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Application of RAPD Molecular Technique to Genetic Diversity Assessment of Citrus in South Sulawesi (Indonesia)

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Abstract*— Indonesia is rich in germplasm resources. A province in Indonesia, namely South Sulawesi is one of the centers for citrus development. The results of the exploration showed that there were various cultivated citrus namely mandarin orange cultivar selayar (seeded selayar, selayar-selayar, JC-selayar), mandarin orange cultivar batu, JC lime (mandarin lime), pummelo pangkep (cultivar pangkep merah, pangkep putih, pangkep golla-golla), tangerine, santang madu, dekopon, lime, and kaffir lime. This study aims to analyze the genetic diversity and similarity among 13 citrus cultivars cultivated in Pangkep, Sidrap, Bantaeng, Malangke, and Selayar Islands, South Sulawesi Province, Indonesia based on five RAPD primers (OPA-05, OPA-09, OPA-17, OPC-09, and OPC-17). The results showed that RAPD primers could be used to characterize the genetic diversity and similarity of 13 citrus cultivars in South Sulawesi. One informative RAPD primer based on its PIC value was OPC-09. The results of the genetic similarity analysis presented in the form of a dendrogram resulted in two main groups. The first cluster consisted of mandarin orange cultivar selayar (seeded selayar, selayar-selayar, JC-selayar), JC lime, mandarin orange cultivar batu, santang madu, and pummelo pangkep (cultivar pangkep merah, pangkep putih, pangkep golla-golla). The second cluster consisted of mandarin orange cultivar selayar (selayar-selayar, JC-selayar), santang madu, tangerine, mandarin orange cultivar batu, dekopon, lime, and kaffir lime. The clusters that have the most distant genetic relationship are cluster A with cluster B, with a genetic similarity of 62%. Meanwhile, the clusters that have the closest genetic relationship are cluster I and II with 79% genetic similarity.

***Keywords*— citrus; South Sulawesi; genetic relationship, genetic diversity, RAPD**

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I. INTRODUCTION

Indonesia is a tropical country and a global megadiverse country consisting of 17,000 islands with various types of habitats and a very complex geological history [1]-[2]. One of Indonesia's leading horticultural commodities is fruit. Indonesia has local superior citrus species and varieties that are spread throughout the archipelago. Citrus is one of the world's most popular and most important fruit crops in terms of global production [3], which has high economic value [4]-[5]. Citrus fruits belong to family Rutaceae, subfamily Aurantioideae, and can be cultivated in subtropical and tropical areas [6]-[8].

Citrus fruits have the potential to be developed to support food security, especially to meet the nutritional needs of the wider community so that it is in line with a healthy lifestyle (by getting back to nature) and so that the consumption of citrus fruits increases along with the population that continues to increase from year to year [9]-[10]. It is a source of vitamin C and minerals [9], phenolic compounds, flavonoids, folic acid, potassium, and pectin, and good source of antioxidant [10]-[14].

There are wide variations in citrus in terms of, for example, fruit shape, quality, embryo types, inflorescence, tree growth and habit, and adaptability [15]. Very high genetic diversity of citrus can be demonstrated by the high number of species and hybrid taxonomic units [16]. However, currently, the genus Citrus is only defined by two different classification systems, namely, Tanaka's, with 156 species, and Swingle's, with only 16 species [17], and seven species of which have been widely cultivated in Indonesia and become commercial citrus species [18]. Citrus fruits that are developed in almost every province in Indonesia are tangerine, mandarin orange, pummelo, sweet orange, lime,

lemon, and kaffir lime [19]. Hybridization, mutation, and phenotypic diversity make the identification and classification of citrus fruits difficult [20]. The results of the exploration of citrus species show that Indonesia, including Sulawesi, is rich in diversity of citrus. South Sulawesi is one of the centers of citrus fruit development. Citrus fruits cultivated in South Sulawesi are mandarin orange (Selayar, Bantaeng), JC lime or mandarin lime (Selayar), orange cultivar Batu (Bantaeng), tangerine (North Luwu), lime and kaffir limes (Sidrap), pummelo (Pangkep), orange cultivar Santang Madu (Bantaeng, North Luwu), and dekopon orange (North Luwu).

