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Genetic Diversity Assessment of Citrus Accessions Grown in Indonesia Using Molecular Markers

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ABSTRACT

The assessment of genetic diversity is crucial in citrus improvement programs that represents the relationship among genotypes, thus determining an effective plant breeding program. A molecular assessment of 52 collected citrus accessions based on ISSR and SSR was conducted at the ICSFRI in 2020. Based on 4 ISSR primers and 3 SSR primers, the citrus genotypes were grouped into 6 major clusters. Cluster I has the largest 25 members, while Cluster II, III, IV, V and VI have 5, 2, 2, 4 and 13 accession members, respectively. Members of Cluster I are the majority in the mandarin type with spheroids fruit shape with truncate on the fruit top. Cluster II contains citrus accessions from naturally occurring hybrids, while orange accession members of Cluster III are originally grown in different climates. Cluster IV and V members are connected to a common ancestor, *Citrus aurantifolia* and *Citrus limonia*. At the same time, Cluster VI is a pumelo group that contains members of *Citrus maxima*. Clustering based on molecular markers (ISSR and SSR) resulting in this study is useful in citrus breeding programs in Indonesia and other countries. The genetic distance of the parents affects the heterosis effect of the progeny.

INTRODUCTION

Citrus plants have high economic and nutritional value, including mandarins, tangerines, oranges, lemons, grapefruits, and limes. This genus belongs to and is considered one of seven subfamilies of the Rutaceae Aurantioideae. This subfamily consists of 2 tribes with 33 genera. The two phyla are the Clauseneae, which includes Micromelinae, Clauseninae, and Merrillinae, and the Citrus phylum, which provides for Triphasiinae, Citrinae, and Balsamocitrinae (Penjor et al., 2013). Compared to other subgroups, the Citrinae subgroup (Phylum Citreae) showed the presence of pulp vesicles in the fruit. Due to these characteristics, these "true citrus trees" are considered the most advanced genus according to their morphological characteristics (Wu et al., 2018). However, classifying citrus fruits based on morphological characteristics remains an open problem. Citrus species are generally classified using two main systems: the Swingle and Reece classification system, which considers 16 species, and the Tanaka classification system, which identifies 162 species in the Citrus genus. More recently, Mabberley proposed a new taxonomy of edible citrus fruits comprising three species and four hybrid populations (Curk et al., 2015).

The large genetic variation among cultivated citrus varieties is due to frequent bud mutations, extensive sexual compatibility between Citrus and related genera, apomixis, wide world distribution, and a long history of cultivation (Maya et al., 2012). Citrus has been widely cultivated under various agroecological conditions in Indonesia. Citrus plantations have been established from low to highlands with dry and wet climates. Through farmer selections, some variants show adaptive growth and bear optimal fruit production in specific sites on lowland or highland (Penjor et al.,

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2014; Tolangara et al., 2020). Local names have also been known for these traditional varieties.

Precise information about the extent of genetic divergence and variation of characters used for differentiation among the population is crucial in the citrus improvement program. Furthermore, assessing genetic diversity becomes even more crucial if we want to maximize the amount of useful genetic variation within a collection (Sharafi et al., 2017). The genetic diversity of collections represented the relationship among genotypes, thus determining an effective plant breeding program. High genetic diversity can facilitate cultivar selection of parents and their inclusion in breeding programs to develop cultivars with high yield potential and achieve diverse plant breeding goals (Omura & Shimada, 2016). The genetic similarity of two parents may have implications for plant breeding. The farther the genetic distance between parents, increased the heterosis effect in their progeny. Heterosis is when hybrids' offspring outperform their parents regarding yield and other desirable traits (Scott et al., 2020).

Since the 1970s, morphological and biochemical studies elucidated the phylogeny of citrus and its wild relatives (Penjor et al., 2013). The assessment of genetic variability, phylogeny, and the genetic maps in certain citrus cultivars has been somewhat controversial because the environment has influenced characterization based on agromorphological traits (Martasari et al., 2013). In particular, molecular marker technology has successfully assessed genetic diversity at inter- and intraspecific levels, distinguished or characterize different cultivars, clones and accessions, and determined genetic relationships within and between populations. Several molecular marker techniques have been used to study the classification of the genus Citrus and the phylogenetic relationships within citrus and with related genera, such as molecular hybridization, PCR, RAPD, AFLP and microsatellite markers (Simple Sequence Repeat-SSR), depending on the purpose and subject of the studies (Ahmed et al., 2017).

