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RAPD primer screening as a preliminary study to analyze the genetic diversity of *Citrus* spp. in South Sulawesi, Indonesia

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Abstract. The typical citrus germplasm collection in South Sulawesi has not been thoroughly characterized, especially in several citrus development centers, which have begun to be promoted again after the decline in productivity due to CVPD infection. The study of citrus diversity is very important to support future citrus breeding programs. Random amplified polymorphic DNA (RAPD) has been widely used for the analysis of genetic diversity among species in populations. In this study, 23 RAPD primers were used on Citrus cultivated in Selayar and Pangkep Regencies, which are citrus development areas in South Sulawesi. A total of 19 primers (OPA-05, OPA-09, OPA-17, OPC-09, OPC-17, OPE-04, OPH-04, OPH-15, OPN-14, OPN-16, OPR-08, OPR-20, OPW-06, OPW-09, OPX-07, OPX-11, OPX-17, UBC-18, and UBC-51) can form polymorphic bands in randomly selected DNA samples. Monomorphic bands were formed by OPA-12 and OPD-07 primer in 12 samples. The primers OPX-13 and OPX-16 produced unclear bands. These 19 primers can be used to amplify DNA and determine the genetic diversity of Citrus in further analysis.

1. Introduction

Citrus is one of the world's fruit crops that have high economic value [1,2], belonging to the Subfamily Aurantioideae, Familia Rutaceae, which can be cultivated in subtropical to tropical areas [3,4]. Citrus contains nutrients and phytochemicals that are beneficial for health. Citrus contains vitamin C, vitamins B, potassium, phosphorus, and other elements [5].

Citrus development centers in South Sulawesi include Pangkep and Selayar Regencies. Pamelo citrus origin from Pangkep has distinctive characteristics, namely large fruit with yellowish-green color, 1-2.5 kg weight, fresh fruit taste, and longer shelf life of up to four months [6]. Citrus origin from Selayar is very synonymous with a distinctive taste, namely fresh sweet with a sour taste and has a fragrant aroma as well as a dense flesh texture and skin character that easily separates from the inside of the orange. In addition to being a superior commodity in the local area, these oranges also have many health benefits.

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Identification of citrus genetic diversity is needed to determine the special characters that will later be used in its development and cultivation [7]. Molecular techniques have been widely used in citrus genetic diversity. One of the most widely used molecular markers in genetic diversity analysis is RAPD [3,7–10]. Several conditions are required to obtain a suitable RAPD marker for a species. Primers can not only amplify DNA samples, but also the resulting bands must be polymorphic and clear. For this reason, RAPD primer screening is needed to obtain suitable markers. This study aims to determine the suitable RAPD primer and annealing temperature to amplify citrus DNA. Primers that produce clear and polymorphic bands will be used later in the analysis of citrus genetic diversity.

2. Materials and methods

2.1. Plant materials

The plant materials used in this study were 50 young leaves from Citrus that were collected from 2 regencies in South Sulawesi, namely Pangkep and Selayar. The samples consisted of 13 samples from Bontomatene, 17 samples from Bontoharu, and 20 samples from Ma'rang (Table 1). Young leaves samples taken were put into envelopes and coded, and then put in a coolbox containing ice gel.

Table 1. Sample used in the RAPD primer screening.

Number of sample	Sample	Origin	Sample code
10	Selayar Biji	Bontomatene	S1
10	Selayar-Selayar	Bontomatene	SS1
10	Selayar-Selayar	Bontomatene	SS3
10	JC-Selayar	Bontoharu	PP4
10	Selayar Biji	Bontoharu	PS1
10	Selayar-Selayar	Bontoharu	PS6
10	JS-Selayar	Bontoharu	PP8
10	Selayar Biji	Bontoharu	SB
10	Pangkep Merah	Ma’rang	GM
10	Pangkep Gula-Gula	Ma’rang	GG
10	Pangkep Gula-Gula	Ma’rang	PG1
10	Pangkep Putih	Ma’rang	GBR1

2.2. DNA isolation

DNA isolation was carried out according to the Genomic DNA Mini Kit (Geneaid) procedure. The leaf sample was weighed as much as 0.1 g, added 400 µl of buffer GP1 then vortexed. They incubated in a water bath at 60°C for 30 minutes (every 10 minutes, the mixture was inverted). A total of 100 µl of GP2 buffer was added, then vortexed and incubated on ice for 10 minutes and centrifuged 1,000 x g for 5 minutes. The filter column was placed in a 2 ml tube, the supernatant was transferred to the filter column and then centrifuged 10,000 x g for 1 minute, and the column was discarded. The solution was added 1.5X buffer GP3 (± 700 l) and immediately inverted. The GD column was placed in a 2 ml tube, all solutions were pipetted into the GD column, then centrifuged for 2 minutes, and the elution was heated. In the GD column, 400 µl of W1 buffer was added and then centrifuged 10,000 x g for 1 minute. The solution was discarded, added 600 µl of wash buffer, and centrifuged 10,000 x g for 1 minute. The solution was discarded in the tube. The GD column was centrifuged 10,000 x g for 3 minutes, and the GD column was transferred to a 1.5 ml tube, then added 100 l of elution buffer, which had been heated right in the center of the column left at room temperature for 5-10 minutes. Then centrifuged 10,000 g for 1 minute. The GD column was discarded, and the solution obtained was DNA solution, then 3 µl of RNAse was added. The DNA solution was stored as stock in a freezer at -20°C.

2.3. RAPD primer screening
There were 23 RAPD primers used in this screening (Table 2). Primer screening was carried out by making 12 PCR reactions using 12 randomly selected samples from 50 samples which can be seen in table 1. Each PCR reaction consisted of 3 µl DNA, 1.25 µl primer, 6.25 µl PCR mix (KAPA 2G Fast), and 3 µl ddH_2O. The DNA amplification process was carried out with the procedure starting from initial denaturation at 95ºC for 3 minutes, first cycle denaturation at 95ºC for 30 seconds, primer annealing (temperature adjusted to each primer pair) for 50 seconds, primer elongation at 72º C for 60 seconds, final elongation 72º C for 5 minutes. The denaturation process was repeated 35 cycles. The PCR product was then electrophoresed using 1% agarose in 1X TAE buffer. Electrophoresis was carried out for 60 minutes at a voltage of 120 volts.

2.4. Data analysis
Data were analyzed descriptively by looking at the number of bands produced from each primer.

3. Results and discussion
The screening results of 23 RAPD primers showed that these primers were able to produce amplification products in the sample DNA. Primer screening is carried out to determine the appropriate attachment temperature to select polymorphic primers [11] and is a basic step for molecular studies, especially in the analysis of genetic diversity [12].

Polymorphic and clear bands were produced by 19 primers, namely primer OPA-05, OPA-09, OPA-17, OPC-09, OPC-17, OPE-04, OPH-04, OPH-15, OPN-14, OPN-16, OPR-08, OPR-20, OPW-06, OPW-09, OPX-07, OPX-11, OPX-17, UBC-18, and UBC-51. Monomorphic bands resulted in primer OPA-12 and OPD-07 with the number of bands 1 and 5, respectively. The results of the 23 primer screenings are presented in table 2.

Table 2. RAPD primer and DNA amplification product of Citrus.

Primer	Primer Sequences 5'-3'	Tm (ºC)	Ta (ºC)	Total bands	Polymorphic band	Monomorphic band	Quality of produced band
OPA-05	AGG GGT CTT G	32.6	35.4	12	1	1	Polymorphic and clear band
OPA-09	GGG TAA CGC C	37.4	35.6	12	3	-	Polymorphic and clear band
OPA-12	TCG GCG ATA G	34.0	-	10	-	1	Monomorphic
OPA-17	GAC CGC TTG T	35.7	40.2	9	4	-	Polymorphic and not clear band
OPC-09	CTC ACC GTC C	36.2	35.6	11	4	-	Polymorphic and clear band
OPC-17	TTC CCC CCA G	37.4	40.2	10	4	-	Polymorphic and not clear band
OPD-07	TTG GCA CGG G	40.9	-	12	-	5	Monomorphic
OPE-04	GTG ACA TGC C	33.2	30.4	12	1	3	Polymorphic and clear band
OPH-04	GGA AGT CGC C	37.5	40.3	12	4	1	Polymorphic and clear band
OPH-15	AAT GGC GCA G	37.1	35.4	12	5	1	Polymorphic and clear band
OPN-14	TCG TGC GGG T	43.2	43.8	11	7	-	Polymorphic and clear band
OPN-16	AAG CGA CCT G	35.1	34.5	12	3	1	Polymorphic and clear band
OPR-08	CCA TTC CCC A	33.2	33.8	11	6	-	Polymorphic and

Primer	Primer Sequences 5'-3'	Tm (ºC)	Ta (ºC)	Total bands	Polymorphic band	Monomorphic band	Quality of produced band
							clear band
OPR-20	TCG GCA CGC A	44.5	45.1	12	3	1	Polymorphic and clear band
OPW-06	AGG CCC GAT G	39.3	37.6	12	3	3	Polymorphic and clear band
OPW-09	GTG ACC GAG T	33.9	37.6	12	4	1	Polymorphic and clear band
OPX-07	GAG CGA GGC T	39.5	41.2	12	8	-	Polymorphic and clear band
OPX-11	GGA GCC TCA G	35.4	36.0	12	4	1	Polymorphic and clear band
OPX-13	ACG GGA GCA A	37.5	-	12	-	-	Smear band
OPX-16	CTC TGT TCG G	31.6	-	12	-	-	Smear band
OPX-17	GAC ACG GAC C	36.8	36.2	12	2	2	Polymorphic and clear band
UBC-18	GGG CCG TTT A	35.0	32.3	12	6	-	Polymorphic and clear band
UBC-51	CTA CCC GTG C	36.9	41.3	12	6	-	Polymorphic and clear band

The number of amplified DNA bands ranged from 1-8, with sizes ranging from 150-500 bp. Figure 1A shows the most polymorphic DNA bands produced by primer OPX-07 as many as 8 with clear band quality. According to [9], the polymorphic alleles observed in each primer in each sample differ in size and number, where polymorphic alleles are alleles that can distinguish individuals. Primers that produce clear, bright, and polymorphic bands can be used in further analysis. Polymorphic primers are needed in the analysis of plant genetic diversity that show the diversity of band patterns resulting from the amplification process [12,].

Primers OPA-17 and OPC-17 produced polymorphic bands but with less clear band quality (Figure 1B). Monomorphic bands were produced by primer OPA-12 with the same allele size as the other samples, namely 250 bp (Figure 1C). Primers OPX-13 and OPX-16 produced less clear DNA bands in the form of smears (Figure 1D). Smear bands are caused by the accumulation of several DNA bands of different sizes but not too large so that they overlap each other continuously [13].

Primer OPX-07 produced the most polymorphic bands of 8. This means that the primer has complementary base pairs with genomic DNA so that it has more attachment sites [14]. Primer OPA-05 and OPE-04 produced only one polymorphic band. The number of bands may differ between primers in each sample. This is caused by differences in the primer sequences and DNA samples. Each primary sequence has a specific attachment site in the genome. The more homologous attachment sites of the primers in the sample genome, the more bands will be generated [9,15]. Primers that have more attachment sites produce more amplified bands [16].

In addition to polymorphism, product band quality is also an important factor in primer selection. Primers that produce unclear bands are not used in genetic diversity analysis because unclear bands can misinterpret the data [9,17].

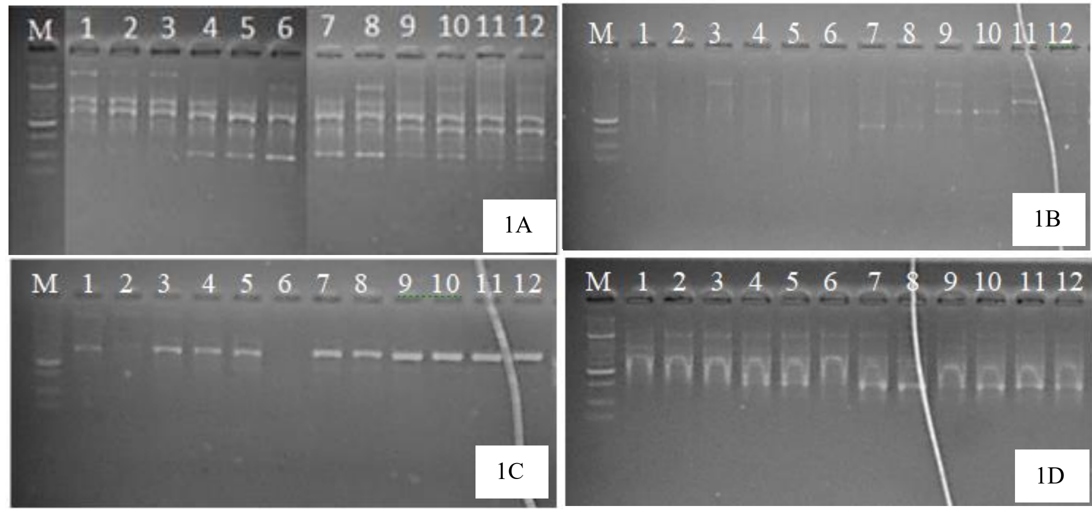

Figure 1. Results of primer screening. 1A) citrus DNA amplification using primer OPX-07; 1B) amplification using primer OPC-17; 1C) amplification using primer OPA-12; 1D) amplification using primer OPX-13. 1=selayar oranges from Bontomatene; 2=selayar oranges from Bontomatene; 3=selayar oranges from Bontomatene; 4=selayar oranges from Bontoharu; 5=selayar oranges from Bontoharu; 6=selayar oranges from Bontoharu; 7=selayar oranges from Bontoharu; 8=selayar oranges from Bontoharu; 9 = pangkep orange from Ma'rang; 10 = orange pangkep from Ma'rang; 11=pangkep oranges from Ma'rang; 12=pangkep oranges from Ma'rang.

4. Conclusion

Primers with polymorphic and clear bands were produced by 19 primers namely OPA-05, OPA-09, OPA-17, OPC-09, OPC-17, OPE-04, OPH-04, OPH-15, OPN-14, OPN-16 , OPR-08, OPR-20, OPW-06, OPW-09, OPX-07, OPX-11, OPX-17, UBC-18, and UBC-51. The annealing temperature of each primer ranged from 30.4-45.1ºC. The selected primers will be used in the analysis of citrus genetic diversity.

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Application of RAPD Molecular Technique to Study the Genetic Variations of Citrus in South Sulawesi, Indonesia

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***Abstract*— Indonesia is a tropical country with superior local citrus species and varieties. Citrus fruits were very useful for cultivation, especially to meet the nutritional needs of the wider community. A province in Indonesia, namely South Sulawesi is one of the centers for citrus development. A preliminary study showed various cultivated citrus in South Sulawesi namely *Mandarin Orange* cultivar *Selayar*, *Mandarin Orange* cultivar *Batu*, *JC* lime, *Pummelo Pangkep*, *tangerine*, *Santang Madu*, *Dekopon*, lime, and *Kaffir* lime. This study aims to evaluate genetic diversity at several citrus plantation centers in South Sulawesi using the RAPD technique. The analysis was carried out using five primers. In this study, RAPD primers could be used to characterize the genetic diversity and similarity of thirteen citrus cultivars in South Sulawesi. One informative RAPD primer based on its PIC value was OPC-09. The results of the genetic similarity analysis are presented in the form of a dendrogram. 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 with the most distant genetic relationship are cluster A with cluster B, with a genetic similarity of 62%. Meanwhile, clusters with the closest genetic relationship are clusters 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 with the second highest level of biodiversity in the world [1] and consists of 16,671 islands that name has been verified as of 2018 [2]. One of Indonesia's leading horticultural commodities is fruit, and Indonesia has local superior citrus species and varieties that are spread throughout the archipelago. Citrus is one of the world's major fruit crops [3], grown in many regions [4], which has high economic value [5]. Citrus fruits belong to the family Rutaceae and subfamily Aurantioideae and can be grown in tropical and sub-tropical climates [6].

Citrus fruits were very useful for cultivation, 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 [7], [8]. It is a source of vitamin C, minerals, phenolic compounds, flavonoids, folic acid, potassium, and pectin, and good sources of antioxidants [7]–[9].

There are differences in citrus in several aspects, such as fruit morphology, quality, embryo, inflorescence, the direction of growth, and adaptability [10], [11]. The diversity of oranges is indicated by the high number of taxonomic units [12]. However, currently in general, the world's citrus classification is still based on the classification system according to Swingle and Tanaka that in the genus Citrus there are sixteen species [13]. Citrus varieties planted in Indonesia are tangerines (75%), mandarin oranges (24%), and other varieties (1%), including grapefruit, orange, lemon, and lime [14]. Citrus fruits that are developed in almost every province in Indonesia are tangerine, mandarin orange, pummelo, sweet orange, lime, lemon, and kaffir lime [15]. The taxonomy of the genus citrus is complex mainly because of sexual compatibility between species and genera [16]. 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 of kinship relationships, breeding programs, and taxonomy [17], [18], [19]. 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, palynological, cytological, biochemical, embryological, and molecular characteristics [20], [21]. Morphological characters are most often used in identification because they are easy to observe. However, morphological characters tend to be unstable because they are influenced by the environment [22]. Morphological characters are still not sufficient to determine a rank in the taxonomic level clearly; thus, it is necessary to complement other methods to evaluate genetic relationships [22]–[24].

Rapid technological developments encourage many molecular diversity studies to be carried out. Molecular markers such as RAPD, RFLP, AFLP, ISSR, and SSR have been used to research germplasm characterization, genetic diversity, and systematic and phylogenetic analysis [25], [26]. Random Amplified Polymorphism DNA (RAPD) has the advantage that with a simple procedure, relatively inexpensive price, and a small amount of DNA for analysis, it can produce highly polymorphic DNA representative of the entire genome [27]–[29]. RAPD has been widely applied to citrus plants, among others, for studying the genetic variations of citrus germplasm [5], [30]. In this study, an evaluation of genetic diversity at several citrus plantation centers in South Sulawesi was conducted using the RAPD technique.

II. Materials 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 the purposive random sampling method.

B. DNA Isolation and PCR

DNA isolation was conducted based on the procedure of Geneaid. The DNA quality was checked by electrophoresis. 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 3 µl; DNA template 3 µl). The steps of PCR refer to Tuwo et al. [31]. 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.

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

C. DNA Isolation and PCR

The PCR results were converted into binary data. The profiles 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 similarity 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 [32], [33]. The heterozygosity value was calculated using the following formula [33], [34].

$$qi = \left(\frac{individuals\ that\ do\ have\ strand}{number\ of\ individuals\ observed}\right)^{1/2} \quad (1)$$

$$pi = 1 - qi \quad (2)$$

$$He = 1 - pi^2 - qi^2 \quad (3)$$

Annotation:

qi = frequency of null allele

pi = frequency of dominant allele

The value of polymorphic information content (PIC) was calculated using the following formula [33], [35]:

$$PIC = 2fi\ (1 - fi) \quad (4)$$

Annotation:
Fi = frequency of allele

There are 3 group of the PIC value, namely highly = > 0.5; moderate = 0.25 > 0.5; and slightly informative = < 0.25 [36], [37].

TABLE II
RAPD PRIMERS SEQUENCE USED IN THIS STUDY

No.	Primer	Primer Sequences 5'-3'	Tm (ºC)	Ta (ºC)	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

III. Result and Discussion

A. RAPD Analysis

The RAPD molecular technique was applied to characterize and determine genetic diversity and genetic similarity (phylogenetic) in 12 citrus cultivars in South Sulawesi. RAPD is widely used on whole genomic DNA and random primers to assess genetic diversity among plants [38]. DNA isolates were obtained through the extraction process from 174 samples of orange leaves using Kit (Geneaid). The DNA isolates were then amplified using five primers (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, where all of these bands are polymorphic bands so that the polymorphic bands produced are 100%, meaning the DNA bands formed are not monomorphic bands (bands that are present in all samples). Thus, this study showed that the 13 citrus cultivars tested had high genetic diversity. As stated by Lizawati et al. [38], the presence of high polymorphic bands means 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). A DNA band that is present or absent among species is called a polymorphic band, while a band is called a monomorphic band if it appears in all analyzed species [38]. Polymorphism results from changes in nucleotide bases that alter the amplification region's primary binding, insertion, or deletion site [39].