Characterization of the various types of citrus fruits is needed as one of the first steps to guarantee the characteristics of citrus fruit varieties. Information on plant diversity is needed in the determination genetic relationships, germplasm characterization, breeding programs, taxonomy, and registration of new cultivars [6][21]. The more available this information is, the easier it is to determine the genetic position or relationship among varieties that can be used as the basis for plant selection.

Diversity can be studied using morphological, physiological, anatomical, palinological, cytological, biochemical, embryological, and molecular characteristics [22]. Morphological characters are most often used in the identification process because they are easy to observe. However, morphological characters tend to be unstable because they are influenced by the environment [6]. Morphological characters are considered still not sufficient to clearly determine a rank in the taxonomic level and thus, it is necessary to complement other methods as a complement to evaluate genetic relationships [23]-[25].

Currently, rapid technological developments encourage many studies of molecular diversity to be carried out. Molecular markers such as RAPD, ISSR, RFLP, SSR, and AFLP have been applied to research on germplasm characterization, genetic diversity, and systematic and phylogenetic analysis [26]. RAPD has the advantage that with a simple procedure, relatively inexpensive price, and a small amount of DNA for analysis, it can produce DNA that is highly polymorphic and representative of the entire genome [27]-[28].

Using the Random Amplified Polymorphism DNA (RAPD) technique, this study was conducted to identify and assess genetic variation in order to map the relationships among citrus cultivars in South Sulawesi. RAPD has been widely applied to citrus plants, among others, for the analysis of the genetic diversity of citrus germplasm [29]-[30] and the genetic characterization of *Citrus maxima* (Burm.) Merrill. F [31]. In Indonesia, RAPD has also been widely applied to analyze the genetic diversity of oranges from East Java, Central Java, North Maluku, Southeast Maluku, and East Kalimantan [9][32], tangerines from Pontianak, Medan, Banjar, Kintamani, Ponorogo, Jember, and Mamuju [33], pummelos from Aceh, East Java, West Java, Central Java, Yogyakarta, East Nusa Tenggara and South Sulawesi [34]-[38], grapefruit [39], lemon from Ternate, North Maluku [40], and some citrus rootstock verieties such as Japansche citroen (JC), citrumelo, and *kanci* orange [41]. In this study, an evaluation of genetic diversity at several citrus plantation centers in South Sulawesi was conducted using the RAPD technique.

II. MATERIAL AND METHOD

A. Sampling

This study was conducted from April to September 2021. Citrus leaf samples were collected from 13 cultivars (Table 1) with the condition that the plants were biologically healthy and growing in citrus growing regions in Pangkep Regency, Sidrap Regency, Bantaeng Regency, North Luwu Regency, North Luwu Regency, and Selayar Islands Regency. Sampling was done by taking 5 young leaves from each of 10 citrus plant cultivars using purposive random sampling method. The leaf samples were then put into a cool box containing ice gel and stored in a freezer until the DNA isolation process was carried out.

TABLE I
CITRUS CULTIVARS AND THEIR REGIONS OF ORIGIN

No.	Cultivars	Sample Code	Origin
1.	Seeded selayar *Citrus reticulata*	S	Bontomatene, Selayar
2.	Selayar-selayar *Citrus reticulata*	SS	Bontomatene, Selayar Bisappu, Bantaeng
3.	JC-selayar *Citrus reticulata*	JS	Bontomatene, Selayar
4.	JC (Japansche Citroen) *Citrus limonia*	JC	Bontomatene, Selayar
5.	Pangkep merah *Citrus maxima*	M	Padang lampe, Pangkep
6.	Pangkep putih *Citrus maxima*	P	Padang lampe, Pangkep
7.	Pangkep golla-golla *Citrus maxima*	G	Padang lampe, Pangkep
8.	Mandarin orange cv. Batu *Citrus reticulata*	B	Bisappu, Bantaeng
9.	Santang madu *Citrus reticulata*	SM, BM	Malangke Barat, Luwu Utara
10.	Tangerine *Citrus nobilis*	JSi, MSI	Malangke Barat, Luwu Utara Bisappu, Bantaeng
11.	Lime *Citrus auratifolia*	N	Pitu Riase, Sidrap
12.	Kaffir lime *Citrus hystrix*	NN	Pitu Riase, Sidrap
13.	Dekopon *Citrus reticulata* Shiranui	D	Malangke Barat, Luwu Utara

B. DNA Isolation and PCR

DNA isolation was completed using the Genomic DNA Mini Kit (Geneaid) procedure. The DNA quality was checked by electrophoresis while the DNA quantity test was performed using the Invitrogen™ Qubit™ 3.0 Fluorometer (Thermo Fisher Scientific). The amplification process was conducted using KAPA2G Fast ReadyMix (KAPA Biosystems). The analysis process was done at the

Laboratory of Biotechnology and Tree Breeding, Faculty of Forestry, Hasanuddin University.