Among these molecular markers, SSR and ISSR represent simple and widely used system because their use does not require any prior information about target sequences and ensure their efficiency and reproducibility (Duhan et al., 2020; Munankarmi et al., 2018; Susandarini et al., 2020). Based on the presented facts, this study aims to analyze genetically 52 collected citrus accessions by SSR and ISSR markers to facilitate the future plant breeding program based on the specific location in Indonesia and other countries. The studied citrus plants comprise mandarin, tangerine, orange, pumelo and functional citrus accessions maintained in the Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI).

MATERIALS AND METHODS

The research was conducted at the Indonesian Citrus and Subtropical Fruits Research Institute (ICSFRI) from January to December 2020. The numbers of citruses used in the study were 52 accessions located at the working collection of ICSFRI. The plants were composed of commercial citrus accessions and included mandarin, tangerine, pomelo, sour orange, and functional citrus accessions, as presented in Table 1.

DNA Extraction, Isolation, and Quantification

The method of DNA extraction, isolation, and quantification followed Doyle (1991) with modification. Young leaf samples (100 g) were ground using mortar in 2 ml extraction buffers (2% CTAB, 100 mM Tris-HCl, 1.4 M NaCl, 20 mM EDTA, 2% PVP, and 0.2% βME). The rinses were then incubated at 65°C for 30 minutes. After the incubation, Na acetate (0.1 x volume) and 1 ml of CHISAM (mixture of chloroform and isoamyl alcohol, 24:1 v/v) were added, and the sample was centrifuged at 12,000 rpm for 10 minutes. After the supernatant was transferred to a fresh tube, Na acetate (0.1 x volume) and cold isopropanol (0.6 x volume) were added. The sample was gently mixed with inversion and centrifuged at 12,000 rpm for 10 minutes to visualize the DNA pellet attached to the bottom of the tube. The liquid phase was drained, and the DNA was washed twice with 50 μL of 70% ethanol. The pellet was allowed to dry for about 12 hours with the tubes inverted on filter paper at room temperature. The pellet was resuspended in 50 μL of TE buffer plus 1 μL of RNase. The solution was then incubated at 37°C for 30 minutes. After 30 minutes of incubation, Na acetate (0.1 x volume) and absolute alcohol (2.5 x volume) were added, and the solution was centrifuged at 12,000 rpm for 10 minutes. The liquid phase was drained, and the DNA was washed twice with 50 μL of 70% ethanol. After the pellet was dried at room temperature, the pellet was resuspended in 50 μL of TE buffer solution. DNA concentration was measured with Nanodrop (Infinite 200Pro Tecan).

DNA Amplification and Separation

PCR reactions were conducted for 50 ng DNA in 20 μl PCR buffers (10 μl Tag Dream-PCR Master Mix, 25 μM forward dan reverse primers, and ddH_2O up to 20 μl). The PCR was performed on an Eppendorf 6331 Nexus Gradient Master Cycler. The reaction conditions were 3 minutes at 94ºC, 28 cycles of 45 seconds at 94ºC, 1 minute at 48ºC, and 1 minute at 72 48ºC. The final extended cycle at 72ºC for 10 minutes (Scarano et al., 2002). The ISSR and SSR primers used in the study are listed in Table 2 and Table 3. The amplification products were separated in 2.5% agarose gel were stained with 10 mg/l ethidium bromide at 0.5 x TBE at

100 volts for 60 minutes. The presence of an amplified band was identified and bio-documented using Bio-Rad Gel Documentation System.

Scoring and Dendrogram Analysis

The presence of a visually amplified product of each accession determined the DNA fragment scoring. The visual DNA fragment represented the DNA locus, and the same migration distance of each visual fragment indicates the homolog loci. The similarity matrix was constructed by binary code based on the presence and absence of DNA bands. The phylogenetic tree genetic relationship was constructed based on cluster analysis of UPGMA (unweighted-pair group method with arithmetic averages).