The difference in polymorphism is caused by the difference in the amount of genetic variation that exists between different accessions [38]. Polymorphic information generated by DNA markers is needed in plant breeding programs to improve plant quality [40]. 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 did 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 [33]. Other possible causes are technical errors, amplification processes, and inappropriate temperatures of certain primers for certain samples [38]. Also influenced by several factors, including PCR conditions, quality/quantity of DNA, and concentration of PCR components [41]. 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 [38].

The PIC value is information to detect primers that are capable of producing polymorphic bands in a population [38], [42]. 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.

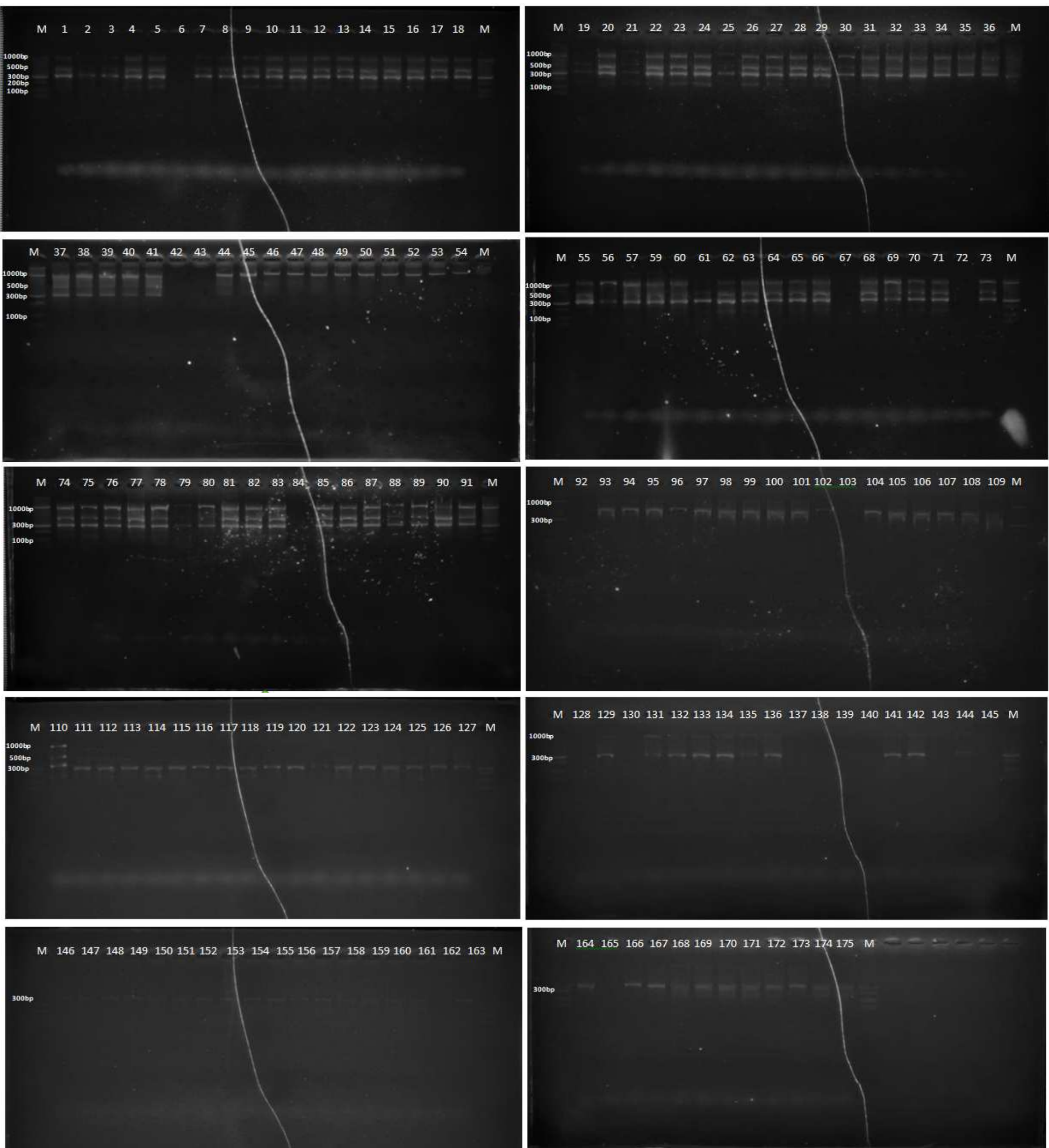

Fig. 1 The resulting RAPD profiles for 175 citrus cultivars on OPC-09 primers. M= 50 bp marker; lanes 1-18 represent the S-SS-coded samples, lanes 19-36 represent the SS-JS-M-coded samples, lanes 37-54 represent the M-P-G-coded samples, lanes 55-73 represent the G-B-coded samples, lanes 74 -91 represents 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 164-17 5 represent the SM-coded samples

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 [43]. Based on 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. 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 [36]. 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. PIC values below 0.25 are not recommended in genetic studies [43]. The PIC value of each primer can be seen in Table 2.

B. *Cluster Analysis*

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 [44].

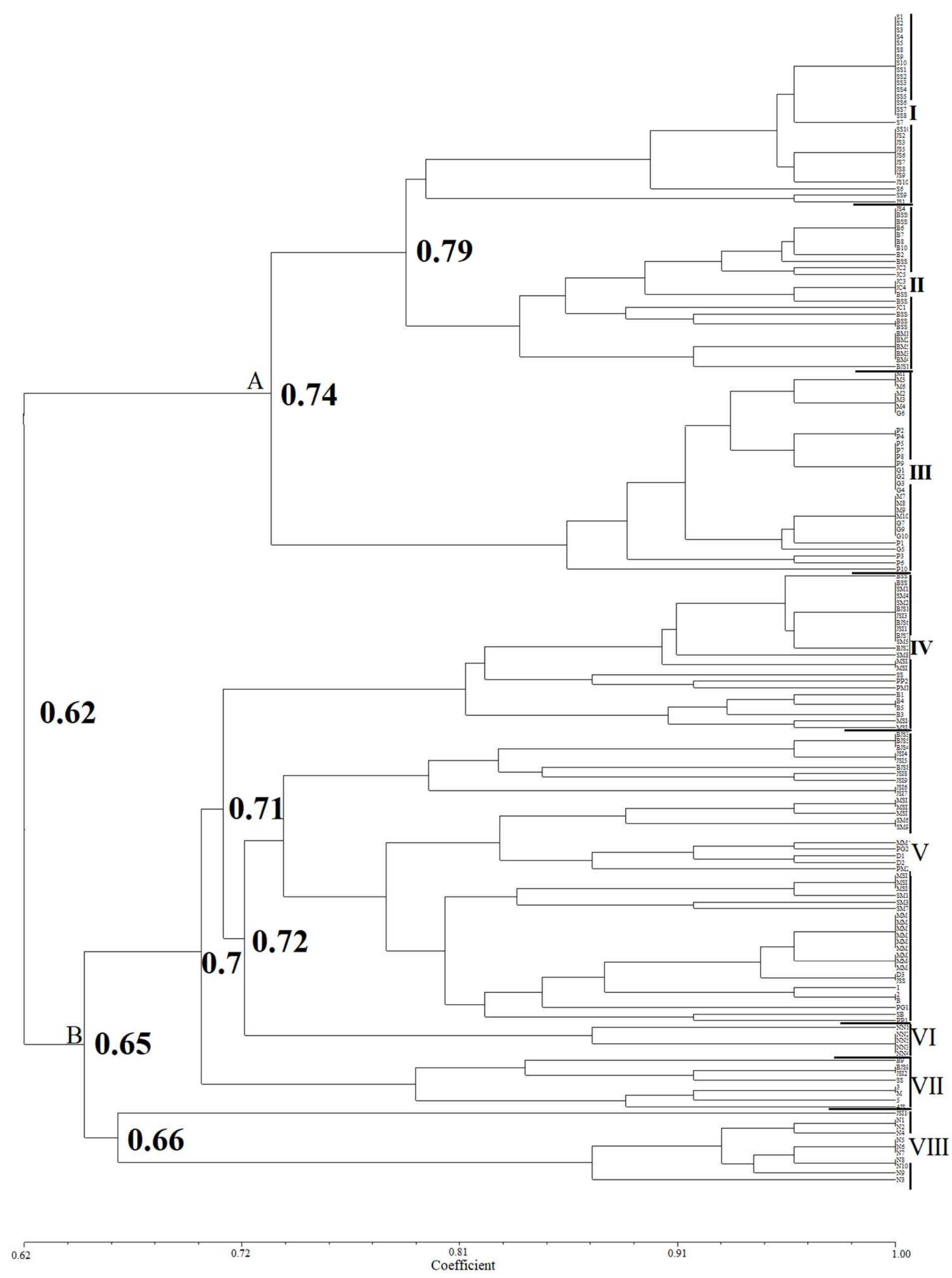

Fig. 2 Dendrogram showing the 174 groups of citrus fruits

Heterozygosity (He) is a fundamental measure of genetic diversity in a population that explains the proportion of heterozygous genotypes under Hardy-Weinberg equilibrium [45]. 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 [46].

Heterozygosity is one of the most important resources in breeding programs because it is associated with genetic variability [45]. Dominant markers such as RAPD can only produce two alleles at each locus [44], and therefore, the maximum heterozygosity value obtained is 0.5. The value of genetic diversity (He) 0.2349 ≤ is categorized as high [47]. The heterozygosity value is obtained from manual DNA visualization scoring results and tabulated into the heterozygosity (He) formula. Each band that appears on the gel is a specific allele [44]. The allele is then translated into binary data, which is assigned a value based on the presence or absence of an allele.

TABLE III
HETEROZYGOSITY VALUES

No.	Cultivar	Sample code	Heterozygosity
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**

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.

The dendrogram that was obtained based on the RAPD banding pattern of the tested citrus cultivars is presented in Figure 2 below. The data above shows that at the level of similarity of 62%, two 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, 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%. Meanwhile, clusters with the closest genetic relationship are clusters I and II with 79% similarity.

TABLE IV
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, 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

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, clusters with the closest genetic relationship are clusters I and II with 79% similarity.

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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Research Article
Diverse Morphology and Anatomy of *Citrus* Spp. (Orange) in South Sulawesi, Indonesia Plantations: A Comprehensive Study

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Abstract
Background and Objective: South Sulawesi, one of the Indonesian provinces, is a producer of oranges with various varieties grown extensively for export and domestic use. Information about the diversity of oranges is crucial for plant breeding and germplasm conservation. This study aims to analyze the diversity of oranges from several plantation centers in South Sulawesi based on morphological and anatomical characteristics. **Materials and Methods:** Orange leaf samples were collected from five plantation locations in South Sulawesi, namely Pangkep, Sidrap, Bantaeng, North Luwu and Selayar Regencies. The morphological characteristics were identified using descriptors from the International Plant Genetic Resources Institute and Tjitrosoepomo. The anatomical characteristics were identified by preparing stomata slides observed under a microscope at a magnification of 200-400x. Similarity analysis between orange varieties was conducted using the NTSYS software and presented in the form of a dendrogram. **Results:** The results of the diversity analysis of 13 orange varieties showed morphological variability in tree form and leaf shape, while anatomical characteristics showed variability in stomata size and stomata index. The similarity analysis showed that morphological characteristics formed clusters consisting of seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), selayar-selayar (SS), batu (B), japansche citroen (JC) and dekopon (D) varieties, which had a 75% similarity with siam (SI) and sweet santang (SM) varieties. Meanwhile, the anatomical cluster analysis showed that the JC and SM orange varieties had a 79% similarity with the D variety. **Conclusion:** The dendrogram diagram can serve as a basis for determining desired plant traits in plant breeding activities.

Key words: Breeding, local germplasm, similarity index, Sulawesi *Citrus* species

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Oranges are fruit plants that belong to the Rutaceae family and are major fruits traded worldwide[1-3]. According to statistical data, nearly 60% of global production of major fruits comes from ten countries, namely China, India, Brazil, the United States, Indonesia, the Philippines, Mexico, Turkey, Spain and Italy[4]. Indonesia has a high diversity of oranges due to the tropical climate that supports the growth and plantation of various types of oranges[5]. In Indonesia, oranges are a favorite fruit highly sought after by consumers and almost every province in the country has orange plantation areas. One of these provinces is South Sulawesi, a major orange producer with a wide range of varieties cultivated for both domestic use and export, orange plantation also contributes to the agricultural economy in the region[6].

The orange species have an extraordinary ability in crossbreeding and producing intra or intergeneric hybrids. In orange plants, apomixis is a common process that allows hybrid propagation through nucellar embryos. This phenomenon poses a challenge for botanists and agronomists who have been striving for centuries to identify orange varieties and define orange taxa. One of the main reasons is that orange taxa often exhibit overlapping morphological characteristics and transitional forms between species frequently occur[5]. Such extensive variability results from field selection, propagation and the diffusion of selected varieties in different plantation areas throughout the year[7]. Information on diversity is necessary for determining genetic relationships, characterizing germplasm, breeding programs, taxonomy and registration of new varieties/cultivars[8,9]. Characterization activities for the existing diversity of orange types are needed as an initial step to ensure the accuracy of the utilized varieties.

Morphological characterization involves studying visible traits[10]. Information obtained from the characterization is crucial as it provides a basis for accurate identification, classification of varieties and differentiation of local orange varieties from those grown worldwide. Understanding the anatomical structure of the orange is equally important. Anatomical analysis involves studying the arrangement, cellular structure and tissue organization of plants[11]. Morphological and anatomical characterization are essential for understanding the diversity and uniqueness of local orange varieties; they can also assist in breeding programs[12]. By identifying and understanding the diversity within and among orange varieties, breeders can select parents with specific morphological or anatomical traits to develop improved varieties[13]. Accurate morphological and anatomical characterization is crucial for the conservation of orange germplasm[14,15].

South Sulawesi is home to a rich diversity of orange varieties, some of which may be unique to the region[6]. The characterization of oranges in South Sulawesi can contribute to the knowledge of orange diversity[16]. This information is valuable for researchers, extension workers and policymakers involved in orange-related studies and programs. The morphological and anatomical characterization of the orange can also facilitate marketing and branding efforts. By identifying distinctive morphological features, farmers can differentiate their products in the market and cater to specific consumer preferences. This creates opportunities for value-added products and marketing strategies that highlight the unique characteristics of South Sulawesi orange. There is a high diversity within the genus in terms of morphology and anatomy and each species has distinguishing characteristics that set it apart[17]. To identify the differences in these species' characteristics, a characterization approach is needed[18]. Research on the morphological and anatomical characteristics of various orange varieties grown in South Sulawesi is still limited. Based on preliminary observations, there are 13 orange varieties cultivated in five orange plantation centers in South Sulawesi. Therefore, this research aims to characterize these 13 orange varieties in terms of morphology and anatomy.

MATERIALS AND METHODS

Sample collection: This research was conducted from March to December, 2022. The orange leaf samples were collected from five orange trees, with ten leaves per tree for each variety gathered from five citrus plantations in South Sulawesi (Table 1). Pangkep Regency with pomelo *Citrus maxima* (Burm.) Merr., Sidrap Regency with lime *Citrus aurantifolia* L. and kaffir lime *Citrus hystrix* D.C, Bantaeng Regency with mandarin orange *Citrus reticulata* Blanco, North Luwu Regency with siam orange *Citrus nobilis* Lour, honey tangerine *Citrus reticulata* and dekopon *Citrus reticulata* Shiranui and Selayar Regency with Selayar tangerine *Citrus reticulata* L. and *Japansche citroen* (JC) *Citrus limonia* Osbeck. Morphological identification was conducted at the Botany Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences, Hasanuddin University. Leaf anatomy analysis was carried out at the Microbiology Laboratory of the Research and Development Agency for Environment and Forestry, Makassar.

Table 1: Orange varieties collected in five orange plantation centers in South Sulawesi, Indonesia

Location	Variety	Sample code	Geographical coordinates	Altitude (m a.s.l.)
Ma'rang, Pangkep	Red pomelo	M	Latitude S-4°42' "Longitude E 119°34"	32
	White pomelo	P		
	Sweet pomelo	G		
Pitu Riase, Sidrap	Lime	N	Latitude S-3.84° "Longitude E 119.81°	205
	Kaffir lime	NN		
Bissappu, Bantaeng	Batu orange	B	Latitude S-5°32' "Longitude E 119°51"	265
Malangke Barat, Luwu Utara	Sweet santang	SM	Latitude S-2°50' "Longitude E 120°19"	17
	Siam orange	SI		
	Dekopon	D		
Bontomatene and Bontona Saluk, Selayar	Seeded selayar	SB	Latitude S-6°8'1" Longitude E 120°27"	268.5
	Selayar-selayar	SS		
	JC-selayar	JS		
	Japansche citroen	JC		

Identification of morphological characteristics: The identification of morphological characteristics of orange plants was conducted using descriptors from the International Plant Genetic Resources Institute[19] and Suariaa *et al.*[20]. Morphological characteristics measured included qualitative and quantitative traits. Qualitative characters included tree form, stem shape, stem growth direction, branching pattern on the stem, branch growth direction, leaf attachment (lamina), leaf shape (circumscription), leaf apex, leaf base, leaf venation, leaf margin, leaf parenchyma, leaf color, leaf surface, leaf arrangement on the stem (phyllotaxis), leaf wing, leaf petiole wing width and leaf petiole wing shape. Quantitative characteristics included average tree height, average stem diameter, leaf length, leaf width, leaf thickness and petiole length. Similarity analysis among orange varieties was performed by processing the morphological data using the Numerical Taxonomy and Multivariate Analysis System (NTSYS)Spc 2.10e software. The results of the similarity analysis were presented in the form of a dendrogram.

Identification of anatomical characteristics: The upper and lower leaf surfaces were treated with acetone while the leaves were still on the tree. Stomatal preparations were examined using a microscope (Nikon 119c Tokyo Japan) at magnifications ranging from 200 to 400x. The anatomical traits observed encompassed the stomata arrangement, stomatal types, stomatal length and width, stomatal size, stomatal index, upper and lower epidermis cell characteristics, upper and lower epidermis cell wall structure, stomatal guard cell morphology, trichome arrangement and form, stomatal opening and stomatal distribution pattern. Photographs of the observed samples were taken. Stomatal size (SS) was quantified using the following formula[21]:

$$SS = L \times B \times K$$

Where:
L = Length
B = Width
K = Franco's constant (0.79)

Stomatal index (SI) is calculated based on the formula as follows[22]:

$$SI\ (\%) = \frac{S}{S+E} \times 100$$

Where:
S = Number of stomata
E = Number of epidermal cells

Statistical analysis: The data obtained from the observation of morphological and anatomical characteristics were analyzed descriptively by presenting the morphological and anatomical features of all orange varieties in tables and figures. The similarity analysis between orange varieties was conducted by processing the data of morphological and anatomical characteristics using the Numerical Taxonomy and Multivariate Analysis System (NTSYS) Spc 2.10e software.

RESULTS AND DISCUSSION

Morphological characteristics: Morphological characterization involves the observation of morphological characteristics of plants based on both qualitative and quantitative properties. Observations were conducted on 24 characteristics. The qualitative characteristics observed included tree shape, stem shape, stem growth direction, branching pattern on the stem, branch growth direction, leaf attachment (lamina), leaf shape (circumscription), leaf apex, leaf base, leaf venation, leaf margin, leaf mesophyll, leaf color,

leaf surface, leaf phyllotaxis, leaf petiole wing, petiole wing width and petiole wing shape. Quantitative characteristics involved observing morphological traits based on size or the number of observed properties using appropriate units. The quantitative characteristics observed were average tree height, average stem diameter, leaf length, leaf width, leaf thickness and leaf petiole length.

Tree shape: Ellipsoid tree shape was found in the following orange varieties: Seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), red pomelo (M), white pomelo (P), sweet pomelo (G), batu orange (B), kaffir lime (NN) and dekopon (D). Meanwhile, the obloid tree shape was found in Japansche citroen (JC), siam orange (SI), sweet santang (SM) and lime (N) citrus varieties (Fig. 1a-m).

Shape, growth direction and branching of the stem: In general, the 13 orange varieties have a round stem shape (teres) with an upright growth direction (erectus). The branching of the sympodial stem, which is the main stem, is difficult to determine as it may cease its growth or exhibit slower and smaller growth compared to its branches (Fig. 1a-m).