DNA amplification employed RAPD markers (Table 2) with 10.5 μl of PCR reaction composition mix (KAPA Mix 6.25 μl; primer 1.25 μl; ddH_2O 2 μl; DNA template 3 μl). The steps of PCR included pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 30 seconds, 35 cycles of annealing (adjusted the temperature of the primers) for 50 seconds, elongation at 72°C for 1 minute, and post-extension at 72°C for 5 minutes. The qualitative test was performed using 1% agarose gel electrophoresis with TAE 1X for 60 minutes at 120 volts and imaging on Gel DOC UV-transilluminator.

C. DNA Isolation and PCR

The PCR results were converted into binary data. The files of DNA bands from the RAPD analysis were scored based on the presence or absence of amplification results. A score of 1 indicates the DNA band that appears and a score of 0 is for the DNA band that does not appear in each primer. The binary data were then converted into a similarity matrix based on the SM (Simple Matching) coefficient. The ilarity value is used for grouping analysis using the SAHN (Sequential Agglomerative Hierarchical Nested Cluster Analysis) function with the UPGMA (Unweighted Pair Group Methods with Arithmetic Average) in the NTSYSpc 2.10e program [42]-[43]. The value of heterozygosity was calculated using the following formula [44].

$$qi = \left(\frac{\text{individuals that do not have strand}}{\text{number of individuals observed}}\right)^{1/2}$$

$$pi = 1 - qi$$

$$He = 1 - Pi^2 - qi^2$$

Annotation:
qi = frequency of null allele
pi = frequency of dominant allele

The value of polymorphic information content (PIC) was calculated using the following formula [45]:

$$PIC = 2\,fi\,(1-fi)$$

Annotation:
Fi = Frequency of allele

The PIC value is standardized for evaluating genetic markers based on DNA bands of PCR amplification results. PIC values are divided into 3 classes: > 0.5 = highly informative; 0.25 > 0.5 = moderately informative; and PIC < 0.25 = slightly informative [46].

III. RESULTS AND DISCUSSION

The RAPD molecular technique was applied to characterize and determine genetic diversity and genetic similarity (phylogenetics) [47] in 12 citrus cultivars in South Sulawesi. RAPD is widely used on whole genomic DNA and random primers to assess genetic diversity among plants [48]-[50]. DNA isolates were obtained through the extraction process from 174 samples of orange leaves using Kit (Genid). The DNA isolates were then amplified using 5 RAPD primers, namely OPA-05, OPA-09, OPA-17, OPC-09, and OPC-17 (Table 1).

The banding pattern of the PCR results was then analyzed through the electrophoresis process. DNA amplification of citrus fruits resulted in a total of 23 bands in 174 samples, ere all of these bands are polymorphic bands so that the percentage of polymorphic bands produced is 100%, meaning that the DNA bands formed are not monomorphic bands (bands that are present in all samples). Thus, in this study, it was shown that the 13 citrus cultivars tested had high genetic diversity. As stated by [51] that the presence of high polymorphic bands means that the genetic diversity of the analyzed species is high.

Each primer produced a different pattern of DNA bands with an amplicon range of 100-1100 bp. The amplicon range of the OPA-05 primer is 400-1100 bp, the OPA-09 primer has an amplicon range of 200-1100, the OPA-17 primer has a range of 100-1100 bp, OPC-09 has a range of 300-1000 bp, and the OPC-17 primer has a range of 300-500 bp (Table 2). According to [49], a DNA band that is present or absent among species is called a polymorphic band, while a band is called monomorphic band if it appears in all analyzed species. Polymorphism is the result of changes in nucleotide bases that alter the primary binding or insertion or deletion site in the amplification region [52].