Table 1. Code and name of citrus accessions used in the study

Code	Citrus Accession	Code	Citrus Accession
1	Mandarin Batu (KBO)	27	Mandarin Topo Putih
2	Mandarin Batu 55	28	Kaffir lime Lemo Kuit
3	Mandarin Batu 231	29	Kaffir lime Monte Hondu Basaulu
4	Mandarin Garut	30	Kaffir lime Monte Kassie
5	Mandarin Kacang Singkarak/Solok	31	Mandarin Kendari
6	Mandarin Madura	32	Pumelo MTR 19
7	Mandarin Pulung	33	Pumelo Jeruk Kelapa
8	Mandarin Soe	34	Lemon BA
9	Mandarin Kisar	35	Lemon Swanggi
10	Mandarin Tejakula	36	Lemon Lisbon
11	Mandarin Selayar	37	Mandarin Topo Hitam
12	Mandarin Kasturi	38	Mandarin Kalele Aceh
13	Tangerine Pontianak	39	Lemo Cina Lamo
14	Tangerine Banjar	40	Lime Borneo
15	Tangerine Kintamani	41	Pumelo Pasaman
16	Tangerine Madu	42	Pumelo Bona Bali
17	Mandarin Slopen	43	Pumelo Cina
18	Mandarin Ponkan	44	Pumelo Pasariki
19	Mandarin Daisy	45	Pumelo Bageng Taji
20	Tangerine Mamuju	46	Pumelo Pamindo
21	Mandarin Kertaji	47	Pumelo Pekalongan
22	Mandarin Topazindo	48	Pumelo Lonceng
23	Mandarin Banten	49	Pumelo Baco
24	Tangerine Pati	50	Pumelo Taliwang Putih
25	Mandarin Tankan	51	Sour orange Valencia Late Orange
26	Kaffir lime Monte Hondu Masariki	52	Sour orange Valencia Orange Coll

Table 2. List of ISSR primers used in the study

No	Primers	Sequences	Polymorphic locus	Number of bands
1	ISSR 1	HVH(CA)7	86%	558
2	ISSR 4	HVH(TCC)5	84%	439
3	ISSR 7	(GT)8YC	82%	364
4	ISSR 8	A(GA)7GYC	83%	763

Table 3. List of SSR primers and their forward and reverse sequences used in the study

No	Primers	Repeat motif	Forward Sequence	Reverse sequence
1	TAA15	TAA	GAAAGGGTTACTTGACCAGGC	CTTCCCAGCTGCACAAGC
2	TAA41	TAA	AGGTCTACATTGGCATTGTC	ACATGCAGTGCTATAATGAATG
3	CAT01	CAT	GCTTCGATCCCTCCACATA	GATCCCTACAATCCTTGGTCC

RESULTS AND DISCUSSION

The study analyzed 52 citrus accessions of 25 mandarin, 6 tangerines, 12 pumelo, and 9 mixed other varieties. All ISSR and SSR markers allowed the identification of the microsatellite sequence regions in the genome in all tested citrus accessions with different frequencies and abundance (Fig. 1). The advantages of both markers are that they are very easy to use, quick to perform, have a small amount of DNA templates (10–30 bp), repeatability and consistency, not much information is required to design primers, and in the ability to distinguish individuals who have a very close relationship (Liu et al., 2013; Suárez-Contreras et al., 2020). The use of ISSR markers in citrus fruit identification is widespread (Kosmiatin et al., 2019; Lombardo et al., 2012; Munankarmi et al., 2018; Sharafi et al., 2017; Uchoi et al., 2017). Likewise, SSR markers have been widely used for genetic studies in citrus fruits (El Zayat et al., 2021; Ollitrault et al., 2010). Microsatellite markers have been used for genetic variability of many crops such as strawberries (Arisah et al., 2022), rose (Zhang et al., 2006), *Lagerstroemia indica* L. (Zheng et al., 2019), Prunus (Barreneche et al., 2021), apple (Lacis et al., 2011) and so on.