Branch growth direction: All varieties have an upright branch growth direction (fastigiatus). The angle between the stem and branches is very small, so the branch growth direction is slightly slanted upwards only at the base, but further up it is almost parallel to the main stem (Fig. 1a-m).

Leaf attachment (lamina): All orange varieties are classified as brevipetiolate, which means that the leaf stalk is shorter than the leaf blade (Fig. 2a-k).

Leaf shape: The leaf shapes of the 13 orange plant varieties vary. The ovate leaf shape was identified in seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), batu orange (B), siam orange (SI), sweet santang (SM), kaffir lime (NN) and dekopon (D). The reverse ovate leaf shape (obovatus) was found in red pomelo (M), white pomelo (P), sweet pomelo (G) and Japansche citroen (JC). These leaf shapes are ovate but wider towards the leaf tip. The ovate-oblong leaf shape (ovalis) is found in the lime (N) variety (Fig. 2a-k).

Leaf tip: A divided leaf tip (retusus) was identified in the varieties of seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), red pomelo (M), sweet pomelo (G) and batu orange (B). A blunt leaf tip (obtusus) was identified in the varieties of Japansche citroen (JC), white pomelo (P) and dekopon (D). A pointed leaf tip (acuminatus) divided (retusus) was found in the varieties of siam (SI) and sweet santang (SM). A sharp-pointed (acutus)-divided (retusus) tip was found in the varieties of lime (N) and a blunt (obtusus)-pointed (acutus)-divided (retusus) tip was identified in the variety of kaffir lime (NN) (Fig. 2a-k).

Leaf base: A blunt leaf base (obtusus) was found in the varieties of seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), white pomelo (P), batu orange (B), siam orange (SI), sweet santang (SM) and kaffir lime (NN). A pointed leaf base (acutus) was identified in the varieties of Japansche citroen (JC), red pomelo (M) and dekopon (D). A blunt (obtusus)-rounded (rotundatus) leaf base was found in the variety of sweet pomelo (G) and a rounded (rotundatus) leaf base was identified in the variety of lime (N) (Fig. 2a-k).

Leaf vein arrangement (nervatio/venation): Orange plants have a pinnate leaf vein arrangement (penninervis), which means the leaf has a single main vein that extends from the base to the tip and serves as an extension of the leaf stalk. From the main vein, smaller branching veins emerge sideways, giving the appearance similar to the fins of a fish (Fig. 2a-k).

Leaf margin (margo folii): Crenate leaf margin was found in the varieties seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), Japanshe citroen (JC), red pomelo (M), batu orange (B), siam orange (SI), dekopon (D), lime (N) and kaffir lime (NN). Sinuate leaf margin was identified in the varieties white pomelo (P), sweet pomelo (G) and sweet santang (SM) (Fig. 2a-k).

Leaf mesophyll (intervenium), color, surface and leaf arrangement on the stem (phyllotaxis): All orange varieties have leaf mesophyll resembling thin paper (papyraceous/chartaceous). The leaf color is dark green with a smooth (laevis) and glossy (nitidus) surface. The leaf arrangement on the stem is alternate (folia sparsa) (Fig. 2a-k).

Width and shape of leaf petiole wings: All orange varieties have leaf petiole wings except for the siam variety (SI). The width of the leaf petiole wings is medium to wide, with obcordate-obdeltate shape in the varieties of red pomelo (M), white pomelo (P) and sweet pomelo (G). They have narrow wings with obdeltate shape in the varieties of seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS), Japanshe citroen (JC), batu orange (B), dekopon (D), lime (N) and kaffir lime (NN). The leaf has narrow to medium wings with obdeltate shapes in the variety of sweet santang (SM) (Fig. 2a-k).

Fig. 1(a-m): Morphological variations of 13 orange plant varieties, (a) Seeded selayar (SB), (b) JC-selayar (JS), (c) Selayar-selayar (SS), (d) Red pomelo (M), (e) While pomelo (P), (f) Sweet pomelo (G), (g) Batu orange (B), (h) Siam orange (SI), (i) Lime (N), (j) Kaffir lime (NN), (k) Dekopon (D), (l) Sweet santang (SM) and (m) Japansche citroen (JC)

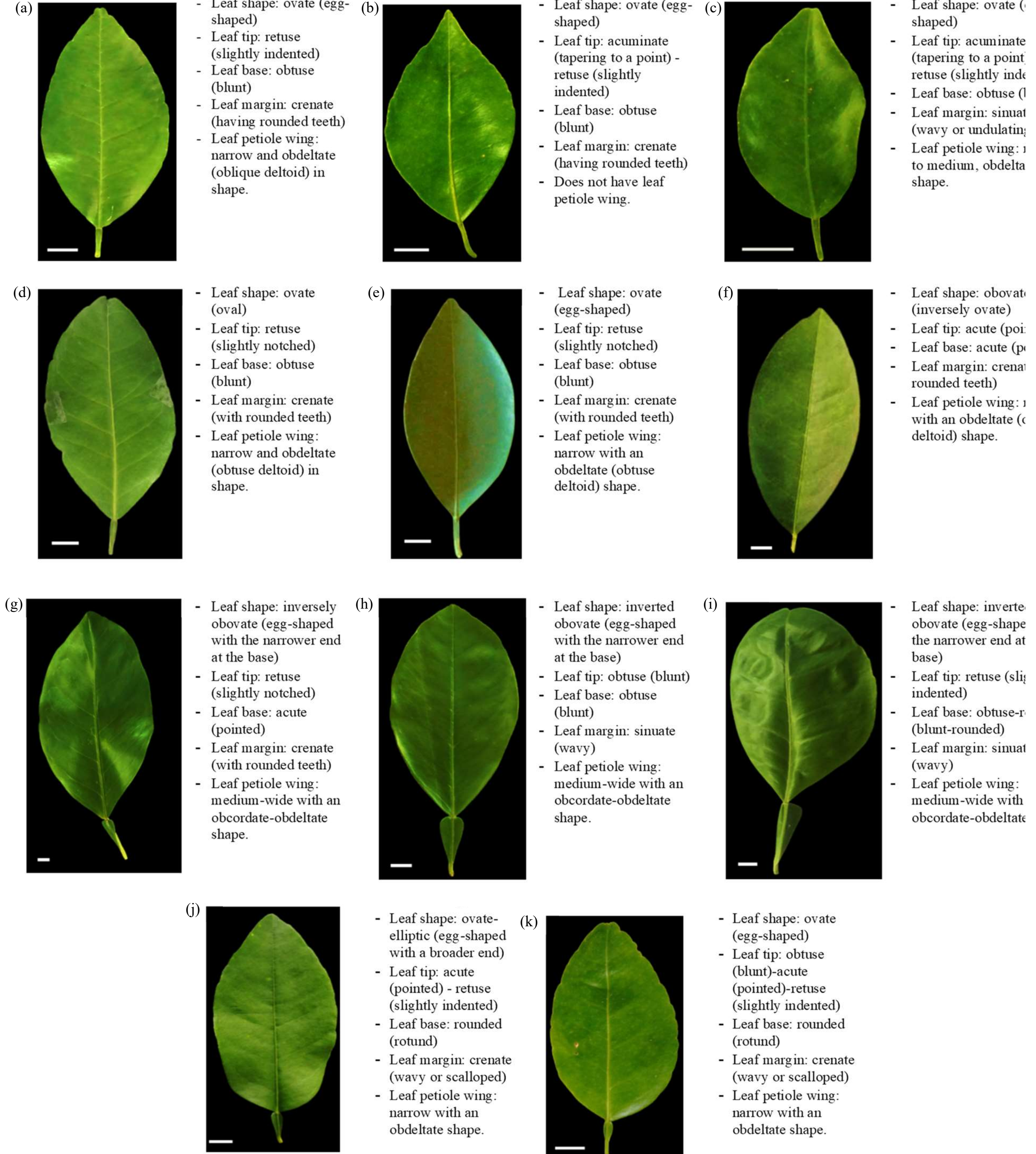

Fig. 2(a-k): Morphological variations of 13 orange plant varieties, (a) Selayar variety [seeded selayar (SB), JC-selayar (JS) and selayar-selayar (SS)], (b) Siam orange (SI), (c) Sweet santang (SM), (d) Batu orange (B), (e) Dekopon (D), (f) Japansche citroen (JC), (g) Red pomelo (M), (h) White pomelo (P), (i) Sweet pomelo (G), (j) Lime (N) and (k) Kaffir lime (NN)

Tree height and stem diameter: The height of orange plant varieties ranges from 1.75 to 8.83 m, with a diameter ranging from 2.30 to 21.34 cm. The tallest trees are found in seeded selayar (SB), JC-selayar (JS) and selayar-selayar (SS) orange varieties, reaching approximately 8 m in height with a diameter of around 9 cm. The shortest tree is the sweet santang (SM) tree, measuring 1.75 m in height with a diameter of 2.60 cm (Fig. 1a-m).

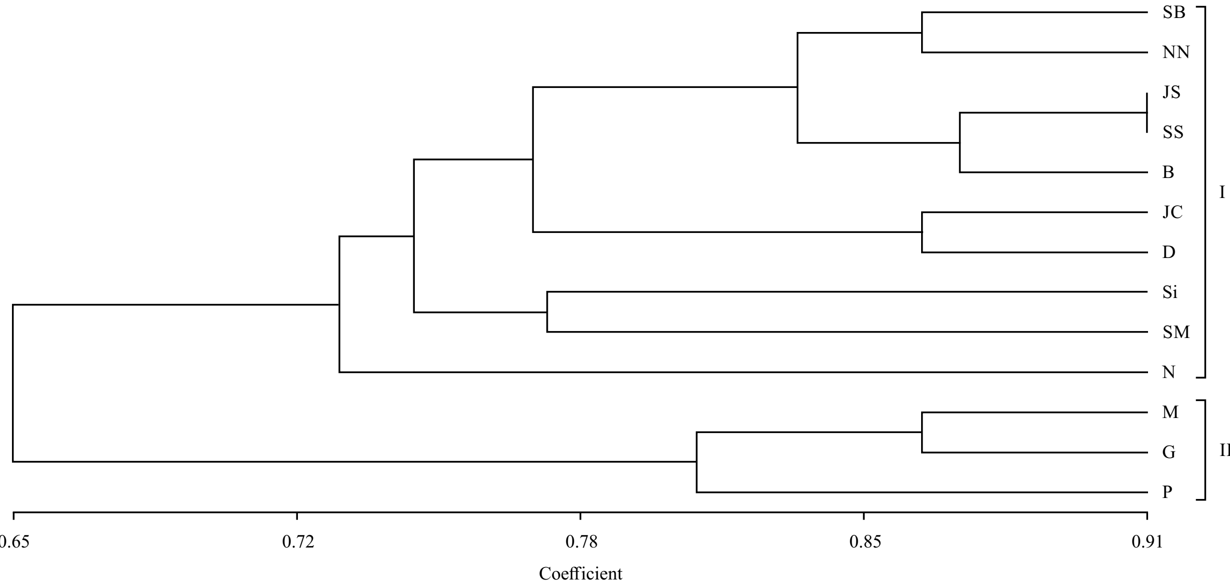

Fig. 3: Dendrogram generated from the cluster analysis of the morphology of 13 orange varieties (Seeded selayar (SB), Kaffir lime (NN), JC-selayar (JS), Selayar-selayar (SS), Batu orange (B), Japansche citroen (JC), Dekopon (D), Siam (SI), Sweet santang (SM), Lime (N), Red pomelo (M), Sweet pomelo (G) and White pomelo (P))

Length, width, thickness and length of leaf stalk: The length of leaves varies among varieties, ranging from 1 to 13.4 cm, with a width of 1.5 to 9.2 cm. The longest leaves are found in red pomelo (M), white pomelo (P) and sweet pomelo (G) varieties, measuring approximately 13 cm in length and around 7.5 cm in width (Fig. 2a-k).

The dendrogram shows two clusters with a similarity coefficient of 65%. Cluster I consists of the varieties seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), selayar-selayar (SS), batu orange (B), Japansche citroen (JC), dekopon (D), siam (SI), sweet santang (SM) and lime (N). On the other hand, Cluster II consists of the varieties red pomelo (M), sweet pomelo (G) and white pomelo (P). Cluster I, with a similarity coefficient of 0.74, is further divided into two sub-clusters, namely sub-cluster 1 and 2. Sub-cluster 1 comprises the varieties seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), selayar-selayar (SS), batu orange (B), Japansche citroen (JC), dekopon (D), siam (SI) and sweet santang (SM). Sub-cluster 2 consists of the variety lime (N). Sub-cluster 1, with a similarity coefficient of 0.75, is further divided into sub-cluster 1.1 and Sub-cluster 1.2. Sub-cluster 1.1 consists of seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), selayar-selayar (SS), batu orange (B), Japansche citroen (JC) and dekopon (D). Sub-cluster 1.2 consists of siam (SI) and sweet santang (SM). Sub-cluster 1.1 and 1.2 have a similarity of 75% (Fig. 3).

The results of the morphological analysis of the 13 orange plant varieties show both similarities and differences in shared characteristics among the varieties. The shared morphological characteristics include the habitus of orange plants, which are generally trees with an upright growth direction (erectus), branching on the sympodial stem (main stem difficult to determine), upright branch growth (fastigiatus) with a very small angle between the stem and branches, nearly parallel to the main stem. The leaf attachment is brevipetiolate (leaf stalk shorter than the leaf blade), the leaf veins are pinnate (penninervis), the leaf tissue is thin like paper (papyraceus/chartaceus), the leaf color is dark green, the leaf surface is smooth (laevis) and shiny (nitidus) and the leaf arrangement on the stem is scattered with single leaves occupying about one-third (folia sparsa). The differing morphological characteristics are observed in the tree and leaf features, such as leaf shape, leaf apex, leaf base, leaf margin, presence of wings on the leaf stalk and width of wings on the leaf stalk.

Leaves are the most diverse vegetative part of plants. Factors contributing to this diversity are adaptations to the environmental conditions in which leaves have evolved and diversified to adapt to various environmental conditions[23]. Different plants inhabit different habitats, each with its own unique challenges. Leaves have adapted to various conditions

through the development of different shapes, sizes, structures and surface features that help plants optimize their interactions with light, water and gases such as carbon dioxide and oxygen[24]. Leaves are primarily responsible for photosynthesis, the process by which plants convert sunlight into chemical energy[25,26]. The diverse forms and structures of leaves reflect adaptations to maximize photosynthetic efficiency[27,28]. For example, broad leaves have a larger surface area for light absorption, while needle-shaped leaves of coniferous trees reduce water loss in cold and dry environments[29]. Leaves also play a crucial role in nutrient acquisition[30,31]. Different plants have developed specialized leaf structures to acquire nutrients from various sources. In terms of defence mechanisms, leaves have developed various defense mechanisms against herbivore attacks and pathogens[32,33]. Some leaves have developed thorns or tough textures to deter herbivores, while others produce chemical compounds or toxins that make them unappetizing or toxic to potential threats[34,35]. For plant variety release, leaf morphology is an important observation component for perennial fruit crops, including orange plants, such as leaf shape, leaf type, leaf characteristics, leaf apex, leaf division, leaf color, leaf type and leaf size.

The differences in morphological characteristics observed in different species are due to their genetic diversity. These genetic differences are not only evident between species but also within a single species, indicating intra-species genetic variability. It is through this genetic diversity that traits within a species vary, known as varieties or even accessions[36]. The differences in characteristics are also influenced by external factors such as the surrounding environment and the plant's growing location. Morphology is the result of the interaction between genotype and environment. It is used to detect the diversity of plants based on their external structures[37,38]. The environment is one of the main factors in the growth and development process of plants, leading to the possibility of morphological and physiological differences even among the same plant species[39]. Environmental factors determine the diversity of a plant population in a specific area, including factors such as elevation, rainfall and humidity[40,41].

Based on the cluster analysis of morphological characters shown in Fig. 3 sub-cluster 1.1 consists of seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), batu orange (B), Japansche citroen (JC) and dekopon (D) varieties. This cluster shows a similarity of 75% with sub-cluster 1.2, which consists of siam (SI) and sweet santang (SM) orange varieties. The high similarity value may indicate a close evolutionary relationship or descent from the same ancestors[42]. It may also indicate that both groups share similar characteristics and may belong to the same group in the context of classification. This indicated that the varieties in these two sub-clusters have many common traits, including stem shape, stem growth direction, branching pattern, branch growth direction, leaf attachment, leaf shape, leaf base, leaf vein arrangement, leaf margin, leaf flesh, leaf color, leaf surface and leaf arrangement.

Anatomical characteristics: Observations of leaf anatomy of the 13 orange plant varieties indicate that stomata are only found on the lower surface (abaxial) of the leaves. All observed varieties have anomocytic stomata type, which means the guard cells are surrounded by a number of certain cells that are not different from other epidermal cells in terms of shape and size. The stomatal guard cells are kidney-shaped and there are trichomes as derivatives of the upper epidermal cells and the distribution of stomata is irregular. Different anatomical characteristics among varieties are found in the size of stomata, stomatal index, upper and lower epidermal cell types and upper and lower epidermal cell walls. The length of the stomata ranges from 12.5-30 μm and the width of the stomata ranges from 7.5-22.5 μm. The stomatal index ranges from 16.07-29.44%, with the lowest stomatal index found in the NN variety (16.07%) and the highest in the batu orange variety (29.44%). The upper epidermal cell type is slightly irregular with 4-6 sides in varieties japansche citroen (JC), sweet pomelo (G), batu orange (B), lime (N), kaffir lime (NN), siam (SI), sweet santang (SM) and dekopon (D) and slightly irregular with 5-6 sides in varieties seeded selayar (SB), Japansche citroen (JS), selayar-selayar (SS), red pomelo (M) and white pomelo (P). The lower epidermal cell type is slightly irregular with 4-6 sides in all varieties except for the variety white pomelo (P), which is slightly irregular with 5-6 sides. The upper epidermal cell walls are slightly undulated-straight in all varieties except for the variety white pomelo (P), which has shallow undulations. The lower epidermal cell walls are slightly undulated-straight in all varieties except for the variety white pomelo (P), which has shallow undulations. The dendrogram results were presented in Fig. 4 show two clusters with a similarity coefficient of 68%. Cluster I consists of selayar keprok varieties, pangkep pomelo and keprok batu. Cluster II consists of Japansche citroen (JC), sweet santang (SM), dekopon (D), siam (SI), lime (N) and kaffir lime (NN) varieties. Cluster II, with a similarity coefficient of 0.79, is further divided into two sub-clusters: Sub-cluster 1 and 2. Sub-cluster 1 consists of Japansche citroen (JC) and sweet santang (SM) varieties, while sub-cluster 2 consists of dekopon (D), siam (SI), lime (N) and kaffir lime (NN) varieties. Sub-clusters 1 and 2 have a similarity of 79% was shown in Fig. 4. The observation results of stomata anatomy indicate that stomata are only found on the lower