The difference in polymorphism is caused by the difference in the amount of genetic variation that exists ween different accessions [53]. According to [54], polymorphic information generated by DNA markers is eded in plant breeding programs to improve plant quality. One of the most important features of the RAPD molecular technique is the ability to detect high levels of polymorphism and this feature has been fulfilled in this study. However, some samples of citrus fruits d not produce bands on certain primers. This is probably due to the absence of homologous primary sequences in the genome. The number of DNA amplification bands depends on the attachment of the homolog to the DNA template [55]-[57]. Other possible causes are technical errors, amplification processes, and inappropriate temperatures of certain primers for certain samples [56]. Also influenced by several factors including PCR conditions, quality/ quantity of DNA, concentration of PCR components [58]. According to [59], the detection of RAPD-based polymorphisms is based on the variation of the annealing primer site in the PCR process. Further analysis regarding primers and samples of certain citrus cultivars needs to be done.

TABLE III
RAPD PRIMERS AND THEIR BASE SEQUENCE, MELTING TEMPERATURE, ANNEALING TEMPERATURE, NUMBER OF BANDS, NUMBER OF POLYMORPHIC BANDS, PERCENTAGE POLYMORPHISM, AMPLICON SIZE RANGE, AND PIC VALUE

No.	Primer	Primer Sequences 5'-3'	Tm (°C)	Ta (°C)	No. of bands	No. polymorphic bands	% polymorphism	Amplicon size range (bp)	PIC
1.	OPA-05	AGG GGT CTT G	32.6	35.4	4	4	100	400-1100	0.22
2.	OPA-09	GGG TAA CGC C	37.4	35.6	7	7	100	200-1100	0.33
3.	OPA-17	TCG GCG ATA G	35.7	40.2	7	7	100	100-1100	0.25
4.	OPC-09	GAC CGC TTG T	36.2	35.6	3	3	100	300-1000	0.45
5.	OPC-17	CTC ACC GTC C	37.4	40.2	2	2	100	300-500	0.35

According to [51], the PIC value is an information to detect primers that are capable of producing polymorphic bands in a population. The high level of genetic diversity is influenced by the level of polymorphism of genetic markers used. Thus, the genetic markers that will be used need to be considered carefully. The value of polymorphic information content (PIC) is standardized for evaluating genetic markers based on DNA bands of PCR amplification results. The maximum PIC value for the RAPD marker is 0.5. The PIC values are used to consider which primer is the best in the RAPD marker and reflect the diversity and allele frequency among the samples.

The higher the PIC value, the better the primer is to be used in analyzing genetic variation [51]. Based on the results of the calculation of the PIC value, each primer had a different value. The highest PIC value was discovered in the OPC-09 primer, which is 0.45, and the lowest PIC value was discovered in the OPA-05 primer. According to [60], PIC value is divided into three classes: PIC > 0.5 = highly informative; 0.25 > PIC > 0.5 = moderately informative; and PIC < 0.25 = slightly informative. Based on this, the PIC values of 0PA-09, OPC-09, and OPC-17 primers were categorized as moderately informative and those of OPA-05 and OPA-17 primers were categorized as slightly informative. According to [61], PIC values below 0.25 are not recommended in genetic studies. The PIC value of each primer can be seen in Table 2.

The RAPD molecular technique using DNA as a template showed a pattern of bands that vary in size and number. The total number of DNA bands is used for cluster analysis where the banding pattern obtained in each species is a score based on the presence or absence of each DNA band that appears. Each banding pattern of DNA amplification products is an informative profile or character to display the construction of genetic diversity and genetic relationships (similarity) between samples. DNA analysis with RAPD marker OPC-09 is shown in Figure 1.

Heterozygosity is one of the parameters that is used to measure the level of genetic diversity in a population based on allele frequency at each locus [62]. According to [63]-[64], heterozygosity (He) is a fundamental measure of genetic diversity in a population that explains the proportion of heterozygous genotypes under Hardy-Weinberg equilibrium. High heterozygosity in a population means that the genetic variability in the population is high, whereas low heterozygosity means that the genetic variability is also low [65].