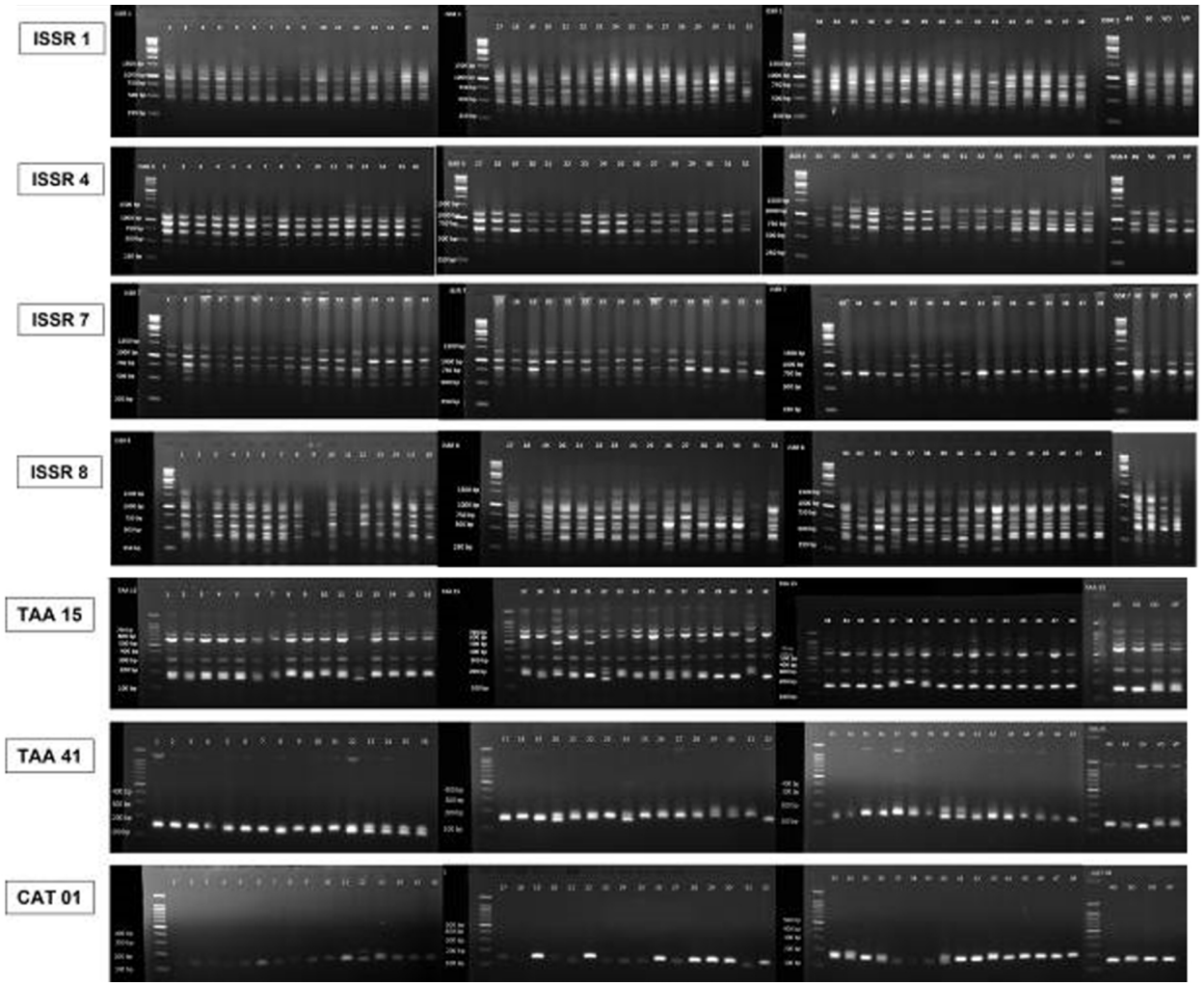

Fig. 1. DNA bands profile of 4 ISSR primers (ISSR 1, ISSR 4, ISSR 7 and ISSR 8) and 3 SSR primers (TAA 15, TAA 41, and CAT 01) resulted from 52 citrus accession samples

The ISSR and SSR alleles represented from 7 markers evaluated were used for genetic diversity analysis. Dice's similarity coefficients were calculated to assess the genetic similarities between the studied citrus accessions, and the similarity coefficients matrix was used for the UPGMA cluster analysis, as shown in Fig. 2. The 52 citrus accessions were divided into 6 major clusters based on genetic similarity. The similarity index among the tested accessions ranged from 65% to 99%. Cluster I had a maximal similarity distance of about 80% and consisted of 25 accessions. Clusters II, III, IV, V, and VI had 5, 2, 2, 4, and 13 accession members, respectively.