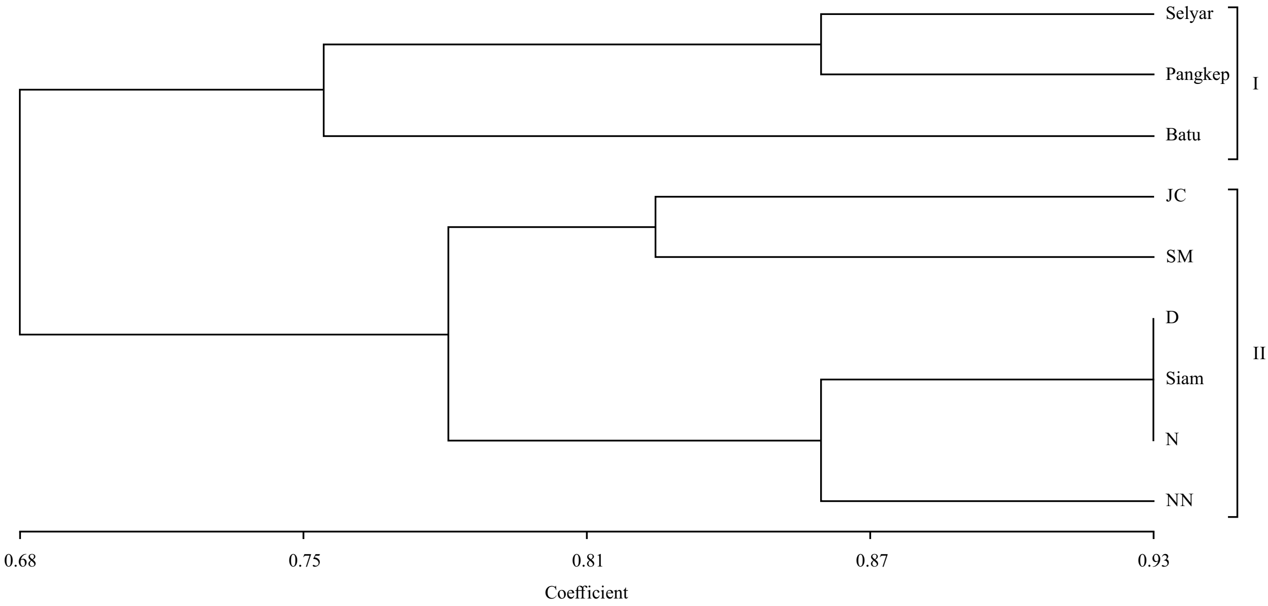

Fig. 4: Dendrogram generated through anatomical cluster analysis of 13 orange varieties
Selayar [seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS)], Pangkep [red pomelo (M), white pomelo (P) and sweet pomelo (G)], batu orange (B), Japanshe citroen (JC), Sweet santang (SM), Dekopon (D), Siam (SI), Lime (N) and Kaffir Lime (NN)

surface (abaxial) of the leaves shown in Fig. 5. The higher density of stomata on the lower leaf surface is an adaptive mechanism of trees to the environment, reducing transpiration[43]. The lower surface of orange leaves tends to be more protected and shaded compared to the upper surface. Stomata located on the lower surface help reduce excessive evaporation due to sunlight exposure and lower temperature. This assists in maintaining hydration balance in orange plants, especially in dry or hot environmental conditions[44]. The upper surface of orange leaves is susceptible to physical damage, especially when exposed to rain or adverse weather. With the presence of stomata on the lower surface, orange plants can protect stomata from direct contact with raindrops or potential mechanical damage. This helps maintain stomatal integrity and ensures smooth gas exchange[45]. Stomatal index, length and width show variation among orange varieties. The stomatal variation among orange varieties is the result of a combination of genetic factors, environment, physiological adaptation and human selection. Genetic factors play a role, where genetic variation among orange varieties can cause differences in stomatal morphology[45,46]. Genes involved in the regulation of stomatal number, size and distribution can differ between orange varieties. Differences in the expression of these genes can result in variations in stomatal index, stomatal length and stomatal width[47]. The growing environment can influence stomatal morphology in plants, including orange varieties. Environmental factors such as temperature, air humidity, light and Carbon dioxide (CO_2) levels can affect stomatal development and size. Orange varieties grown in different environments may show variations in stomatal index, stomatal length and stomatal width. Physiological adaptation comes into play, where stomata play a role in gas exchange, including Carbon dioxide (CO_2) exchange and water vapor transpiration. Variations in stomatal index, stomatal length and stomatal width among orange varieties may result from physiological adaptations to different environmental conditions[48]. Orange varieties grown in dry or humid environments, with different light levels, or with different water requirements may have different stomatal morphologies to optimize gas exchange and hydration balance[49]. Different orange varieties have undergone human selection for centuries to obtain desired traits such as taste, fruit size, disease resistance or productivity. In this selection process, some varieties may have undergone changes in stomatal morphology as a result of desired genetic changes or side effects of selection. This can lead to variations in stomatal index, stomatal length and stomatal width among orange varieties. The results of the diversity analysis of 13 orange varieties showed morphological variability in tree form and leaf shape, while anatomical characteristics showed variability in stomatal size and stomatal index. Similarity analysis revealed that morphological traits formed clusters consisting

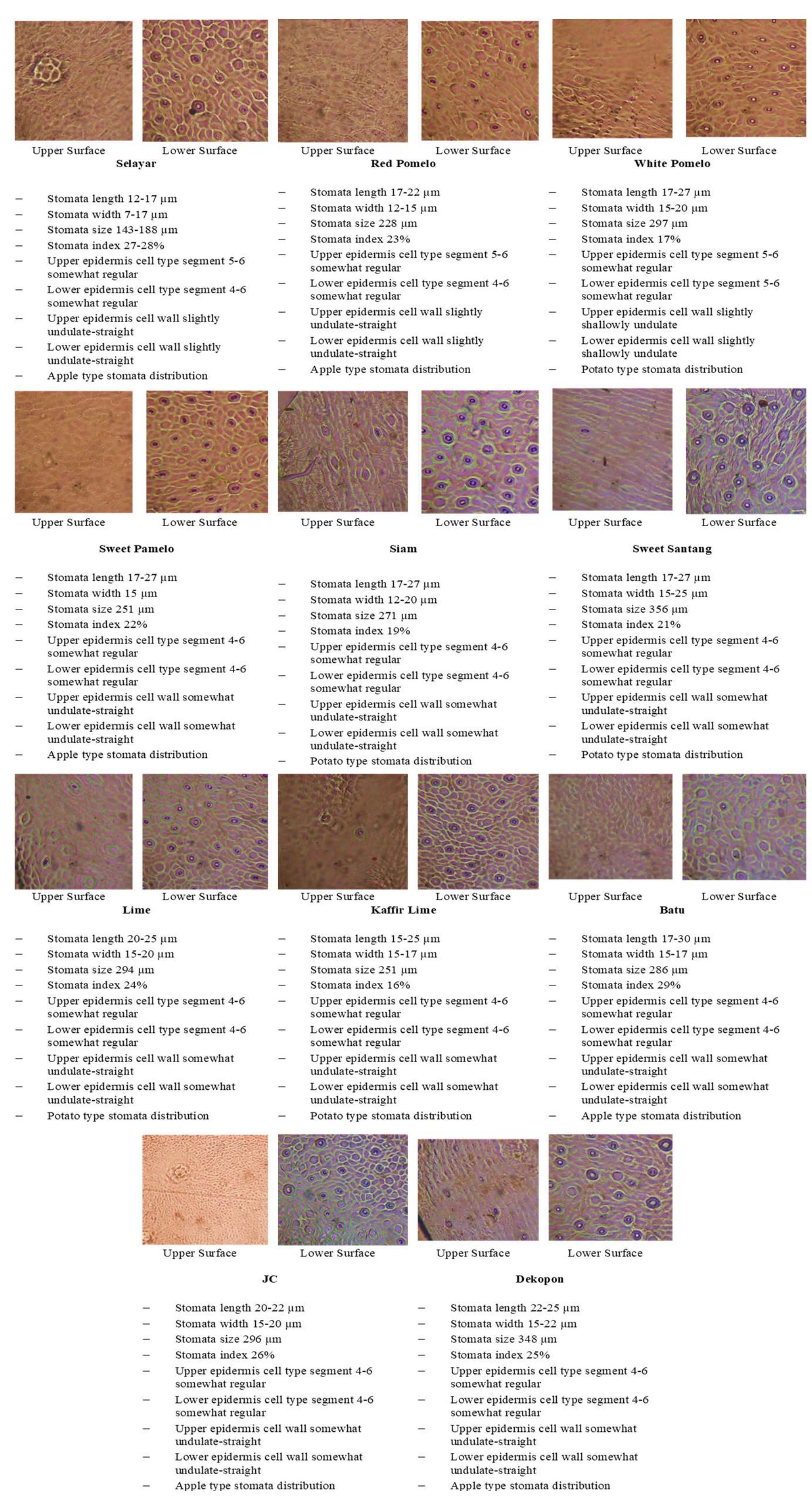

Fig. 5: Anatomical characteristics of leaf stomata of 13 orange varieties in South Sulawesi. Selayar [seeded selayar (SB), JC-selayar (JS), selayar-selayar (SS)], Pangkep [red pomelo (M), white pomelo (P), sweet pomelo (G)], Siam (SI), Sweet santang (SM), Lime (N), Kaffir Lime (NN), batu orange (B), Japansche citroen (JC) and Dekopon (D)

of seeded selayar (SB), kaffir lime (NN), JC-selayar (JS), selayar-selayar (SS), batu (B), Japansche citroen (JC) and dekopon (D) orange varieties with a similarity of 75% to siam (SI) and sweet santang (SM) orange varieties. Meanwhile, anatomical cluster analysis showed that Japansche citroen (JC) and sweet santang (SM) orange varieties had a similarity of 79% to dekopon (D) orange variety. The dendrogram diagram can serve as a basis for determining desired plant traits in plant breeding activities. However, further genetic analysis is needed to strengthen the interpretation and gain a more comprehensive understanding of plant relationships and trait inheritance.

CONCLUSION

The analysis of 13 orange varieties revealed significant variations in both morphological and anatomical traits. Morphological features, such as tree form and leaf shape, showed diversity. Notably, the similarity analysis revealed that morphological characteristics grouped varieties SB, NN, JC-JS, SS, B, JC and D together, sharing a 75% similarity with SI and SM varieties. Concerning anatomical traits, JC and SM orange varieties demonstrated a 79% similarity with D. The resulting dendrogram diagram can be a valuable resource for selecting specific plant traits in future breeding initiatives.

SIGNIFICANCE STATEMENT

In plant breeding efforts, data on the characteristics of the cultivated plants are essential. Plant characterization can be conducted through morphological and anatomical traits. With the availability of this characterization data, it becomes easier to determine the position or relationship among varieties, which can serve as the basis for plant selection. This research aims to identify the diversity of oranges in South Sulawesi based on morphological and anatomical characteristics. The results of this study are expected to serve as a guide in the selection of desired traits in plant breeding.

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Research Article

Estimating the Genetic Diversity of Oranges *Citrus* spp. in South Sulawesi, Indonesia, Using RAPD Markers

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Oranges hold significant economic importance, being cultivated extensively worldwide and having a large global market. Indonesia, ranked eighth globally as a producer of oranges, is one of the countries with high genetic diversity of oranges. This diversity is distributed across various regions of Indonesia, including South Sulawesi. Despite the advancements in DNA-based molecular marker techniques for assessing genetic diversity, information on orange diversity in South Sulawesi is currently unavailable and under-researched. In this study, random amplified polymorphic DNA (RAPD) markers were utilized to analyze the genetic diversity of oranges in five production centers in South Sulawesi. Leaf samples of 13 orange varieties were collected from the five production centers: Pangkep, Sidrap, Bantaeng, North Luwu, and Selayar in South Sulawesi, Indonesia. Genomic DNA extraction from the orange leaves followed the protocol of the DNA Mini Kit Geneaid. DNA amplification was carried out using the RAPD method with 14 primers: OPE-04, OPH-04, OPH-15, OPN-14, OPN-16, OPR-08, OPR-20, OPW-06, OPW-09, OPX-07, OPX-11, OPX-17, UBC-18, and UBC-51. The RAPD primers yielded 109 amplified fragments ranging in size from 200 to 2000 base pairs (bp), and all RAPD primers showed 100% polymorphism. The genetic diversity value (He) of oranges in South Sulawesi was moderate (0.236). Cluster analysis based on a similarity coefficient of 77% divided the 175 orange genotypes into five groups. The most closely related genotypes were SB6 and SB7, exhibiting 100% similarity, followed by genotypes JS8 and JS9 and JS13 and JS17, with genetic similarities exceeding 99% for each pair. Genotypes P9 and SI5 displayed the highest genetic distance, with a similarity coefficient of 57%. The dendrogram diagram can serve as a basis for selecting desired plant traits in the improvement of plant characteristics through both conventional breeding and genetic engineering activities.

1. Introduction

Oranges are considered the most important fruit commodity worldwide, both in fresh and processed forms [1, 2]. According to data from the Food and Agriculture Organization (FAO) of the United Nations, global orange production exceeded 75 million tons in 2019 [3]. This is in line with data from the Central Statistics Agency (BPS) of Indonesia, which reported that orange (tangerine/mandarin) production in Indonesia reached 2.72 million tons in 2022, representing a 13.2% increase compared to the previous year's production of 2.4 million tons [4]. These figures position Indonesia as the eighth largest orange producer in the world, with Brazil, India, and China as the top three orange-producing countries [5]. Indonesia has high genetic diversity in *Citrus* fruits, with production centers relatively dispersed throughout the country, including South Sulawesi. In South Sulawesi, the production centers for oranges are in the districts of Selayar, known for its tangerines, and Pangkep, known for its pomelos, which have been established as long-standing production areas. In addition to these two districts, there are three other districts that have emerged as new production areas: North Luwu for siam oranges, Bantaeng for batu oranges, and Sidrap for lime and kaffir lime. The increase in global orange production is proportional to the high demand for oranges worldwide, indicating that oranges are a favored fruit among the population. This is supported by the nutritional content of

oranges, which is beneficial for health. Oranges are a source of energy and carbohydrates (sucrose, glucose, and fructose), providing good dietary fiber that helps prevent gastrointestinal diseases. They are also rich in vitamin C and antioxidants [6]. Oranges are a source of phytochemicals, including phenols, carotenoids, phytoestrogens, and sulfides, which have potential antioxidant properties and health benefits for the human body [7].

The abundance of orange varieties and cultivars makes it difficult for researchers to differentiate them, necessitating the use of numerical taxonomy for grouping [8]. Diversity represents a valuable resource in the national orange germplasm. However, if this diversity does not reflect genetic diversity, it can lead to confusion in *Citrus* breeding activities, considering that the seed sources used by farmers in national *Citrus* centers are interrelated. Genotypic variation becomes important as genetic information that can be identified and analyzed through molecular marker applications. Despite advancements in DNA-based molecular marker techniques for studying genetic diversity, information regarding orange diversity in South Sulawesi is currently unavailable and under-researched. To address the lack of genetic diversity data for oranges in South Sulawesi, this study utilized the random amplified polymorphic DNA (RAPD) molecular marker. RAPD technology remains relevant and can be used to assess genetic diversity among cultivars originating from the same ancestors [8]. RAPD is the first and simplest PCR-based molecular marker developed for assessing genetic diversity among plant species [9], genetic diversity within populations [10, 11], selection of cultivars with genetic tolerance to salt [12], genetic conservation programs [13], and analysis of ecological aspects [14]. This study aims to analyze the genetic diversity of orange plants in five production centers in South Sulawesi using the RAPD molecular marker. The results of this study are expected to support more accurate characterization of oranges, which can serve as a basis for further research, plant breeding, and development of oranges, particularly in South Sulawesi, Indonesia.

2. Material and Methods

2.1. Plant Materials and DNA Isolation. Leaf samples of *Citrus* plants were collected from five *Citrus* cultivation centers located in the South Sulawesi Province at different elevations. Sampling was conducted from July 2021 to February 2022. Ten young leaves were collected from each plant, and leaves were taken from 10 plants for each orange variety. Detailed information of the leaf samples collected from the five locations is provided in Table 1 and Figure 1.

The extraction of DNA genomes from young *Citrus* leaves was performed following the DNA Mini Kit Geneaid protocol. The DNA quantity was measured using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific) with the Invitrogen QubitTM dsDNA BR Assay Kit, 100 assay (2–1000 ng). The quality of the DNA was assessed using a 1% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml) at 100 V for 90 minutes.

2.2. PCR Amplification and Electrophoresis. The RAPD amplification reactions were analyzed using 14 primers to generate reproducible bands (Table 2). The PCR reaction mixture (13.5 μl) consisted of 3 μl genomic DNA, 3 μl ddH2O, 1.25 μl of each RAPD primer, and 6.25 μl KAPA2G Fast ReadyMix. The PCR process was performed using a SensiQuest PCR machine. The PCR amplification steps included an initial denaturation at 95°C for 30 seconds, followed by 35 cycles of annealing (adjusted to the primer temperature) for 50 seconds, extension at 72°C for 1 minute, and a final postextension at 72°C for 5 minutes. The amplified products were analyzed on a 1% (w/v) agarose gel containing ethidium bromide (0.5 μg/ml) at 100 V for 90 minutes, alongside a 50 bp DNA ladder, and documented using a Gel DOC UV-transilluminator. All PCR results were tested for reproducibility repeated three times.

2.3. Data Analysis. The DNA band profiles obtained from RAPD analysis were scored based on the presence or absence of amplification bands observed on the agarose gel, taking into consideration clear and reproducible DNA bands selected for analysis. A score of 1 was assigned to bands that appeared, while a score of 0 was given to bands that did not appear for each primer. The presence or absence of bands was manually observed through the electropherogram. The calculated data included the percentage of polymorphism, heterozygosity value, and polymorphic information content (PIC). The percentage of polymorphism was calculated as the percentage of polymorphic loci out of the total loci obtained per primer.

The heterozygosity value was calculated using the following formula [15, 16]:

$$\text{He} = 2^{*} p^{*} q, \tag{1}$$

where for binary diploid data and assuming Hardy–Weinberg equilibrium, $q = (\text{1-Band Freq.})^{\wedge}0.5$ and $p = 1 - q$ [15].

The value of polymorphic information content (PIC) was calculated using the following formula [17]:

$$\text{PIC} = 2\,\text{fi}\,(1 - \text{fi}). \tag{2}$$

Annotation: fi = frequency of allele.

A similarity matrix of the binary data was used for cluster analysis using the UPGMA (unweighted pair group method with arithmetic averages) and SAHN (sequential agglomerative hierarchical and nested) algorithms to obtain a dendrogram using NTSYS-pc version 2.10e software [18, 19]. Principal coordinate analysis (PCoA) was performed based on random amplified polymorphic DNA (RAPD) data to further understand the similarity among cultivars using the PCoA package in NTSYS-pc 2.1 [20].

3. Results

3.1. RAPD Analysis. A total of 14 primers, selected based on previous studies [21], were used (listed in Table 3).

Table 1: *Citrus* spp. cultivar collected from South Sulawesi, Indonesia.

Sample site	Varieties of oranges	Geographical coordinates	Altitude (m asl.)
Ma'rang (Pangkep Regency)	Red pomelos, white pomelos, and sweet pomelos	Lat S-4o42′ ″long E 119°34″	32
Pitu Riase (Sidrap Regency)	Lime and kaffir lime	Lat S-3.84° long E 119.81°	205
Bisappu (Bantaeng Regency)	Batu orange	Lat S-5o32′ ″long E 119°51″	265
Malangke Barat (North Luwu)	Sweet santang, siam orange, and dekopon	Lat S-2o50′ ″long E 120°19″	17
Bontomatene and Bontona Saluk (Selayar Regency)	Seeded selayar, selayar-selayar, JC-selayar, and JC	Lat S-6°8′1 ″long E 120°27″	268.5

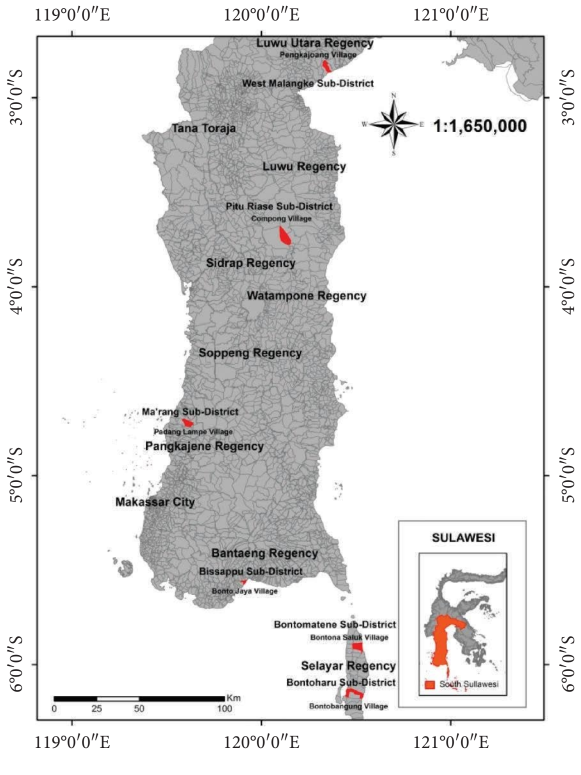

Figure 1: Sampling locations of *Citrus* leaf specimens in South Sulawesi, Indonesia.

Table 2: List of RAPD primers used in this study.