TABLE IIIII
HETEROZYGOSITY VALUES

No.	Cultivar	Sample code	Heterozigosity
1.	Seeded selayar	S	0.33
2.	Selayar-selayar	SS	0.38
3.	JC-selayar	JS	0.39
4.	JC	JC	0.36
5.	Pangkep merah	M	0.31
6.	Pangkep putih	P	0.28
7.	Pangkep golla-golla	G	0.31
8.	Batu	B	0.24
9.	Santang madu	SM	0.16
10.	Dekopon	JSI	0.22
11.	Tangerine	MSI	0.20
12.	Lime	N	0.27
13.	Lime kaffir	NN	0.26
Average			**0.29**

Heterozygosity is one of the most important resources in breeding programs because it is associated with genetic variability [66]. According to [67], dominant markers such as RAPD can only produce two alleles at each locus and therefore, the maximum heterozygosity value obtained is 0.5. [68] states that the value of genetic diversity (He) 0.2349 ≤ is categorized as high. The heterozygosity value is obtained from the results of manual DNA visualization scoring which is then tabulated into the heterozygosity (He) formula. According to [69], each band that appears on the gel is a specific allele. The allele is then translated into binary data which is assigned a value based on the presence or absence of an allele. A value of 1 will be assigned if there is an allele, and a value of 0 will be assigned if there is no allele. The He value of each citrus population is quite diverse, ranging from 0.16-0.39. The average He value of the citrus population in South Sulawesi is 0.29. Based on the results of DNA analysis in this study, it can be said that the genetic diversity of the citrus population is high.