Cluster I was observed to have the largest accession members and most of them were mandarin-type citrus. The members were accessions with codes 1-11, 13– 18, 20, 23, 24, 25, 27, 37, 38 and 39. In subcluster A, the closest genetic distance was observed on Mandarin Batu (accession code 1) and Batu 55 (accession code 2), while the furthest was found in tangerine Kintamani and Madu (Fig. 3.). The close genetic distance between Mandarin Batu and Batu 55 indicated that these accessions were very similar and had a close relationship (Hazarika et al., 2014). This genetic relationship is also supported by the similar morphological character of both accessions regarding fruit shape. However, most all members of this subcluster had the fruit shape of spheroids with truncate on the fruit top. The fruit performance of mandarin Batu (KBO), Batu 55, tangerine Kintamani and Madu are presented in Fig. 4.

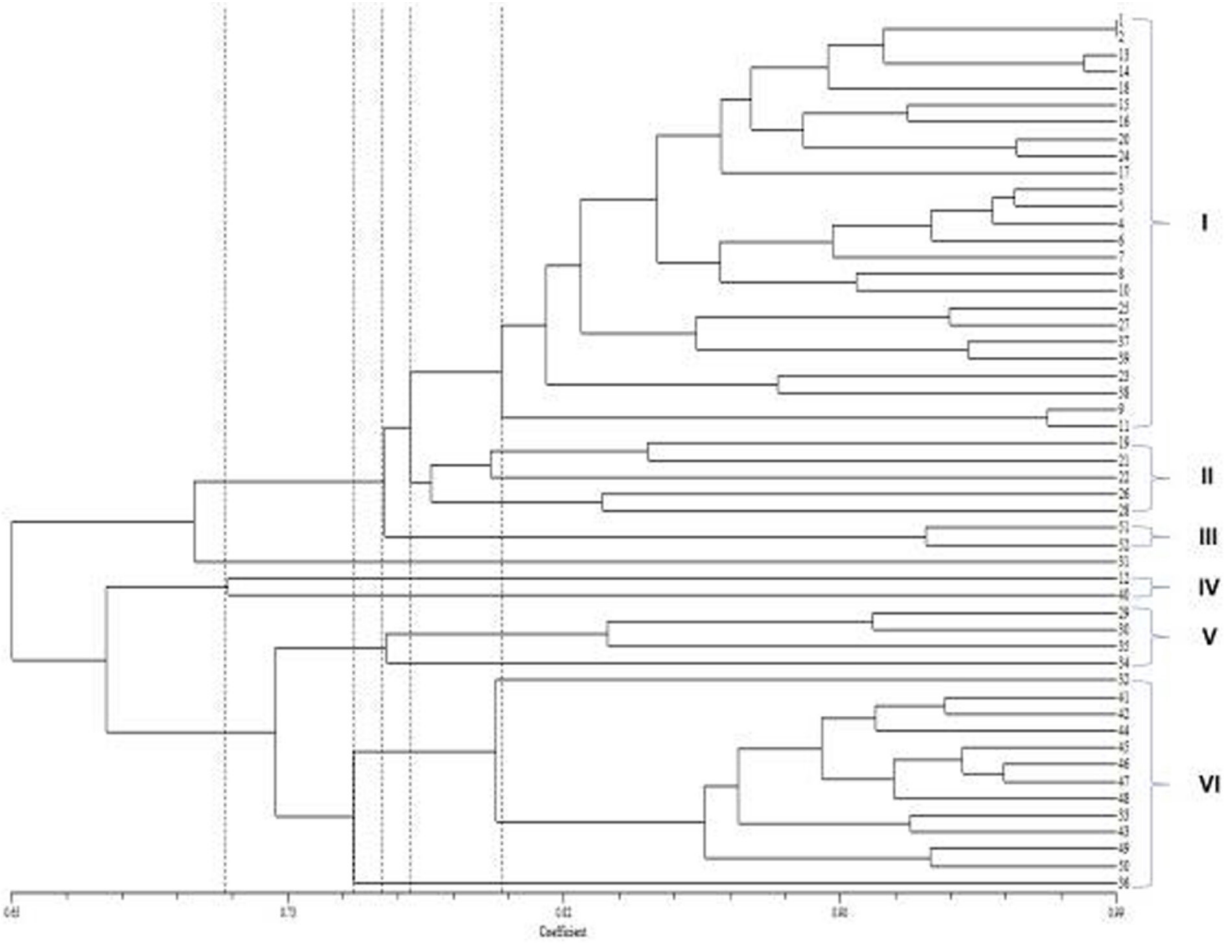

Fig. 2. Dendrogram among 52 citrus accessions based on 4 ISSR and 3 SSR markers

Subcluster B (cluster 1) had 7 accession members, all categorized as Mandarin type. While subcluster C had 4, subcluster D and E had each 2 accession members with different citrus types. Similar morphological features of large fruit size supported the grouped accessions in subcluster B. All accession members of subcluster C tended to have thicker fruit skins than