malasia, la variedad Limau Madu está extendida vegetativamente, por lo que se presume que su variabilidad es mínima. Para nuestro estudio, se evaluaron la variabilidad genética y el parentesco genético de la muestra de material de Limau Madu, entre las muestras recolectadas en diferentes estados de Malasia, mediante el uso de cebadores de repetición de secuencia única (SSR). **Material y métodos**. Se examinaron treinta pares de cebadores de SSR derivados de *Citrus unshiu* y se emplearon 22 pares de cebadores de SSR para evaluar la variabilidad genética y el parentesco entre 118 muestras de Limau Madu cultivadas. **Resultados y discusión**. El porcentaje de la transferibilidad de SSR de *C. unshiu* hacia *C. reticulata* (Limau Madu) fue del 73,33%. Esto indicaría que las secuencias de cebador que delimitan las repeticiones de secuencia única se preservaron entre las especies de cítricos. La mayoría de los loci de SSR mostraron un gran exceso de heterocigotos. En nuestro estudio, se registró una diversidad alélica floja con una media de 5,227 alelos por locus. El valor de la información polimorfa varió de 0,048 a 0,674. En base al análisis de clases mediante el método UPGMA, se identificaron cuatro grupos, a partir de estos genotipos de cítricos, con una distancia genética media de 0,170. La floja variabilidad genética en el seno de la especie podría atribuirse o bien a la multiplicación vegetativa o bien a la incapacidad de detectar diferencias entre las muestras con el uso de nuestros cebadores.

Malasia / *Citrus reticulata* / recursos genéticos / variación genética / marcadores genéticos / microsatélites