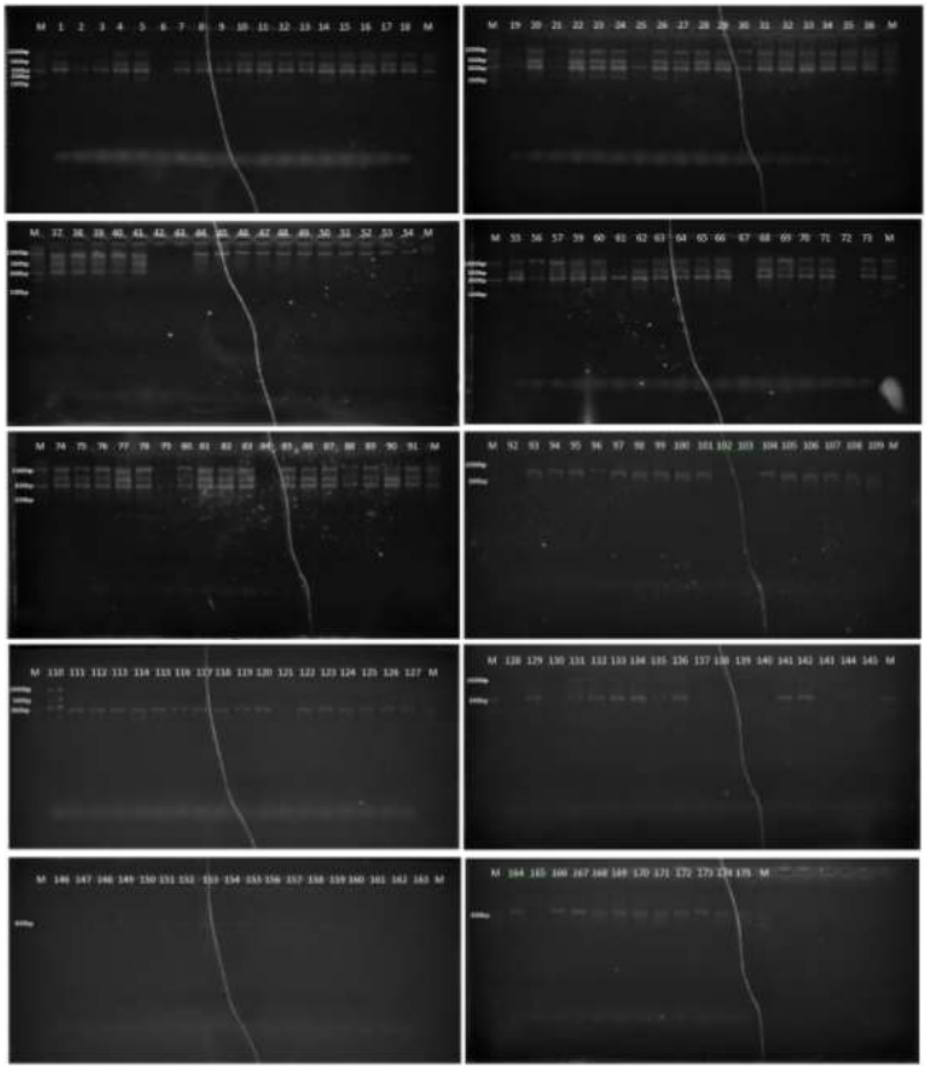

Fig. 1 The resulting RAPD profiles for 175 citrus cultivars on OPC-09 primers. M= 50 bp marker; lanes 1-18 represent the S-SS-coded samples, lanes 19-36 represent the SS-JS-M-coded samples, lanes 37-54 represent the M-P-G-coded samples, lanes 55-73 represent the G-B-coded samples, lanes 74 -91 represents the B-JS-coded sample, lanes 92-109 represents the JS-JSI-coded sample, lanes 110-127 represents the JSI-N-NN coded sample, lanes 128-145 represents the MSI-SM-coded samples, lanes 146-163 represents the SM-coded samples, lanes 164-175 represent the SM-coded samples

The dendrogram that was obtained based on the RAPD banding pattern of the tested citrus cultivars is presented in Figure 2 below. The [13]e above shows that at the level of similarity of 62%, 2 main clusters, namely cl[14]r A and cluster B, were obtained. Cluster A consisted of sub-clusters I, II, and III. Sub-cluster I consisted of seeded mandarin orange cultivar selayar, mandarin orange cultivar selayar-Selayar, and mandarin orange cultivar JC-selayar. Sub-cluster II consisted of JC lime, mandarin orange cultivar batu, and orange cultivar santang madu while sub-cluster III consisted of pummelo cultivar pangkep merah, pummelo cultivar pangkep putih, and pummelo cultivar pangkep golla-golla cultivars. Meanwhile, cluster B consisted of sub-clusters IV, V, VI, VII and VIII with the number of individuals in each cluster varying. Cluster IV consisted of orange cultivar santang madu and tangerine, cluster V consisted of dekopon orange and tangerine, cluster VI consisted of kaffir lime, cluster VII consisted of tangerine, and cluster VIII consisted of lime. The clusters that have the most distant genetic relationship are cluster A and cluster B with a similarity of 62%. Meanwhil[34]e clusters that have the closest genetic relationship are cluster I and cluster II with 79% similarity.

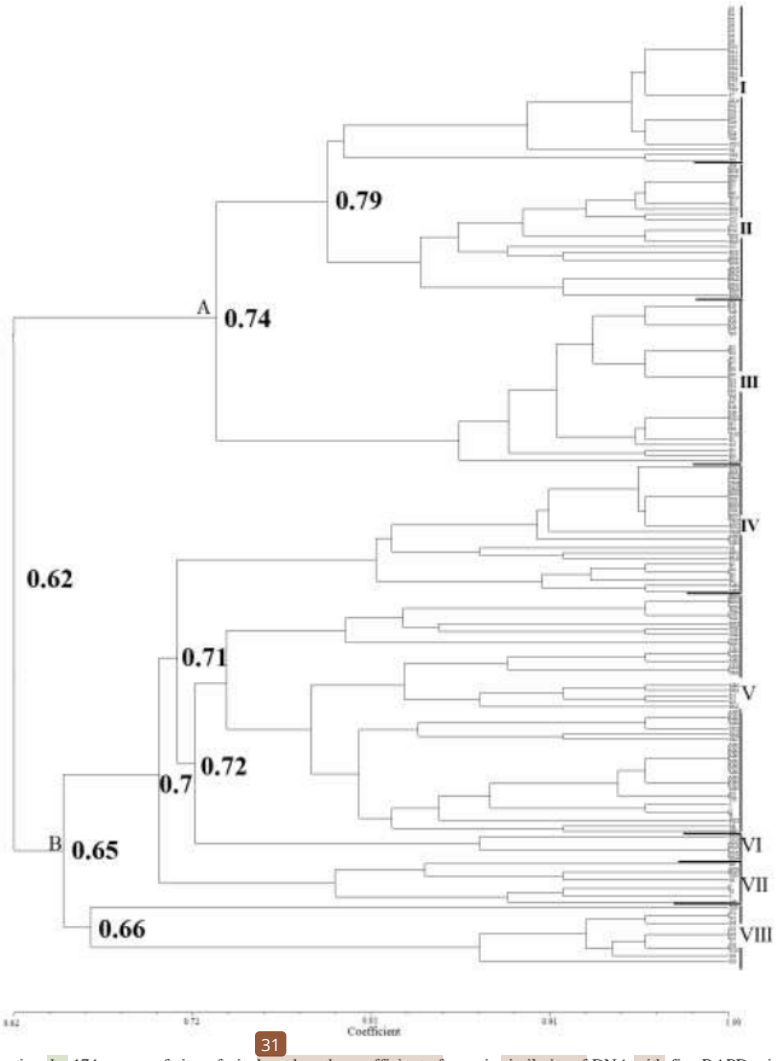

Fig. 2 Dendrogram showing the 174 groups of citrus fruits based on the coefficient of genetic similarity of DNA with five RAPD primers