typical mandarin. While subcluster D had small fruit sizes, and subcluster E contained members with obloid fruit shapes. The sample fruit performance of each subcluster is presented in Fig. 5, Fig. 6, Fig. 7 and Fig. 8.

Fig. 2 shows that the constructed dendogram also confirmed 5 other clusters of the studied citrus accessions, i.e., II – V. Cluster II consisted of 5 accessions. At the same time, Cluster III and IV had 2 accession members each. Cluster V comprised 4 accessions, and Cluster 6 had 13 accession members. One accession (code 33, pumelo Jeruk Kelapa) did not belong to any clusters. Unfortunately, this accession has not been characterized morphologically.

The accession grouping in every cluster based on ISSR and SSR markers is related to the common character of the members (Hassanzadeh Khankahdani et al., 2018; Kashyap et al., 2021; Shahsavar et al., 2007; Uzun et al., 2010). Members of Cluster II are naturally occurring hybrid genotypes. Cluster III consisted of 2 orange accessions originally grown in different countries (France and Indonesia). Cluster IV members were connected to a common ancestor, *Citrus aurantifolia*, as reflected in Cluster V from *Citrus limonia*. Cluster VI was designated for pumelo type since it contained members of *Citrus maxima*. The fruit performance of several accession members is presented in Fig. 9, Fig. 10, and Fig. 11.