Primer	Primer sequences (5′-3′)	Tm (°C)
OPE-04	GTG ACA TGC C	33.2
OPH-04	GGA AGT CGC C	37.5
OPH-15	AAT GGC GCA G	37.1
OPN-14	TCG TGC GGG T	43.2
OPN-16	AAG CGA CCT G	35.1
OPR-08	CCA TTC CCC A	33.2
OPR-20	TCG GCA CGC A	44.5
OPW-06	AGG CCC GAT G	39.3
OPW-09	GTG ACC GAG T	33.9
OPX-07	GAG CGA GGC T	39.5
OPX-11	GGA GCC TCA G	35.4
OPX-17	GAC ACG GAC C	36.8
UBC-18	GGG CCG TTT A	35.0
UBC-51	CTA CCC GTG C	36.9

Primer screening was conducted to determine the appropriate annealing temperature and select polymorphic primers. This was done by amplifying PCR reactions using different primers and DNA samples under the same conditions [22]. A total of 109 amplified fragments were obtained using the 14 primers, and all fragments generated were found to be polymorphic. Each primer yielded an average of 7.79 amplified fragments, with a minimum of 4 fragments produced by primer OPX-17 and a maximum of 12 fragments with primer OPH-15 (Figure 2). The size of the amplified products ranged from 200 to 2000 bp. The polymorphic information content (PIC) values of the primers ranged from 0.143 for primer OPX-11 to 0.388 for primer OPH-04, with an average value of 0.253. The dendrogram was obtained from the UPGMA analysis of the binary RAPD data, resulting in five clusters.

3.2. Genetic Diversity. Genetic diversity can be defined as the variation within and between species in terms of genetic composition. Populations with high genetic diversity are more likely to exhibit enhanced adaptation [23]. Genetic diversity can be assessed based on the values of heterozygosity. Heterozygosity is a parameter used to measure the level of genetic diversity within a population. The average value of heterozygosity (He) is 0.236 (Table 4). The highest heterozygosity value was observed in type red pomelos (M), which is 0.299, while the lowest was observed in type JC-selayar (JS), which is 0.167. The values of He among the *Citrus* cultivar populations varied considerably, ranging from 0.167 to 0.299. The average heterozygosity value for the *Citrus* population is 0.236.

Cluster analysis results of 175 *Citrus* genotypes using 14 primers can be seen in Figure 3. At a similarity level of 0.69, all analyzed *Citrus* genotypes can be separated into 2 main clusters. Cluster 1 can be further divided into subclusters with different genetic distances. Based on the genetic distance at a coefficient of genetic similarity of 0.77, 5 clusters were identified, each having distinct genetic relationships. Cluster 1 consists of 54 genotypes (SB, SS, JS, B, P, JSI, SI, SM, JC, and M), cluster 2 consists of 40 genotypes (SB, SS, JS, D, SI, MSI, SI, and SM), cluster 3 consists of 41 genotypes (JC, SS, B, SM, JS, and NN), cluster 4 consists of 30 genotypes (M, P, and G), and cluster 5 consists of 10 genotypes (N) (Table 5).

Principal coordinate analysis (PCoA) is an analysis used to determine the proximity of individuals based on the similarity of their characteristics through dimensionality reduction. Figure 4 shows the results of the principal coordinate analysis derived from the binary RAPD data. The PCoA analysis grouped the *Citrus* genotypes based on their types, including red (M), white (P), and sweet (G) pomelo cultivars, as well as the lime (N) cultivar. This indicates that each *Citrus* cultivar is distinct from the others. The pomelo group exhibits higher diversity compared to other cultivars as evidenced by the scattered distribution of points within the group compared to the tendency of other cultivar groups to cluster together.

4. Discussion

The application of molecular markers is an appropriate strategy for analyzing the genetic diversity of *Citrus* species and cultivars. Molecular markers such as RAPD have been widely used in germplasm characterization, genetic diversity studies, systematic analysis, and phylogenetic analysis [24]. RAPD has proven to be quite efficient in detecting genetic variations [25]. For the purpose of identifying genetic diversity, the choice of primers is crucial in distinguishing between species varieties or cultivars [26]. Amplification of

Table 3: Details of RAPD primers and obtained polymorphisms.

Primer	Primer sequences (5′-3′)	Ta (°C)	Fragment size (bp)	Number of band	Number of fragment polymorphic	% fragment polymorphic	PIC
OPE-04	GTG ACA TGC C	30.4	400–1300 bp	5	5	100	0.378
OPH-04	GGA AGT CGC C	40.3	250–1600 bp	6	6	100	0.388
OPH-15	AAT GGC GCA G	35.4	200–1200 bp	12	12	100	0.196
OPN-14	TCG TGC GGG T	43.8	200–1800 bp	7	7	100	0.258
OPN-16	AAG CGA CCT G	34.5	250–1100 bp	9	9	100	0.291
OPR-08	CCA TTC CCC A	33.8	300–2000 bp	8	8	100	0.192
OPR-20	TCG GCA CGC A	45.1	250–1200 bp	10	10	100	0.178
OPW-06	AGG CCC GAT G	37.6	250–1400 bp	9	9	100	0.274
OPW-09	GTG ACC GAG T	37.6	200–700 bp	7	7	100	0.220
OPX-07	GAG CGA GGC T	41.2	200–1600 bp	8	8	100	0.288
OPX-11	GGA GCC TCA G	36.0	350–1000 bp	8	8	100	0.143
OPX-17	GAC ACG GAC C	36.2	350–1000 bp	4	4	100	0.350
UBC-18	GGG CCG TTT A	32.3	350–1000 bp	5	5	100	0.198
UBC-51	CTA CCC GTG C	41.3	200–1500 bp	11	11	100	0.188
Total				109	109		3.542
Mean						100	0.253

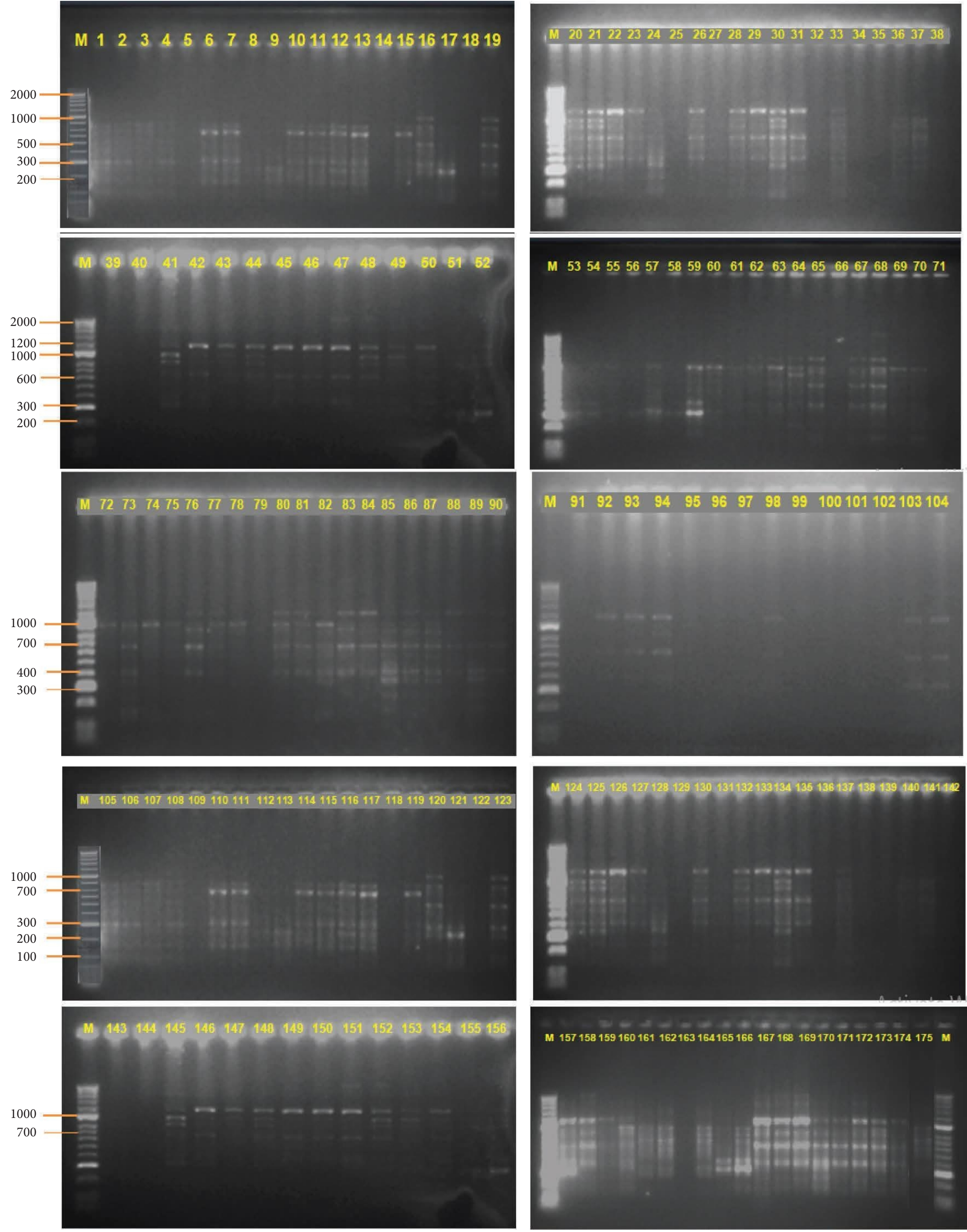

Figure 2: RAPD profile using primer OPA-15. M = marker, 1–10 = SB (seeded selayar), 11–20 = SS (selayar-selayar), 21–30 = JS (JC-selayar), 31–40 = M (red pomelo), 41–50 = P (white pomelo), 51–60 = G (sweet pomelo), 61–70 = JC (japanche citroen), 71–90 = B (batu orange), 91–110 = SI (siam orange), 111–130 = N (lime), 131–140 = NN (kaffir lime), 141–160 = SM (sweet santang), and 161–175 = D (dekopon orange).

the total genomic DNA of 175 *Citrus* genotypes was performed using 14 primers (Figure 2). Each primer produced a different number of DNA fragments. The appearing fragments exhibited variations in base size and fragment intensity. Differences in fragment intensity are influenced by the distribution of primer binding sites on the genome, as well as the purity and concentration of the genomic DNA in the reaction. The number of fragments generated by each primer depends on the distribution of homologous sites in the genome [25]. The presence of differences in DNA fragment patterns (in terms of quantity and size) reflects the existence of a highly complex plant genome [27].

Table 4: Heterozygosity values.

No.	Varieties of oranges	Heterozygosity (He)
1	Seeded selayar (SB)	0.204
2	Selayar-selayar (SS)	0.202
3	JC-selayar (JS)	0.167
4	Red pomelo (M)	0.299
5	White pomelo (P)	0.268
6	Sweet pomelo (G)	0.290
7	JC orange (JC)	0.211
8	Sweet santang (SM)	0.195
9	Batu orange (B)	0.233
10	Siam orange (SI)	0.229
11	Lime (N)	0.268
12	Kaffir lime (NN)	0.289
13	Dekopon orange (D)	0.212
	Average	0.236

Despite the newly developed genetic markers, the use of RAPD as a genetic marker remains a preferred choice for rapid estimation of genetic diversity status. The key feature of the RAPD technique is its high polymorphism detection capability [28], which is consistent with the findings of this study, where each primer produced a varying number of DNA fragments, up to 12 fragments. The percentage of polymorphic bands for all RAPD primers was 100%, indicating that the utilized RAPD markers had a high level of polymorphism. This is in line with previous primer selection results [21], where these primers were identified as generating polymorphic band patterns for *Citrus* varieties. This indicates that the used RAPD markers possess a high level of polymorphism (>50%). RAPD profiles reveal that each primer can yield prominent bands that can serve as RAPD markers to detect differences among the 13 varieties. Polymorphic bands can depict the genomic state of the plant, with a greater number of polymorphic bands indicating higher genetic diversity [29].

The success of a primer in amplifying template DNA is determined by the presence of nucleotide sequence homology between the primer and the template sequence. Other factors that also influence amplification include the quantity and quality of DNA, the concentration of MgCl2, Taq DNA polymerase enzyme, and annealing temperature [30]. The quality of RAPD markers is evaluated through the polymorphic information content (PIC) value. RAPD primers yielded PIC values ranging from 0.143 to 0.388, indicating that all the primers used in this study are suitable for the genetic characterization of *Citrus*. According to Botstein et al. [31], PIC classification is highly informative if $PIC > 0.5$, moderately informative if $0.5 > PIC > 0.25$, and weakly informative if $PIC < 0.25$. RAPD markers can be recommended for use in *Citrus* breeding programs. To date, RAPD is still widely used to assess genetic diversity in various plant species [8, 10, 32–36]. In *Citrus* plants, RAPD markers have been used for cultivar identification, mapping, genetic diversity assessment, and other breeding programs [37]. The application of RAPD has been successful in characterizing sweet orange varieties, enabling the differentiation and distinction of each variety from one another [28]. The utilization of RAPD has proven effective in analyzing phylogenetic relationships and genetic diversity among *Citrus* varieties [38].

DNA markers commonly used to reveal genetic diversity and relationships are RAPD markers, which are one of the many techniques used in molecular biology research. RAPD is considered a simple DNA marker because it does not require prior information from DNA sequence data [39], it is simple in preparation [40], it is fast and easy to analyze, it can be distributed throughout the genome [41], and it can be performed at any stage of plant development [42]. Additionally, RAPD does not require highly pure DNA, meaning it is tolerant to varying levels of DNA purity [43]. RAPD markers are effective and reliable molecular markers for assessing genetic variation accurately [44]. RAPD generates a higher number of genetic loci compared to phenotypic and biochemical markers [45]. One drawback of RAPD markers is their low reproducibility [46]. However, this can be minimized by optimizing PCR conditions, testing the reproducibility of selected primers by repeating PCR amplification three times under the same amplification conditions [47], choosing suitable primers [48], and ensuring optimal extraction methods [49]. Reproducibility in RAPD refers to the extent to which the results of RAPD analysis can be consistently reproduced when performed by different laboratories or individuals. In this study, efforts have been made to minimize factors affecting reproducibility, such as the quality of DNA obtained, which averaged between 39.93 and 85.20 ng/μg. This range is considered more than sufficient for RAPD analysis, where the required DNA concentration is typically 10–100 ng/μg [50]. The PCR technique, including the PCR reaction conditions (temperature, time, and cycle number), the choice of primers, and the electrophoresis conditions, has been standardized, and researchers followed the same protocol during repetitions. Internal reproducibility was also conducted within this study, with experiments repeated three times in the same laboratory, and external reproducibility involved collaboration with three different laboratories: the Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Hasanuddin University; the Laboratory of Research and Development in Sciences, Faculty of Mathematics and Natural Sciences, Hasanuddin University; and the Microbiology Laboratory, Hasanuddin University.

One of the parameters used to assess genetic diversity is genetic variation or heterozygosity (He) [51]. The highest genetic diversity is found in the red pomelo (M) population with a He value of 0.299. The lowest genetic diversity is observed in the JC-selayar population (JS) with a He value of 0.167. This is likely due to the fact that the JS population originates from the same parent. Low genetic diversity is estimated to have a negative impact on species survival and is a major concern for conservation efforts [52]. The average He value for all tested genotypes is 0.236. Dominant markers like RAPD can only produce two alleles at each locus. Therefore, the maximum He value is 0.5 [24]. Based on the analysis of He values, the genetic diversity of *Citrus* in South Sulawesi is considered moderate. According to the criteria, He values greater than 0.30 indicate high diversity, values between 0.20 and 0.30 indicate moderate diversity, and

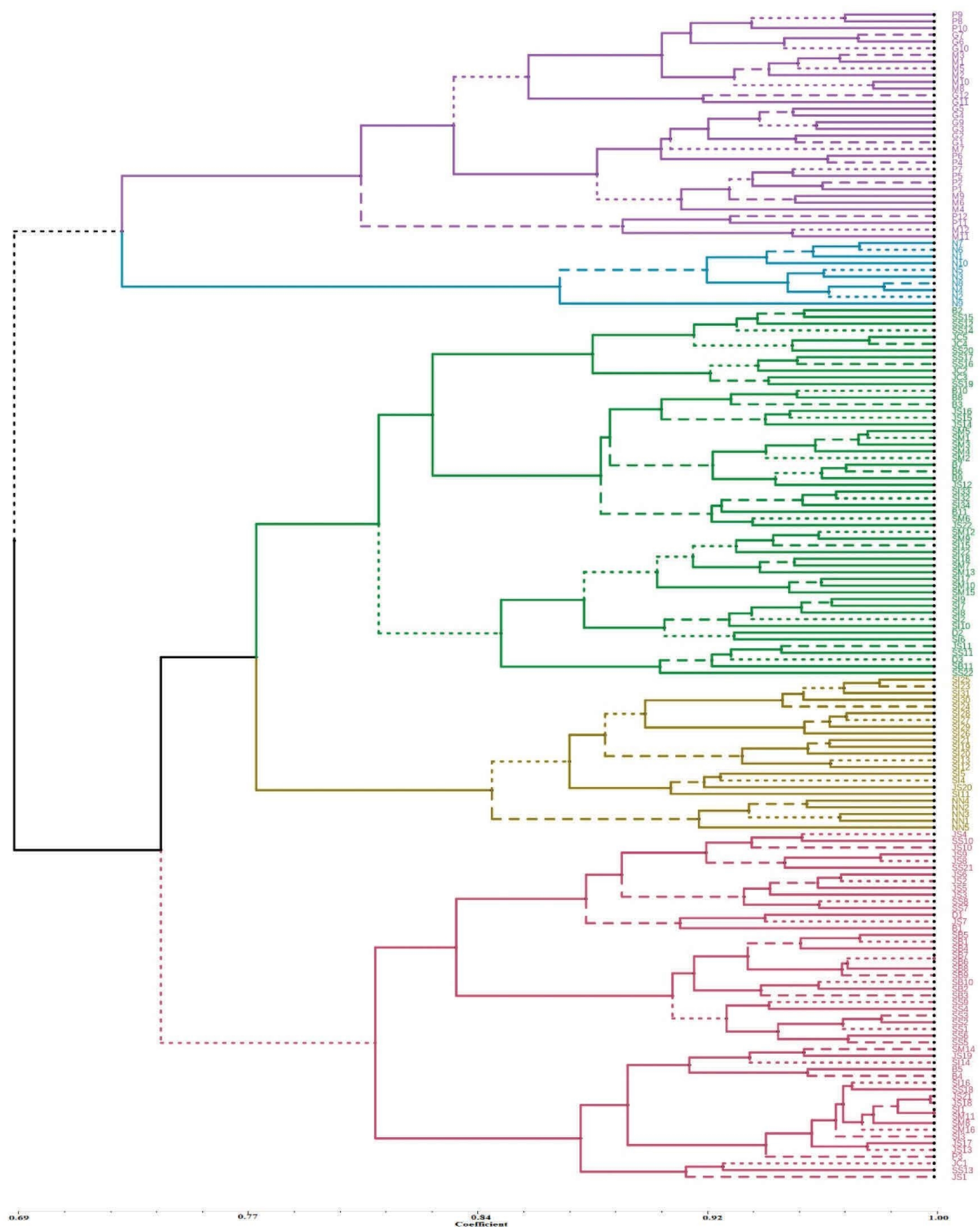

Figure 3: Dendrogram generated from UPGMA cluster analysis of 175 *Citrus* genotype samples.

values less than 0.20 indicate low diversity [53]. Populations with high genetic diversity have the ability to withstand diseases and extreme climatic changes, allowing them to persist over multiple generations [23]. The high genetic diversity in the red pomelo (M) population is likely influenced by the larger population size compared to other locations [49]. The high diversity may also be attributed to cross-pollination facilitated by pollinator agents, which play a significant role in successful fertilization. In such conditions, the likelihood of inbreeding is reduced. Cross-pollination can lead to genetic material mixing among different parent trees [54, 55].

Populations with high genetic diversity are highly valuable as they provide a diverse gene pool for genetic conservation and plant breeding programs [11]. According to the previous studies [56], populations with high genetic diversity can be attributed to several factors: (i) the population already had high genetic diversity since its formation, (ii) the population has been minimally disturbed by human activities, preserving its condition, and (iii) random

Table 5: Grouping of 175 *Citrus* cultivar genotypes at a genetic similarity coefficient of 70%.