The data above shows that at the level of similarity of 62%, 2 main clusters, namely cluster A and cluster B, were obtained. Cluster A consisted of sub-clusters I, II, and III. Sub-cluster I consisted of seeded mandarin orange cultivar selayar, mandarin orange cultivar selayar-selayar, and mandarin orange cultivar JC-selayar. Sub-cluster II consisted of JC lime, mandarin orange cultivar batu, and orange cultivar santang madu while sub-cluster III consisted of pummelo cultivar pangkep merah, pangkep putih, and pangkep golla-golla cultivars. Meanwhile, cluster B consisted of sub-clusters IV, V, VI, VII and VIII with the number of individuals in each cluster varying. Cluster IV consisted of orange cultivar santang madu and tangerine, cluster V consisted of dekopon orange and tangerine, cluster VI consisted of kaffir lime, cluster VII consisted of tangerine, and cluster VIII consisted of lime. The clusters that have the most distant genetic relationship are cluster A and cluster B with a similarity of 62%. Meanwhile, the clusters that have the closest genetic relationship are cluster I and cluster II with 79% similarity.

TABLE IVII
CLUSTERS AND CODES OF CITRUS FRUIT SAMPLES

No.	Sub-cluster	Sample code
1	I	S1, S2, S3, S4, S5, S8, S9, S10, SS1, SS2, SS3, SS4, SS5, SS6, SS7, SS8, S7, SS10, JS2, JS3, JS5, JS6, JS7, JS8, JS9, JS10, S6, SS9, JS1
2	II	JS4, BSS9, BSS10, B6, B7, B8, B10, B2, BSS8, JC2, JC5, JC3, JC4, BSS1, BSS3, JC1, BSS4, BSS5, BSS6, BM1, BM2, BM5, BM3, BM4, BJS1
3	III	M1, M5, M6, M2, M3, M4, G6, P2, P4, P5, P7, P8, P9, G1, G2, G3, G4, M7, M8, M9, M10, G7,

No.	Sub-cluster	Sample code
		G9, G10, P1, G5, P3, P6, P10
4	IV	BSS2, BSS7, SM10, SM4, SM2, BJS10, JSI3, BJS6, JSI1, BJS7, SM5, BJS2, SM8, MSI3, MSI5, SS, PP2, PM1, B1, B4, B5, B3, MSI4, MSI6
5	V	BJS3, BJS5, BJS4, JSI4, JSI5, BJS8, JSI9, JSI6, JSI7, MSI1, MSI2, MSI7, SM6, SM9, MM9, PG2, D1, D2, PM2, MSI8, MSI10, MSI9, SM1, SM3, SM7, MM1, MM10, MM7, MM5, MM8, MM2, MM3, MM4, MM6, D3, JSS1, SS, SB, PG1, SB, PP1
6	VI	NN1, NN2, NN5, NN3, NN4
7	VII	B9, BJS9, JSI2, SS3, MM5, JSI4, JS6, M7, N8, O
8	VIII	JSI10, N1, N2, N4, N5, N6, N7, N8, N10, N9, N3

IV. Conclusion

By applying the RAPD molecular technique to 12 citrus fruit cultivars in South Sulawesi, it was found that the diversity of citrus fruits in South Sulawesi is high, making it possible for plant breeding activities to be conducted. Five primers (OPA-05, OPA-09, OPA-17, OPC-09, and OPC-17) that were used succeeded in producing polymorphic bands and were suitable to be used as markers in detecting genetic diversity of citrus fruits where OPC-09 primer was the most effective one. A total of 12 citrus cultivars tested were grouped into 2 main clusters with a genetic distance of 62%. It is necessary to do further analysis using larger amount of primers to complete the genetic information of citrus fruits in South Sulawesi.

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