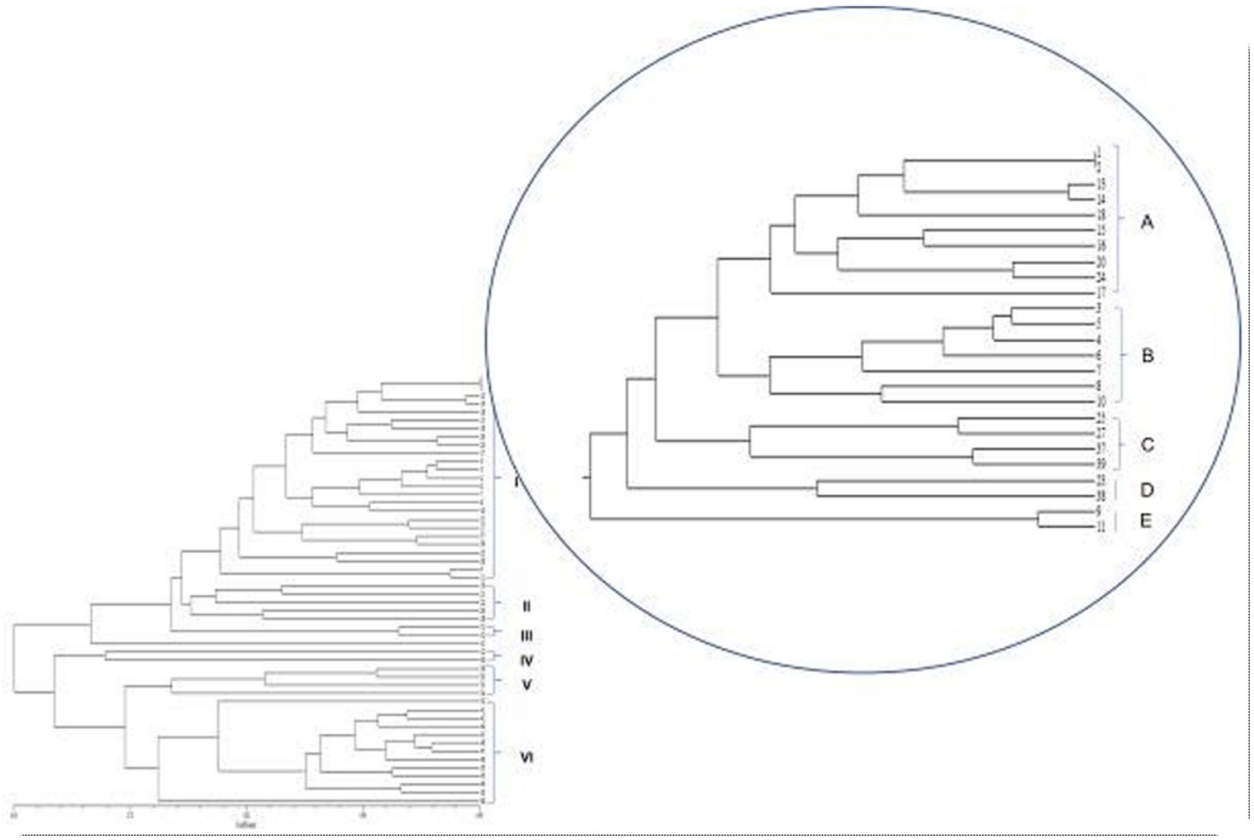

Fig. 3. Dendogram of 5 subclusters derived from cluster 1 mandarin type group

Fig. 4. Fruit performace of mandarin (a) Batu (KBO), (b) Batu 55, (c) tangerine Kintamani and (d) tangerine Madu

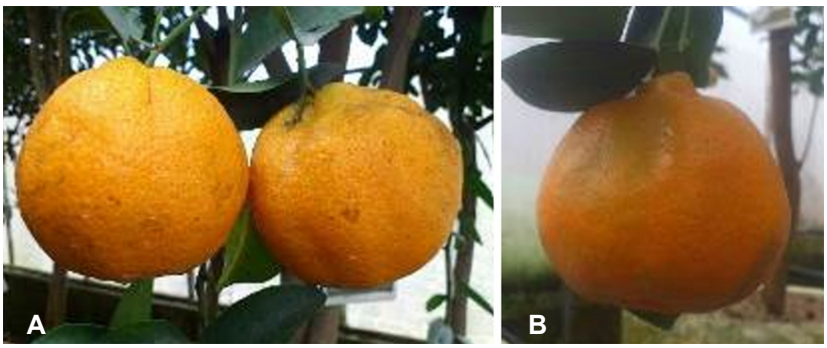

Fig. 5. Sample fruit performace of subcluster B members; mandarin (a) Kacang Singkarak and (b) Pulung

Fig. 6. Sample fruit performace of subcluster C members; mandarin (a) Tankan and (b) Topo Putih

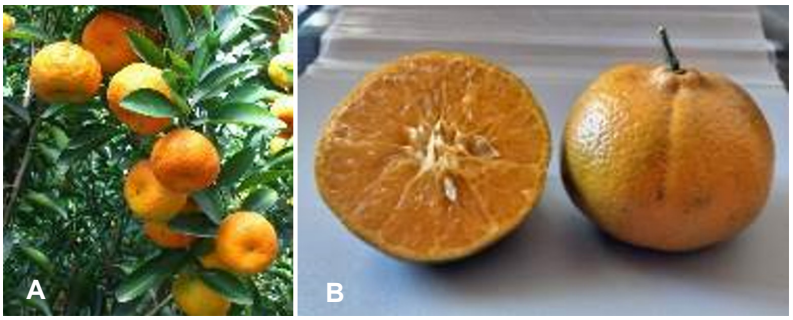

Fig. 7. Sample fruit performace of subcluster D members; mandarin (a) Banten and (b) Kalele Aceh