Cluster	Genotype
I	SB1, SB5, SB6, SB7, SB8, SB9, SB4, SB2, SB3, SB10, SS1, SS2, SS3, SS7, SS8, SS10, SS9, SS5, SS6, JS2, JS5, JS6, JS3, JS8, JS9, JS10, JS4, JS7, SS4, SS21, B1, JS1, P3, SS18, JSI3, JSI17, JSI18, JSI21, SI1, SM11, SM8, SI3, SM16, SI16, JSI9, SM14, JC1, B4, B5, SS13, M11, M12, P11, and P12
II	SB11, SS11, JS11, D1, D2, D3, SI2, SI4, SI5, SI10, SI6, SI7, SI8, SI9, SI12, SI13, SI18, SI19, MSI9, SI20, SI21, SI14, SI17, SI15, SM9, SM10, SM15, SM12, SM13, SM7, SI22, SI23, SI24, SI25, SI26, SI27, SI29, SI28, SI30, and SI31
III	JC2, SS16, SS17, SS19, JC3, JC4, JC5, SS12, SS20, SS14, SS15, SS22, B2, SM2, B6, B7, B9, SM1, SM3, SM5, SM4, JS12, B3, B10, B8, B11, JS22, SI32, SI33, SI34, SM6, SI11, JS14, JS15, JS16, JS20, NN1, NN2, NN3, NN4, and NN5
IV	M1, M3, M2, M5, M6, M7, M9, M8, M10, M4, P1, P2, P4, P6, P7, P5, P8, P9, P10, G1, G2, G10, G3, G9, G6, G7, G4, G5, G11, and G12
V	N1, N7, N3, N6, N2, N5, N4, N8, N9, and N10

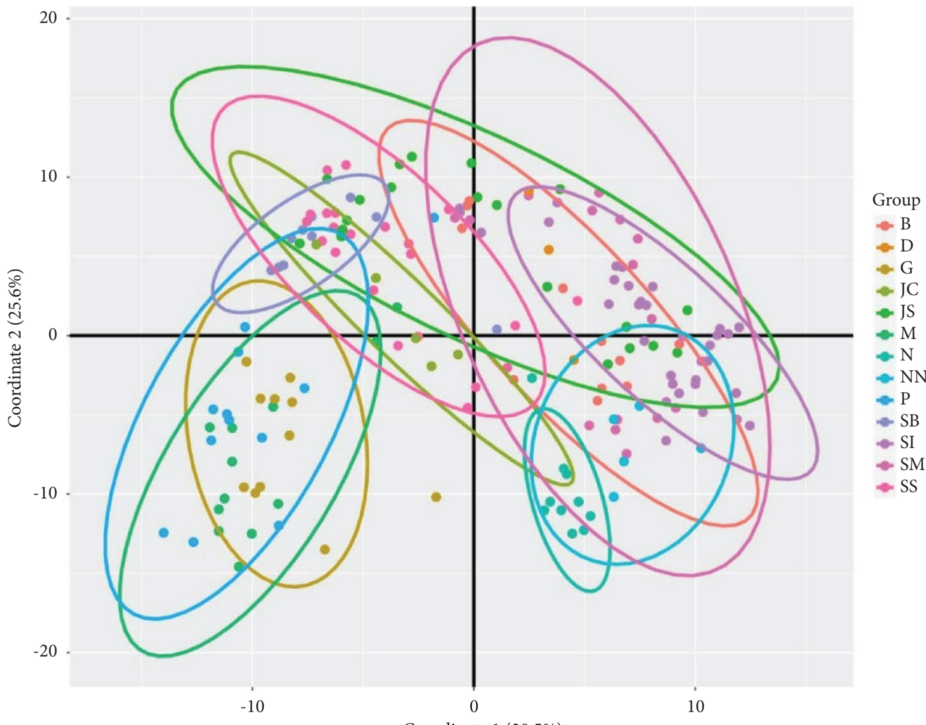

Figure 4: Results of principal coordinates analysis (PCoA) on 175 *Citrus* plant genotypes.

mating between individuals leading to genetic recombination and increased genetic diversity within the population. Conversely, low genetic diversity in a population indicates that it is threatened, fragmented, and damaged by human activities. The genetic diversity of a plant serves as the foundation for plant breeders to identify germplasm for trait improvement, viability analysis, rootstock purity, and enhancing fruit production quality and quantity [57]. Understanding phylogenetic relationships and genetic variability plays a crucial role in determining relatedness, characterizing germplasm, and establishing *Citrus* breeding programs [58]. The analysis of relatedness aims to cluster plant populations based on shared characteristics to determine their distant or close relationships [59]. To determine the genetic relationships among the 13 *Citrus* varieties, scoring data were used to calculate a similarity matrix, which was subsequently used in cluster analysis to generate a dendrogram.

The dendrogram (Figure 3) shows the separation of *Citrus* varieties into several clusters, with some clusters based on their populations. Some populations are also randomly grouped as their distribution patterns are not influenced by geographic location. This is evident in the dendrogram where the populations from Selayar Regency are grouped with varieties from other regencies such as Sidrap, Bantaeng, and North Luwu. Based on the genetic distance calculated using Nei's coefficient [60], with a similarity coefficient of 0.77, the 175 *Citrus* genotypes are

divided into 5 distinct groups with separate genetic relationships. The clustering results show that several genotypes belonging to the pomelo varieties, namely, red pomelo (M), white pomelo (P), and sweet pomelo (G), are grouped together. The PCoA analysis also confirms that the pomelo varieties M, P, and G form separate clusters, as shown in Figure 4. PCoA can be used for further confirmation of genetic diversity. The same pattern is observed for the kaffir lime genotype (N) which forms a separate cluster. However, not all genotypes with the same parentage are grouped together randomly, such as seeded selayar (SB), selayar-selayar (SS), JC-selayar (JS), Japanshe citron (JC), Siam (SI), sweet santang (SM), dekopon (D), batu (B), and kaffir lime (NN). This is likely due to the high heterozygosity of *Citrus* plants, resulting in different characteristics among genotypes derived from the same parent combination. The dendrogram reveals 5 distinct main clusters. The first cluster consists of 54 genotypes, the second cluster consists of 40 genotypes, the third cluster consists of 41 genotypes, the fourth cluster consists of 30 genotypes, and the fifth cluster consists of 10 genotypes. There is some mixing of varieties collected from three regions (Selayar, North Luwu, and Bantaeng), as seen in clusters I, II, and III. This is likely due to *Citrus* breeders using desired plant material and grafting or propagating it onto different plants or selling it to different locations. Clusters IV and V consist only of varieties collected from Pangkep and Sidrap.

The relationship among the tested genotypes ranges from 0.69 to 1, indicating that the 13 varieties exhibit varying degrees of genetic relatedness, from close to distant. All genotypes can be differentiated among the different varieties. High genetic distances indicate relatively distant relationships between varieties, and while small genetic distances indicate close genetic relatedness. Genetic distance is used to detect relationships among populations and between species. Based on the RAPD marker analysis, the *Citrus* genotypes SB6 and SB7 exhibit the closest genetic relationship, with a similarity coefficient of 100%. This is followed by genotypes JS8 and JS9, as well as JS13 and JS17, with genetic similarity values exceeding 99%. The high genetic similarity between SB6 and SB7 suggests that they are likely the same genotype. Both genotypes belong to the Keprok *Citrus* type originating from Selayar. Similarly, genotypes JS13 and JS17 have a genetic similarity value of >99% and both belong to *Citrus* varieties obtained through grafting the JC rootstock with the Selayar Keprok scion. The genotypes P9 and SI5 exhibit the furthest genetic relationship, with a similarity coefficient of 57%. These two genotypes belong to different types. SI5 is a Siam *Citrus* variety from North Luwu characterized by its greenish-yellow and shiny fruit skin, as well as a smooth fruit surface texture. On the other hand, P9 is a white pomelo from Pangkep, characterized by its large fruit size with an average diameter of 15–22 cm, and in some cases even larger than 30 cm. The fruit has a relatively thick skin measuring 2.1–3.73 cm and a strong adhesion to the flesh. Increasing genetic distance between genotypes leads to a higher heterosis effect. However, to produce desirable recombinants, agronomic characteristics should also be considered. One factor influencing genetic variation in nature is the mating system in plants [61]. This mechanism depends on flower structure, mutations, migration, and mating systems [50, 62, 63]. Genetic variation is a key factor in the conservation of biodiversity [64], as the loss of genetic variation can hinder a species' ability to respond to natural selection [65]. The observed genetic variation among samples taken from different regions with varying ecological conditions and elevations may be attributed to differences in seed sources or the influence of mutations and natural crossbreeding [66].

5. Conclusion

The genetic diversity value (He) of *Citrus* in South Sulawesi is moderate (0.236). Genetic diversity plays an important role in improving plant traits through plant breeding. Cluster analysis based on a similarity coefficient of 77% divided the 175 *Citrus* genotypes into 5 groups. The most closely related genotypes are SB6 and SB7 with a similarity coefficient of 100%, followed by JS8 and JS9, as well as JS13 and JS17, with genetic similarity values exceeding 99% for each pair. Genotypes P9 and SI5 exhibit the furthest genetic relationship, with a similarity value of 57%. The dendrogram diagram can serve as a basis for selecting desired plant traits in improving plant characteristics through both conventional breeding and genetic engineering activities.

Data Availability

The data used to support this study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

In vitro Culture Optimization of Pomelo Seeds (*Citrus maxima* (Burm.) Merr.): A South Sulawesi Orange

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Abstract

Background and Objective: Indonesia boasts a variety of delicious tropical fruits, including pomelo, mainly grown in Pangkep Regency, South Sulawesi Province. However, in this region, some challenges hinder such as inadequate care, aging trees and limited seed supply hinder productivity in this region. *In vitro* culture methods present a solution by rapidly producing high quality, disease-free pomelo seeds. This study aims to determine the optimal concentration of the BAP added to the culture medium to induce shoots from pomelo seeds. **Materials and Methods:** The seeds were planted on MS media with the addition of BAP hormone (0.5, 1, 1.5, 2 and 2.5 ppm) and 0 ppm as the control. The experimental units were arranged in a CRD and analyzed using SPSS 20.0 software, employing the Shapiro-Wilk normality test and Levene's Statistic for homogeneity. If the data met the normality and homogeneity assumptions, ANOVA was applied, followed by the DMRT for a parametric test. Otherwise, a non-parametric test namely the Kruskal-Wallis was conducted and differences were further analyzed using the Mann-Whitney test at a 5% significance level. **Results:** The application of the BAP accelerated shoot emergence, with the most rapid development occurring on the 10th day after planting (DAP), at a BAP concentration of 2.5 ppm for red pomelo. For white pomelo and sweet pomelo, shoots appeared on the 19th and 20th days, respectively at a 2 ppm BAP. Interestingly, root development was fastest between the 4th and 6th DAP in 0 ppm BAP (control). **Conclusion:** The addition of the BAP at a concentration of 1.5 ppm in the culture medium promotes faster shoot emergence and has a significant impact on the number of shoots in red pomelo.

Key words: Cytokinin, local pomelo oranges, micropropagation, regeneration, shoot induction

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Indonesia is a tropical fruit-producing country renowned for the diversity and superior flavor of its produce compared to tropical fruit from other producing countries. Among these fruits, oranges are a particularly promising product for development. They offer high nutritional content and an appealing taste, making them popular among consumers[1]. Orange cultivation is widespread across 14 Indonesian provinces, including South Sulawesi, where the heart of the industry lies in the Pangkep Regency. This region is also recognized as the largest producer of *Citrus maxima* (Burm.) Merr., commonly known as pomelo in Eastern Indonesia with some varieties such as red pomelo, white pomelo and sweet pomelo.

Previous research indicates that pomelo production in the Pangkep Regency remains low due to insufficient maintenance by farmers. This lack of care, stemming from budget constraints, affects important aspects such as irrigation, pruning, fertilization and orchard sanitation. Inadequate maintenance has negative effects on both the productivity and quality of pomelo fruit[2]. Another contributing factor to declining productivity is the presence of pomelo trees aged over 20 years[3]. Traditional seed propagation methods also face limitations in providing a large-scale supply of seedlings. Aging pomelo trees are inherently unable to bear fruit optimally, necessitating a focus on plant regeneration[4]. The implementation of *in vitro* culture offers a viable solution to the challenges faced in the Pangkep Regency.

Plant propagation through *in vitro* culture provides many advantages, including the regeneration of mature pomelo plants with identical characteristics to their parent trees[5]. It also accelerates the production of a substantial quantity of superior, disease-free pomelo seeds[6,7]. *In vitro*, seed culture is a fitting method for the propagation of endemic plant species, particularly for conservation purposes aimed at preserving genetic diversity[8,9]. One pivotal factor influencing the success of *in vitro* seed culture is the use of plant growth regulators. This study, focuses on optimizing the media through the addition of the BAP (6-benzylaminopurine) plant growth regulator to induce shoots from superior South Sulawesi pomelo seeds. This research yields a method and procedure that can be repeatedly applied for the production of high-quality pomelo seedlings through shoot induction *in vitro* seed culture.

MATERIALS AND METHODS

Study site and sample collection: This research was conducted from July to December, 2022. The plant material consisted of 162 pomelo seeds from *Citrus maxima* (Burm.) Merr., including red pomelo, white pomelo and sweet pomelo varieties, obtained from the pomelo plantations in Pangkep Regency. The research was carried out in the Plant Tissue Culture Laboratory, Department of Biology, Faculty of Mathematics and Natural Sciences and the Biotechnology and Tree Breeding Laboratory, Faculty of Forestry, Universitas Hasanuddin.

Equipment sterilization: All equipment used for planting must always be in a sterile condition. Glass and metal equipment were thoroughly cleaned with liquid soap and hypochlorite, then dried and sterilized in an oven at 121°C, 17.5 psi pressure for 15 min.

Media preparation: The medium used was Murashige and Skoog medium with vitamins (MSP09-50LT, Caisson Labs). To prepare 1 L of media, 4.43 g was needed, mixed with 30 g L^{-1} of sucrose and 7 g L^{-1} of agar (phytagel, Sigma). Then, 1 L of distilled water was added and the pH was adjusted to 5.8. Next, it was dissolved in a chemical glass, stirred with a stirring rod and heated until it became homogeneous and the solution turned clear. The medium was then distributed into culture bottles and sealed with aluminum foil before being sterilized with an autoclave.

Plant material sterilization: The sterilization of plant materials was performed by thoroughly washing red pomelo, white pomelo and sweet pomelo seeds (Fig. 1) with running water. They were then washed with sunlight liquid soap (commercial liquid soap, Unilever Indonesia) for 5 min, rinsed until the foam disappeared and the outer peel was peeled. The pomelo seeds were then soaked in a tween 80 solution for 15 min and rinsed with sterile distilled water three times. The seeds were soaked in a 20% NaOCl solution for 15 min, rinsed with sterile distilled water three times and soaked in 90% alcohol for 15 min, followed by rinsing with sterile distilled water[9,10].

Culturing seeds on media with various BAP concentrations: The sterilized seeds were planted on media with five different concentrations of BAP (6-Benzylaminopurine) plant growth regulator, which were 0.5, 1, 1.5, 2 and 2.5 ppm and one control medium with 0 ppm. Three seed explants were planted in each culture bottle and the culture bottles were stored on culture racks. Each treatment consisted of three replicates with five culture bottles per replicate. The cultures were kept at a temperature of 23±2°C with a lighting schedule of 16 hrs of light and 8 hrs of darkness.

Fig. 1(a-d): Morphology of the pomelo fruit *Citrus maxima* (Burm.) Merr., (a) White pomelo, (b) Red pomelo, (c) Sweet pomelo and (d) Seed
Scale bar = 0.5 cm

Observation and data analysis: The experimental units were arranged in a completely randomized design. Observations of shoot induction from seed cultures were conducted for 8 weeks after planting (WAP). Data was analyzed using Statistical Package for Social Science (SPSS) 20.0 software with tests for normality (Shapiro-Wilk) and homogeneity (Levene Statistic)[11]. If the data met the assumptions of normality and homogeneity, an Analysis of Variance (ANOVA) test was performed and if there was an effect or difference, Duncan's Multiple Range Test (parametric test) was conducted. If the data was not normal and not homogenous or one of them, a non-parametric test Kruskal-Wallis was carried out and if differences were found, a Mann-Whitney test was performed at a 5% significance level[12-14].

RESULTS AND DISCUSSION

Shoot emergence time: Observations of shoot emergence time and root emergence were counted from the day after the planting (DAP) of explants. Data from each treatment was then calculated and the values from each replicate were averaged as presented in Table 1. The emergence of shoots is an indicator in tissue culture studies that shows that plants can grow with the given treatment[15]. The fastest shoot emergence time was observed in treatment 5 (MS+BAP 2 ppm) for white and sweet pomelos, which occurred on day 20 and day 19 after planting, respectively. In red pomelos, it was observed in treatment 6 (MS+BAP 2.5 ppm) on day 10. This may be attributed to the higher endogenous hormone content in red pomelos compared to white and sweet pomelos. The endogenous hormone content in each explant varies, so the addition of exogenous cytokinins to the culture medium will result in varying responses. The addition of the BAP in the medium stimulates faster shoot growth because one of BAP's functions is to induce shoot formation in plants. However, it should be noted that excessive use of cytokinin-class growth regulators, such as BAP in concentrations exceeding 2 ppm, can be toxic to the plant. This corresponded to the findings of a previous study which indicated that if the BAP level exceeds the optimum limit of 2 ppm, it can be toxic to plant tissues. Cytokinins like BAP can reduce the dominance of apical meristems and induce the formation of axillary and adventitious shoots in plants[16,17].

Root emergence time: The fastest root emergence time was observed in treatment 1 (control) for all varieties of red, white and sweet pomelos, with each of them appearing on day 6, 5 and 4 after planting, respectively. This is because the medium without the addition of the growth regulator BAP more quickly stimulates root growth, as pomelo plants generally already have higher levels of endogenous auxin hormone. This aligns with the statement by Mahadi *et al.*[18], that in pomelo plants grown in a medium without the addition of cytokinin hormones, the formation of roots is better than in media containing cytokinins. This is also supported by Márquez *et al.*[19], who stated that cytokinin hormones have been proven to inhibit root growth in pomelo plants.

Table 1: Days when the roots and shoots of pomelo oranges emergence after planting

Variety	Treatments	Shoot emergence time (DAP)	Root emergence time (DAP)
Red pomelo	1 (MS+0)	16.00	6.00
	2 (MS+0.5)	21.67	8.00
	3 (MS+1)	30.67	8.33
	4 (MS+1.5)	21.67	8.00
	5 (MS+2)	32.00	8.33
	6 (MS+2.5)	10.00	8.33
White pomelo	1 (MS+0)	25.00	5.00
	2 (MS+0.5)	25.00	6.00
	3 (MS+1)	25.00	9.00
	4 (MS+1.5)	25.67	11.00
	5 (MS+2)	20.67	12.00
	6 (MS+2.5)	24.67	13.00
Sweet pomelo	1 (MS+0)	31.67	4.00
	2 (MS+0.5)	24.67	4.67
	3 (MS+1)	27.67	8.00
	4 (MS+1.5)	20.00	8.00
	5 (MS+2)	19.67	8.67
	6 (MS+2.5)	23.33	10.33

1: MS+BAP 0 ppm, 2: MS+BAP 0.5 ppm, 3: MS+BAP 1 ppm, 4: MS+BAP 1.5 ppm, 5: MS+BAP 2 ppm and 6: MS+BAP 2.5 ppm

Influence of BAP on the number of shoots, leaves and roots: Observations of the number of shoots, leaves and roots in pomelo fruit were conducted in the final week of observation, which was 8 weeks after planting (WAP). The data was then analyzed using a normality test to determine whether the observed data were normally distributed or not. Subsequently, a homogeneity test was conducted to identify significant treatment differences[12,14]. In the normality test, if the obtained significance value is greater than 0.05, the data is considered normally distributed. Conversely, if the significance value is less than 0.05, the data is not normally distributed[20]. The normality test used in this study is the Shapiro-Wilk test, which is suitable for small-scale samples[21,22]. Furthermore, Levene's test was performed to assess the equality of population variances in the research[23]. In the homogeneity test, if the significance value is greater than 0.05, the data is considered homogeneous. Conversely, if the significance value is less than 0.05, the data is not homogeneous[24]. If the normality and homogeneity test results do not meet the parametric test requirements (normal and homogeneous), a non-parametric Kruskal-Wallis test is performed[25,26]. If the Kruskal-Wallis test results in a significance value less than 0.05, it indicates an effect of treatment and a further Mann-Whitney test is conducted.