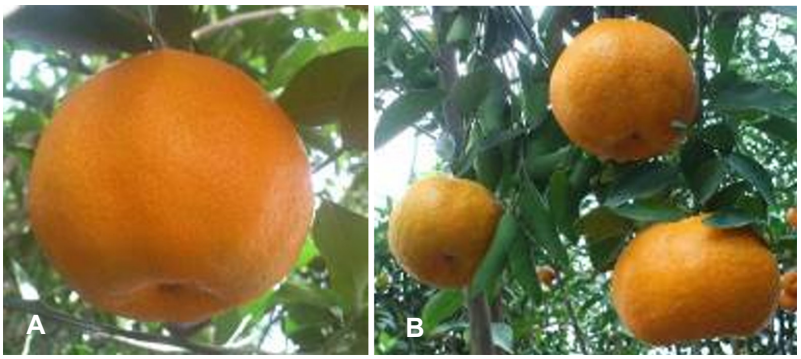

Fig. 8. Sample fruit performace of subcluster D members; mandarin (a) Kisar and (b) Selayar

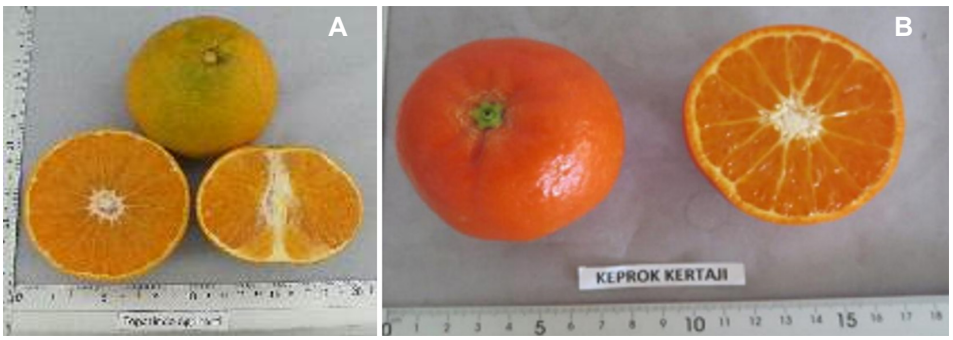

Fig. 9. Sample fruit performace of Cluster II members; mandarin (a) Topazindo and (b) Kertaji

Fig. 10. Sample fruit performace of Cluster IV members; (a) Kasturi and (b) Lime Borneo

Fig. 11. Sample fruit performace of Cluster VI members; pumelo (a) Pamindo and (b) MTR 19

This study's clustering results based on molecular markers (ISSR and SSR) are also useful for citrus breeding programs in Indonesia and other countries. In many citrus species, conventional breeding methods like mutagenesis, inter- and intraspecific crossings, and clonal selection have resulted in several new varieties (Salonia et al., 2020). Interspecific crossings have been used in citrus to create new varieties with improved traits. An example of interspecific crosses is Citrange, a hybrid between the sweet orange and the trifoliate orange that is cold hardy and used as a rootstock for other citrus trees (Wu et al., 2018), and Tangelo, a hybrid between the orange tangerine and grapefruit, which are sweeter than grapefruit and have a thinner skin (Cuenca et al., 2018). Interspecific crosses by protoplast fusion have also been performed to create seedless citrus cultivars (Sa'adah et al., 2022). Technological advances have made it possible to carry out plant breeding on cultivars with large genetic distances.

CONCLUSION

The molecular analysis of citrus accessions based on 4 ISSR primers and 3 SSR primers revealed that 52 citrus genotypes were grouped into 6 major clusters based on genetic similarity, with a similarity index ranging from 65% to 99%. Cluster I had the largest 25 members, while Cluster II, III, IV, V, and VI had 5, 2, 2, 4, and 13 accession members, respectively. One accession (between clusters III and IV) did not belong to any clusters. Members of Cluster I were the majority in Mandarin type with spheroids fruit shape with truncate on the fruit top. Cluster II contained citrus accessions from naturally occurring hybrids. Two orange accession members of Cluster III were originally grown in different climates. Cluster IV and V members were connected to a common ancestor, *Citrus aurantifolia* and *Citrus limonia*.

In comparison, Cluster VI was pumelo group that contained members of *Citrus maxima*. Clustering based on molecular markers (ISSR and SSR) resulting in this study is useful in citrus breeding programs in Indonesia and other countries. The farther the genetic distance between parents, increased the heterosis effect in their progeny.

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