The results of the normality test for the effect of BAP on the number of shoots, leaves and roots in pomelo fruit were presented in Table 2. Based on the normality test results, the significance values were less than 0.05 for the number of shoots, leaves and roots of red, white and sweet pomelo fruit, indicating that the data was not normally distributed. In the homogeneity test presented in Table 3, the significance values were less than 0.05 for the number of shoots and roots in red and white pomelo fruit, indicating that the data was not homogeneous. However, for the number of leaves in red and sweet pomelo fruit, the significance values were greater than 0.05, indicating homogeneous data. The significance value for white pomelo was 0.05, indicating homogeneous data. Based on the results of the normality and homogeneity tests for the number of shoots, leaves and roots in red, white and sweet pomelo fruit, none of the data met the requirements for parametric tests (normal and homogeneous). Therefore, a non-parametric Kruskal-Wallis test was performed.

Based on the Kruskal-Wallis test results presented in Table 4, significant results were obtained for red pomelo, with significance values of 0.016 for shoots and 0.011 for roots, both less than 0.05. This indicates an effect of the concentration of the BAP on the number of roots and shoots. Therefore, a Mann-Whitney test was conducted as a follow-up. For the number of leaves, a significance value of 0.082 was obtained, which is greater than 0.05, so no further test was conducted. For white pomelo, the Kruskal-Wallis test resulted in significance values of 0.106, 0.0472 and 0.053 for the number of shoots, leaves and roots, respectively. These values are greater than 0.05, indicating no significant effect of the addition of the BAP plant growth regulator on the number of shoots, leaves and roots in white pomelo, so no further test was conducted. For sweet pomelo, the number of shoots had a significance value of 0.066 and the number of roots had a significance value of 0.284, both of which are greater than 0.05, so no further test was conducted. However, the number of leaves had a significance value of 0.018, which is less than 0.05, indicating an effect of the application of the BAP on the number of leaves in sweet pomelo (Fig. 2). Therefore, a Mann-Whitney test was conducted to determine significant differences between treatments.

Table 2: Normality test results of the effect of BAP on the number of shoots, number of leaves and number of roots of pomelo oranges

		Red pomelo				White pomelo				Sweet pomelo			
Parameter	Treatment	Statistic	df	Sig.	Decision	Statistic	df	Sig.	Decision	Statistic	df	Sig.	Decision
Number of shoots	1	0.75	3	0.305	Non-normally distributed	0.75	3	0	Non-normally distributed	0.75		0	Non-normally distributed
	2	0.75	3	0	Non-normally distributed		3		Non-normally distributed				Non-normally distributed
	3		3		Non-normally distributed	0.75	3	0	Non-normally distributed				Non-normally distributed
	4	0.75	3	0	Non-normally distributed		3		Non-normally distributed				Non-normally distributed
	5	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed	0.87		0.295	Normally distributed
	6	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed	1		1	Normally distributed
Number of leaves	1	0.873	3	0.305	Normally distributed	0.866	3	0.283	Normally distributed	0.893		0.363	Normally distributed
	2	0.964	3	0.637	Normally distributed	0.75	3	0	Non-normally distributed				Non-normally distributed
	3	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed	0.923		0.463	Normally distributed
	4	1	3	0.984	Normally distributed	1	3	0.992	Normally distributed	1		1	Normally distributed
	5	0.922	3	0.459	Normally distributed	0.75	3	0	Non-normally distributed	1		1	Normally distributed
	6	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed	0.99		0.808	Normally distributed
Number of roots	1	0.873	3	0.305	Normally distributed	0.994	3	0.848	Normally distributed	0.964		0.637	Normally distributed
	2	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed	0.963		0.63	Normally distributed
	3	0.75	3	0	Non-normally distributed	0.871	3	0.298	Normally distributed	0.75		0	Non-normally distributed
	4	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed				Non-normally distributed
	5	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed				Non-normally distributed
	6	0.75	3	0	Non-normally distributed	0.75	3	0	Non-normally distributed				Non-normally distributed

Table 3: Homogeneity test results of the effect of BAP on the number of shoots, number of leaves and number of roots of pomelo oranges

	Red pomelo					White pomelo					Sweet pomelo				
Parameter	Levene statistic	Dfl	Df2	Sig.	Decision	Levene statistic	Dfl	Df2	Sig.	Decision	Levene statistic	Dfl	Df2	Sig.	Decision
Number of shoots	3.202	5	12	0.046	Non-homogenous	8.694	5	12	0.001	Non-homogenous	5.720		12	0.006	Non-homogenous
	0.200	5	12	0.956		0.543	5	12	0.740		1.196		12	0.368	
	0.200	5	9.994	0.955		0.543	5	4.177	0.741		1.196		5.080	0.423	
	2.522	5	12	0.088		6.848	5	12	0.003		5.195		12	0.009	
Number of leaves	2.442	5	12	0.095		3.100	5	12	0.050	Homogenous	5.720		12	0.006	
	0.428	5	12	0.821		0.496	5	12	0.773		1.196		12	0.368	Homogenous
	0.428	5	6.806	0.816	Homogenous	0.496	5	6.170	0.771		1.196		5.080	0.423	
	2.179	5	12	0.125		2.743	5	12	0.071		5.195		12	0.009	
Number of roots	6.833	5	12	0.003	Non-homogenous	5.842	5	12	0.006	Non-homogenous	9.574		12	0.001	Non-homogenous
	0.830	5	12	0.552		0.517	5	12	0.759		1.471		12	0.270	
	0.830	5	3.685	0.592		0.517	5	4.288	0.757		1.471		2.296	0.433	
	5.819	5	12	0.006		4.846	5	12	0.012		8.429		12	0.001	

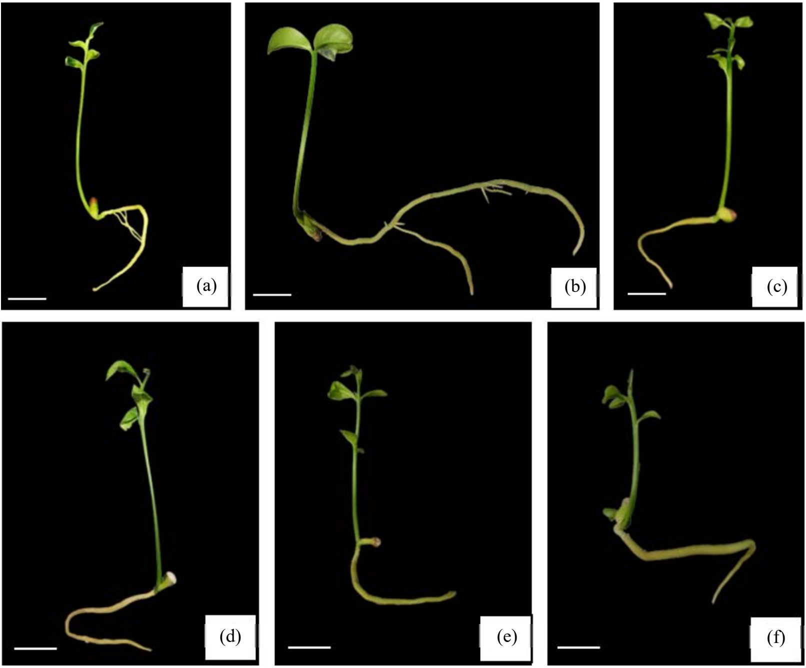

Fig. 2(a-f): Comparison of the number of sweet pomelo leaves, (a) MS+0, (b) MS+0.5, (c) MS+1, (d) MS+1.5, (e) MS+2 and (f) MS+2.5

Table 4: Kruskal-Wallis test results of the effect of BAP on the number of shoots, number of leaves and number of roots of pomelo oranges

	Red Pomelo			White pomelo			Sweet pomelo		
	Number of shoots	Number of leaves	Number of roots	Number of shoots	Number of leaves	Number of roots	Number of shoots	Number of leaves	Number of roots
Kruskal-Wallis	13.971	9.757	14.959	9.071	4.558	10.909	10.362	13.712	6.235
Df	5	5	5	5	5	5	5	5	5
Asymp. Sig.	0.016	0.082	0.011	0.106	0.472	0.053	0.066	0.018	0.284

The results of the Mann-Whitney test were presented in Table 5. For red pomelo, there were significant differences in the number of roots in treatment 6:1 with a value of 0.018, which is less than 0.05. As for the number of shoots, there were significant differences in treatment 6:4 with a value of 0.036, which is less than 0.05. However, none of the other treatments showed significant differences in the growth of red pomelo. In the case of sweet pomelo, all treatments had significance values exceeding 0.05, indicating that none of the treatments significantly affected the number of leaves in sweet pomelo.

The addition of the BAP plant growth regulator affected the number of shoots in red pomelo in treatment 4 (MS+BAP 1.5 ppm) and there were no shoots in treatment 6 (MS+BAP 2.5 ppm). Media without the addition of BAP (control) had an effect on the number of roots in red pomelo, with the fewest roots in treatment 6 (MS+BAP 2.5 ppm) (Fig. 3). Cytokinin hormone regulates cell division and increases cell expansion during proliferation, lateral shoot growth and leaf cell development[27,28]. The BAP as a plant growth regulator with various concentrations can affect plant growth, especially in red pomelo[29,30]. Some studies have shown that the optimal concentration of the plant growth regulator BAP for the growth of pomelo is between 1-2 ppm[13,31].

For red pomelo, the optimal concentration for the number of roots was found in treatment 1 (control) with 2.11 roots. This is because media without the addition of cytokinin hormone (BAP) stimulates endogenous auxin hormone to work optimally, promoting root growth. If the content of endogenous auxin hormone is higher, it will result in more roots[32]. Even though endogenous hormones are synthesized in small amounts by plants, they are highly active physiologically[33]. Shoot and root formation is regulated by the

Fig. 3(a-b): Comparison of the number of red pomelo roots, (a) MS+0 and (b) MS+2.5
Scale bar = 0.5 cm

Table 5: Mann-Whitney test results of the effect of BAP on the number of shoots, number of leaves and number of roots of red pomelo and sweet pomelo oranges

	Red Pomelo		Sweet Pomelo	
Treatment	Number of roots	Number of shoots	Treatment	Number of leaves
6:3	1.000	0.129	1:2	1.000
6:5	1.000	1.000	1:3	1.000
6:4	0.921	0.036	1:6	0.323
6:2	0.108	0.491	1:5	0.087
6:1	0.018	0.491	1:4	0.054
3:5	1.000	0.730	2:3	1.000
3:4	1.000	1.000	2:6	1.000
3:2	1.000	1.000	2:5	0.578
3:1	0.291	1.000	2:4	0.394
5:4	1.000	0.258	3:6	1.000
5:2	1.000	1.000	3:5	1.000
5:1	91	1.000	3:4	1.000
4:2	1.000	1.000	6:5	1.000
4:1	1.000	1.000	6:4	1.000
2:1	1.000	1.000	5:4	1.000

balance between auxin and cytokinin, with high auxin and low cytokinin promoting root formation, low auxin and high cytokinin promoting shoot formation and a balance between auxin and cytokinin promoting callus formation. In treatment 6 (MS+BAP 2.5 ppm), the fewest roots were produced among all the treatments, with only 0.11 roots. This was because the culture medium had a high concentration of BAP at 2.5 ppm, resulting in a higher level of cytokinin hormones compared to auxin hormones, which inhibited root growth. This aligns with the statement by Kurepa and Smalle[34] that the function of cytokinin hormones is to promote shoots and inhibit root growth, while auxin does the opposite by promoting root growth and inhibiting shoot growth. This is also supported by Khan *et al.*[35], stated that, BAP concentrations higher than 2 ppm can inhibit the extension of adventitious meristems and their transformation into complete plants. Some studies have also indicated that media with low cytokinin concentrations, i.e., below 1 ppm BAP are suitable for plant root growth[36].

The number of shoots is an important indicator in determining the potential of tissues regulated by genetic factors and growth hormones[15]. Based on the data obtained for the number of shoots in red pomelo, the optimal concentration was found in treatment 4 (MS+BAP 1.5 ppm) with 2.33 shoots. This was because one of BAP's functions is to stimulate shoot growth in plants. This aligned with Devsharmma *et al.*[37] statement that media supplemented with the cytokinin BAP can produce many shoots. This was also supported by Pereira *et al.*[38] stated that BAP significantly stimulates the growth of axillary shoots, adventitious shoots

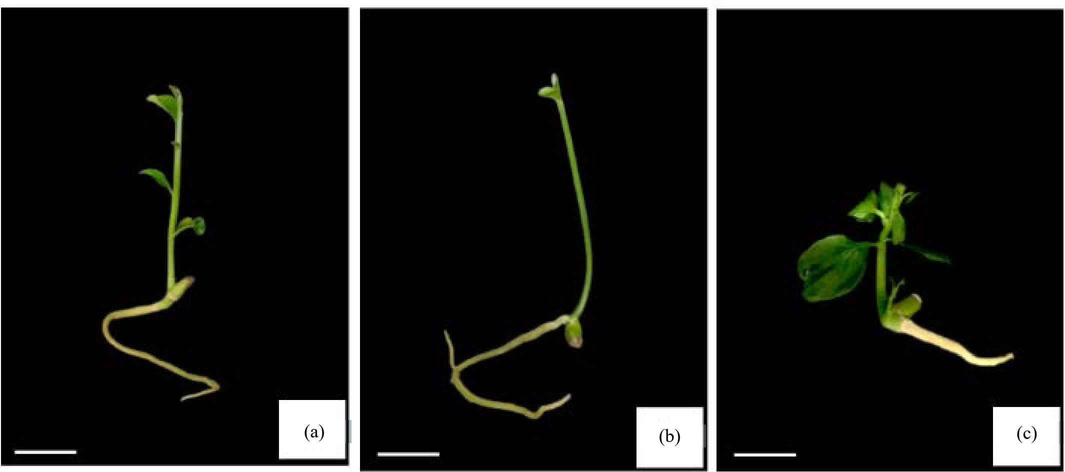

Fig. 4(a-c): Comparison of the number of red pomelo leaves, (a) MS+1.5, (b) MS+0 and (c) MS+2.5
Scale bar = 0.5 cm

and leaves. The BAP is a cytokinin hormone that plays a role in cell division and when combined with auxin, it can also promote cell expansion[39]. However, if the concentration of BAP exceeds the optimum level, it can be toxic to plants. The BAP plays a role in cell division and plant regeneration by stimulating seeds to differentiate into shoots, but if it exceeds the optimum level, it can be toxic to plant tissues[16]. This was evident in treatment 6 (MS+BAP 2.5 ppm), as shown in Fig. 4, where red pomelo growing in media with a concentration of 2.5 ppm resulted in abnormal plants with many leaves but dwarfed growth. This aligned with the study by da Silva *et al.*[40] stating that higher BAP concentrations lead to explants forming shoots without the development of new shoots, resulting in dwarfed and abnormal shoots.

Future recommendations are drawn from the significant implications across various domains found in this study. They offer promising avenues for agricultural advancement by employing *in vitro* cultivation techniques for pomelo seeds. These methods facilitate accelerated and superior plant growth, potentially elevating fruit yield. Moreover, this research underscores the importance of genetic conservation by employing *in vitro* culture to safeguard rare or indigenous pomelo varieties. Additionally, the study highlights its role in driving technological innovation, particularly in adopting *in vitro* technology for propagating region-specific fruit plants, paving the way for future advancements in this field. The outcomes of this study offer practical applications in promoting sustainable agriculture. *In vitro* techniques showcased here hold immense potential for widespread adoption in agriculture, expediting plant reproduction and bolstering overall productivity. Additionally, these methods can significantly contribute to regional progress. Specifically, implementing *in vitro* methodologies in pomelo cultivation across South Sulawesi stands to invigorate and strengthen the local economy. Based on this research, it is recommended to explore and expand the application of *in vitro* methods in pomelo cultivation through further investigation. Additionally, fostering a deeper understanding of *in vitro* techniques in agricultural practices necessitates training and educational initiatives for farmers. However, it's crucial to note limitations in *in vitro* culture research, particularly regarding costs and accessibility. The considerable investment of resources and the challenge of scaling production hinder its widespread adoption. Moreover, reliance solely on *in vitro* technology could pose obstacles to its practical implementation in the field.

CONCLUSION

The results indicate that the BAP treatment stimulates the faster emergence of shoots and has a significant effect on the number of shoots in red pomelo (*Citrus maxima* (Burm.) Merr.) at a concentration of 1.5 ppm. Understanding this precise concentration can serve as a cornerstone for refining agricultural methods, ultimately enhancing output. Furthermore, leveraging BAP in *in vitro* plant propagation forms the foundation for pioneering technological advancements in pomelo orange plant multiplication endeavors.

SIGNIFICANCE STATEMENT

This study proposes a method and process for propagating pomelo plants through *in vitro* seed culture. The research will assist researchers in determining seed surface sterilization procedures and in establishing the optimal concentration of the cytokinin hormone, especially BAP for faster shoot growth. The results of this research make a fundamental contribution to the field of crop cultivation and plant breeding, as it allows for the production of high-quality pomelo plants through plant tissue culture technology, thereby supporting the continuous availability of plant seedlings.

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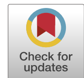
Uncovering the presence of CVPD disease in citrus varieties of South Sulawesi, Indonesia: A molecular approach



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ABSTRACT

Background: The citrus vein phloem degeneration (CVPD) disease is one of important diseases that infects citrus plants and threatens global citrus production and quality due to its severe symptoms and rapid spread. In the 2000s, South Sulawesi Province as one of the citrus producers in Indonesia reported CVPD infection. To date, it is still uncertain as to whether the citrus production center has already been rid of the CVPD infection, keeping in mind the low prevalence of certified citrus saplings use and sub-optimal management of plantations by farmers.
Results: Field observation results revealed varied chlorosis symptoms from young to productive cultivation, which certainly makes it appealing to find out the presence of the causative bacterium, as it has yet to be known whether all the leaves with positive chlorosis symptoms carry the bacterium *Candidatus* Liberibacter asiaticus. Citrus saplings that appear healthy may carry CVPD pathogens as the incubation period of CVPD pathogens in the host plant spans three to five months. Thus, it is necessary to find the right, rapid way to detect the presence of CVPD pathogens in the citrus plant. The most effective method to use is PCR as the bacterium *Candidatus* L. asiaticus is non-culturable *in vitro*, but it is detectable using 16S rDNA. Sampling of leaves with CVPD symptoms was conducted purposively from eight varieties in five citrus cultivation locations, i.e., Pangkep, Sidrap, Bantaeng, Luwu Utara, and Kepulauan Selayar Regencies. DNA isolation was carried out following the Genomic DNA Kit (Geneaid) procedure, followed by detection using the specific pathogenic primer pair OI1 (5′ GCG CGT ATG CAA TAC GAG CGG C 3′) and OI2c (5′ GCC TCG CGA CTT CGC AAC CCA T 3′).
Conclusion: The PCR visualization result shows seven positive samples with DNA fragments measuring 1160 bp. The seven samples were samples of the Key lime, tangerine, Mandarin (cv. batu 55), and Mandarin (cv. selayar), each being derived from Sidrap, Luwu Utara, and Bantaeng. The average disease incidence rate was 66.56 %. Based on the field observation results, the insect vector *Diaphorina citri* was nowhere to be found in the five citrus cultivation locations in South Sulawesi.

1. Background

Citrus vein phloem degeneration (CVPD) is the primary threat to global citrus production and quality.[1,2] It is variably known as citrus greening, yellow shoot, leaf mottle (the Philippines), likubin or decline (Taiwan), citrus dieback (India), and blotchy-mottle or mottling disease (Africa), but its international name is huanglongbing (HLB) (China).[3] In Indonesia, CVPD infected citrus crops when there was a report of severe damages to citrus cultivation at production centers.[4,5] HLB was first encountered in Indonesia in 1964 in coincidence with the naming of citrus vein phloem degeneration (CVPD) which caused growth disorder and even death to grafted citrus plants.[4] The disease is caused by the Gram-negative non-culturable bacterium in the α-proteobacteria group, *Candidatus* Liberibacter spp.[6–8] It can be transmitted through vegetative propagation material, whose spread is geographically caused by infected sapling transport, or through vector in its spread between plants in the same cultivation area.

Indonesia houses various local citrus species and varieties with nationally acknowledged superiority throughout the archipelago from Sabang to Merauke. The Indonesian Ministry of Agriculture has issued a decree on national agricultural estate locations and established national citrus development estates, one of which is South Sulawesi

Abbreviations: CVPD, Citrus vein phloem degeneration; PCR, Polymerase chain reaction; 16S rDNA, 16S ribosomal DNA; DNA, Deoxyribonucleic acid; cv, Cultivar; TE, tris-EDTA; bp, base pair; RNAse, Ribonuklease; CLas, Candidatus liberibacter asiaticus; P, Phosphorus; Mn, Manganese; B, Boron; K, Kalium; Ca, Calcium; Mg, Magnesium; Zn, Zinc.

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Province. However, some citrus development centers in South Sulawesi with their respective superior citrus varieties were reported to be CVPD-infected, i.e., Jeneponto, Sidrap, Bantaeng, and Luwu Utara Regencies.[9,10] To date, it is still uncertain as to whether the citrus production center has already been rid of the CVPD infection, keeping in mind the low prevalence of certified citrus saplings use[11] and sub-optimal management of plantations by farmers. The symptoms triggered by this disease infection resemble those caused by nutritional deficiencies. Field observation results revealed varied chlorosis symptoms from young to productive cultivation. The symptom variation in pattern from mild to severe chlorosis in citrus cultivation has led to an interest in finding out the presence of the causative bacterium as it has yet to be known whether all the leaves with positive chlorosis symptoms carry the bacterium *Candidatus* Liberibacter asiaticus. Citrus saplings that appear healthy may carry CVPD pathogens as the incubation period of CVPD pathogens in the host plant spans three to five months.[12] Thus, it is necessary to find the right, rapid way to detect the presence of CVPD pathogens in the citrus plant. To the present day, CVPD has yet to receive any significant resistance from the citrus species. There has been no effective therapy,[7] and even the formulated methods of CVPD mitigation have yet to be well-established up until now. However, correct detection enables preventive measures.[13]

CVPD disease detection can be performed in numerous ways, including visual observation, chemical testing, shield budding and grafting, and molecular detection. However, the most effective method to use is the PCR one[14] as the bacterium *Candidatus* L. asiaticus is non-culturable *in vitro* but can be detected with its 16S rDNA using specific primers.[15,16] The PCR method has high levels of accuracy and capacity, involves a small amount of DNA, and offers the best alternative for effective and efficient performance.[17–19] This research detected the presence of the bacterium *Candidatus* L. asiaticus in citrus leaves with signs of chlorosis with the PCR method using specific primers to ensure that the signs point to the CVPD disease.

2. Methods

2.1. Plant sampling

Sampling of citrus leaves with CVPD symptoms was conducted purposively at citrus cultivation locations in Pangkep, Sidrap, Bantaeng, Luwu Utara, and Kepulauan Selayar Regencies (Fig. 1). Symptomatic leaves were collected from citrus plants assumed to be CVPD-infected. The symptomatic leaf samples were wrapped in plastic bags and coded by tree point and name of place of origin. The samples extracted were stored in an ice-gel-containing coolbox (Fig. 2).

2.2. Disease incidence

Disease incidence observation was conducted on citrus cultivation exhibiting CVPD disease symptoms over an expanse in each location. The observation data collected were tabulated and calculated to obtain a disease incidence rate. The disease incidence rate is given by the formula below[20,21]:

$$\text{Disease Incidence}\,(\%) = \frac{n}{N} \times 100$$

Where:
P = Disease incidence
n = Number of infected plants
N = Total number of observed plants

2.3. DNA isolation

The DNA extraction of the citrus plants was conducted following the Genomic DNA Kit (Geneaid) protocol. A young leaf sample was weighed at 50–100 mg and added with 400 µl of GP1 buffer. It was then vortexed and incubated in a water bath at 60 °C for 30 min. The mix was turned upside down every 10 min. As much as 100 µl of GP2 buffer was added, followed by vortexing, incubation in ice for 10 min, and centrifugation at 10,000 g for 5 min. A filter column was set in a 2 ml tube. The supernatant was pipetted and transferred to the filter column and then centrifuged at 1,000 g for 1 min. The column was then discarded. The supernatant was added with 700 µl of GP3 buffer and quickly turned upside down. A GD column was set in a 2 ml tube, and all solution was pipetted into it before being centrifuged for 2 min. To the GD column 400 µl of W1 buffer was added, followed by centrifugation at 10,000 g for 1 min. The supernatant was then removed. The GD column was centrifuged for 3 min and then

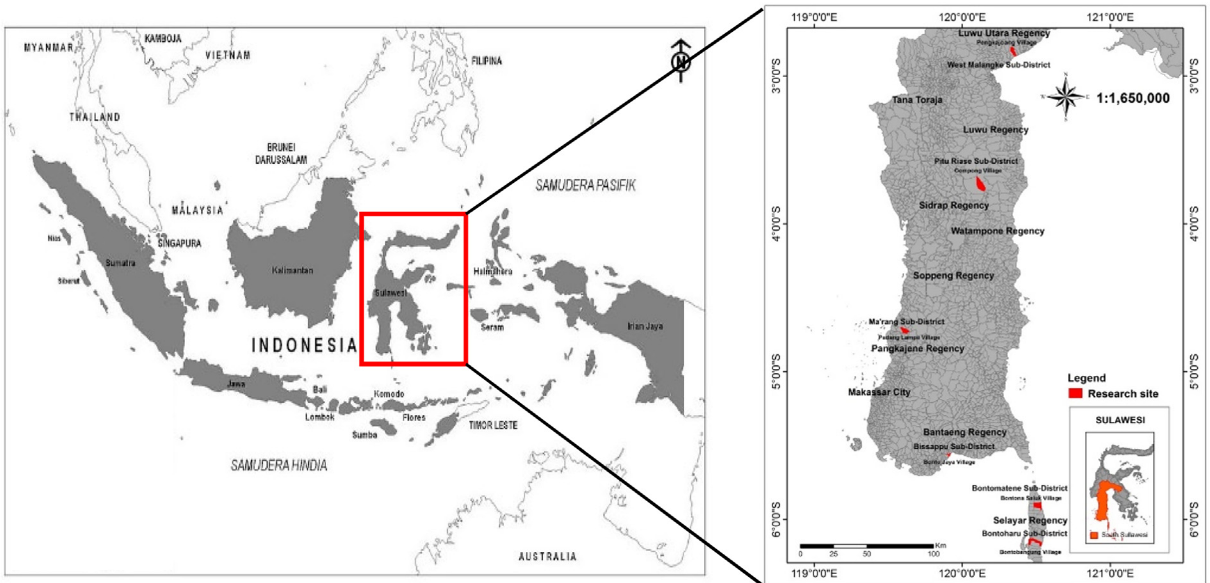

Fig. 1. CVPD disease survey locations in South Sulawesi. The red areas represent locations of sampling of leaves with CVPD symptoms. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

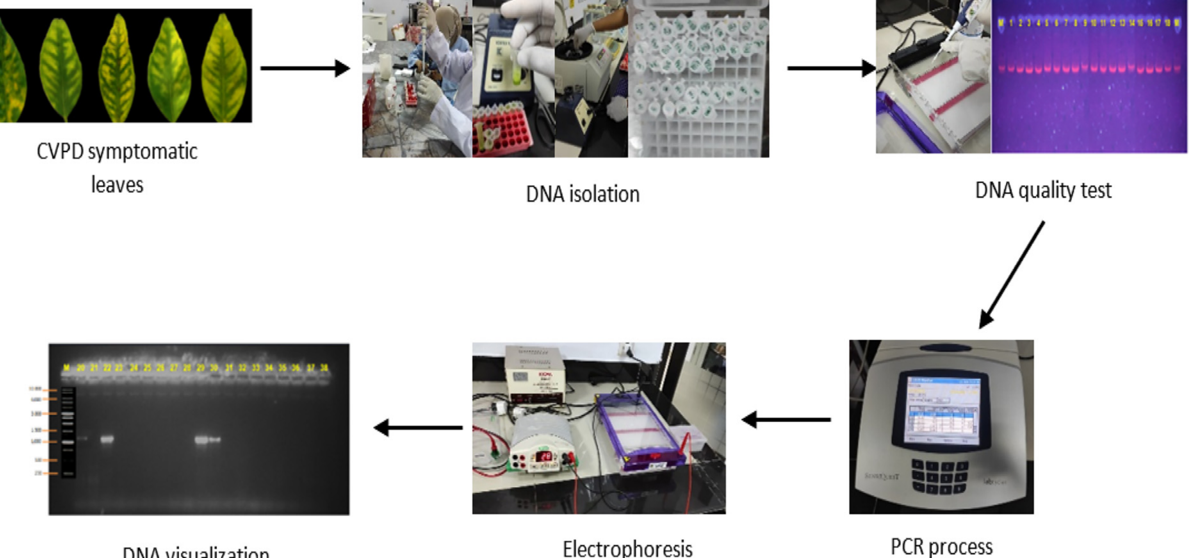

Fig. 2. The research schematic stages.

transferred to a 1.5 ml tube, added with 100 µl of pre-heated elution buffer/TE precisely at the center of the column for 5 min, and centrifuged at 10,000 g for 1 min. The GD column was then discarded. The solution derived was a DNA solution, which was later added with 3 µl of RNAse.

2.4. DNA amplification and electrophoresis

The isolated DNA of the symptomatic sample was used in the PCR reaction. The tube with materials for the PCR reaction contained 3 µl of target DNA, 1 µl of OI1 primer, 1 µl of OI2c primer, 6.25 µl of KAPA PCR Mix, and 3 µl of ddH_2O. The tube was inserted into a PCR machine. The 16 s rDNA fragment was amplified with the pair of CVPD pathogen-specific primers OI1 (5′ GCG CGT ATG CAA TAC GAG CGG C 3′) and OI2c (5′ GCC TCG CGA CTT CGC AAC CCA T 3′). The DNA amplified with the primers measured approximately 1160 bp.[22] The PCR program utilized was a combined modification of[23–25] method and[26] method. The DNA amplification consisted of initial denaturation at 92 °C for 30 s, followed by 40 cycles each at 92 °C for 60 s (denaturation), 55 °C for 30 s (annealing), and 72 °C for 90 s (extension), and a final cycle at 72 °C for 10 min.

Electrophoresis was carried out by first weighing agarose at 3.6 g, added with 180 ml of 1x TE buffer. The solution was heated in a microwave for 5 min and then added with 1.5 µl of red gel. The solution was poured into an agar cast to be coupled with a comb and then left to solidify. After the agar solidifies, the comb was removed. The agar was then inserted into a tank containing 1x TE buffer. The DNA sample that had gone through the PCR stage was inserted into each agar well. On the right side and left side of the well, a marker solution was added. Electrophoresis was performed at 120 v for 70 min. The agar was inserted into gel documentation for visualization. The electrophoresed PCR product was documented and analyzed through a DNA band measuring 1160 bp.

2.5. Data analysis

A descriptive data analysis was conducted with the indicator of the CVPD bacterium presence: if a DNA band (i.e., the 16S rDNA of *Candidatus* L. asiaticus) measuring 1160 bp was present, then it was declared that the bacterium was present or the sample was positive (+), and if no DNA band in that size was present, then the bacterium was said to be absent or the sample was declared negative (−).

3. Results

3.1. CVPD disease infection symptoms

The visual observation results showed varied chlorosis symptoms across five regencies in South Sulawesi. The morphologies of symptomatic leaves in Selayar Regency were as follows: a leaf was dark green in the lamina, with irregular yellowish green blotchy mottles, and dark green in both the midrib and veins (Fig. 3a); a leaf was dark green in the lamina, with irregular yellowish blotchy mottles, and yellowish green in both the midrib and veins (Fig. 3b); a leaf was dark green in the lamina, with irregular bright yellow spots, and dark green in both the midrib and veins (Fig. 3c); a leaf was yellowish green in the

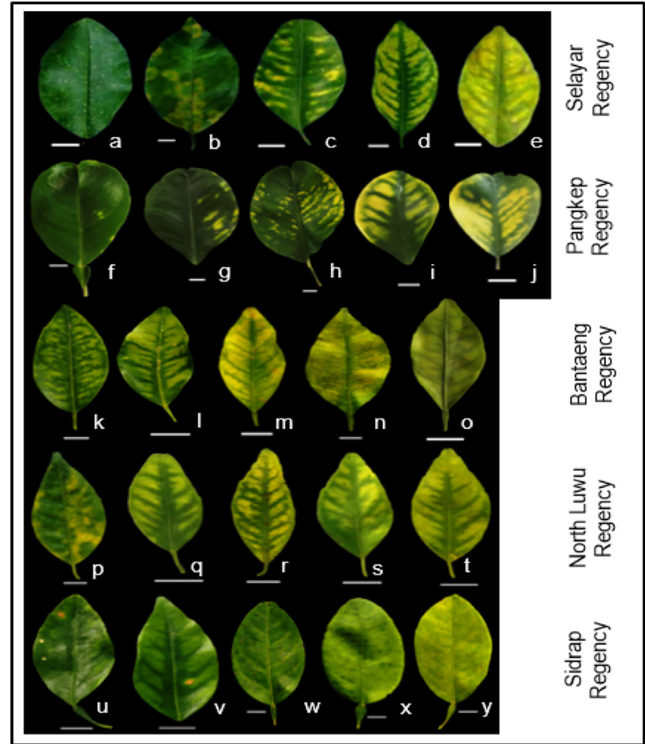

Fig. 3. The morphologies of symptomatic leaves in five regencies.

lamina and dark green in both the midrib and veins (Fig. 3d); and the last leaf was pale yellow in the lamina and yellowish green in both the midrib and veins (Fig. 3e).

The morphologies of symptomatic leaves in Pangkep Regency were as follows: a leaf was dark green in the lamina, with yellowish green blotchy mottles, and dark green in both the midrib and veins (Fig. 3f); a leaf was dark green in the lamina, with bright yellow blotchy mottles in the area on the right side of the midrib, and dark green in both the midrib and veins (Fig. 3g); a leaf was dark green in the lamina, with irregular bright yellow spots, and dark green in both the midrib and veins (Fig. 3h); a leaf was predominantly with an uneven bright yellow color in the lamina, with a small part of it being in dark green, and the midrib and veins were both dark green (Fig. 3i); and the last leaf was dark yellow in the lamina and dark green in both the midrib and veins (Fig. 3j).

The morphologies of symptomatic leaves in Bantaeng Regency were as follows: a leaf were yellowish green in both the lamina and the midrib and dark green in the veins (Fig. 3k-l); a leaf was yellow in the lamina, yellowish green in the midrib, and dark green in the veins (Fig. 3m); a leaf was irregular yellow in the lamina and yellowish green in the midrib and veins, with some of the veins being identical with the lamina in the yellow color (Fig. 3n); and the last leaf was pale yellow in the lamina and yellowish green in the midrib and veins (Fig. 3o).

The morphologies of symptomatic leaves in Luwu Utara Regency were as follows: a leaf was dark green in the lamina, with irregular dark yellow blotchy mottles, and yellowish green in the midrib and veins (Fig. 3p); a leaf was yellowish green in the lamina and dark green in the midrib and veins (Fig. 3q); a leaf was bright yellow in the lamina, yellowish green in the midrib, and dark green in the veins (Fig. 3r); a leaf was irregular yellowish green in the lamina, yellowish green in the midrib, and pale green in the veins (Fig. 3s); and the last leaf was yellow in the lamina and midrib and pale green in the veins (Fig. 3t).

The morphologies of symptomatic leaves in Sidrap Regency were as follows: a leaf was dark green in the lamina, with yellow blotchy mottles, and dark green just like the lamina in the midrib and veins (Fig. 3u); a leaf was pale green in the lamina and midrib and dark green in the veins (Fig. 3v); a leaf was black-spotted irregular yellowish green in the lamina and yellowish green in the midrib and veins (Fig. 3w); a leaf was irregular yellowish green in the lamina and yellowish green in the midrib and veins (Fig. 3x); and the last leaf was irregular pale yellow in the lamina, midrib, and veins (Fig. 3y).

3.2. Disease incidence

The mean disease incidence in citrus plants that showed CVPD symptoms was 6.56 % (Table 1).

3.3. CVPD disease detection

The detection of CVPD disease requires specialized methods as the bacteria are difficult to identify. Table 2 present the methods used to detect CVPD disease.

The DNA isolated from symptomatic citrus plants was shown to contain DNA fragments in the agarose gel electrophoresis (Fig. 4). Total DNA isolation was performed to obtain a good-quality DNA template for subsequent PCR amplification processes. The DNA quality obtained was fairly good. DNA fragments appeared in all samples with average thickness nearly identical to one another in all columns and with clarity, except in columns 1, 9, and 14 where the fragments appeared thin. No smear was discovered in all sample columns.

The PCR amplification results from the symptomatic citrus leaf DNA samples showed that 7 of the 70 samples contained DNA in parallel with the positive control, namely in columns 22 (Mandarin (cv. Batu 55)), 29 (Mandarin (cv. Batu 55)), 30 (Mandarin (cv. Batu 55)), 40 (Key lime), 51 (tangerine), 66 (Mandarin (cv. Selayar)), and 69 (Mandarin (cv. Selayar)) (Fig. 5). This marks that the 7 samples contained the pathogenic bacterium *Candidatus* L. asiaticus causative of the CVPD disease, as detected from the 16S rDNA primers. The positive samples were from four citrus cultivars, namely, Mandarin (cv. Batu 55), Key lime, tangerine, and Mandarin (cv. Selayar).

4. Discussion

The citrus plants observed were of eight varieties from five regencies, i.e., pomelo from Pangkep Regency, Key lime and kaffir lime from Sidrap Regency, Mandarin (cv. Batu 55) from Bantaeng Regency, tangerine, sweet santang orange, and dekopon from Luwu Utara Regency, and Mandarin (cv. Selayar) from Selayar Regency. In each regency, a district whose majority of residents engaged in citrus agribusiness and in which a citrus development center with the largest potential area and the highest number of citrus trees in the regency was selected. The citrus plant age in the five regencies ranged between 5 and 15 years. The farmers in the locations conducted citrus propagation on generative and vegetative bases. Seed-based generative propagation is typically designated for the sowing of rootstocks, which constituted an important factor in the provision of citrus rootstocks given that in Indonesia it still relies on sowing. Vegetative propagation through bud shielding and grafting requires scions and rootstocks.

The CVPD disease may vary in terms of symptoms, types, and modes of transmission, and the symptoms may resemble the symptoms of other diseases. Based on the observation in the five research locations, the symptoms varied from mild to severe across the citrus varieties and cultivation locations. The diagnoses of the patterns of CVPD symptom expressions may also vary for each variety. According to,[9] there are four types of CVPD symptoms in citrus foliage, i.e., type I (mottling), type II (mild chlorosis with green veins), type III (severe chlorosis with green veins), and type IV (yellowing in the veins). However, there are other characteristics of infected citrus trees in cultivation: one yellow shoot or more, infected leaves generating unclearly bordered blotchy mottles, asymmetrical blotchy mottles, thickened leaves with enlarged veins, and possible Zn-deficiency-like symptom followed by leaf-fall and twig dieback.[43,44] As pointed out by,[44] CVPD symptoms include blotchy mottle leaf (BML), little leaf chlorosis (LLC), and nutritional deficiency, which typically occur simultaneously. The

Table 1
Mean disease incidence percentage.

Cultivation area in south sulawesi	Total number of trees	Number of observed trees	Number of infected trees	Disease incidence (%)
Pangkep Regency	224	72	48	66.67
Sidrap Regency	352	52	35	67.31
Bantaeng Regency	144	40	29	72.50
Luwu Utara Regency	530	107	74	69.16
Selayar Regency	108	35	20	57.14
Average				**66.56**

Table 2
Summary of CVPD detection methods.

No.	Detection method used	Name of citrus variety	Area	Citation
1.	Histological analysis	Mexican lemon *Citrus aurantifolia*	Colima, Mexico	[27]
2.	Loop-mediated isothermal amplification (LAMP)	Sweet orange *Citrus sinensis*	Sao Paulo, Brazil	[28]
3.	Specific 16S rDNA primers	Tangerine *Citrus nobilis* Lime *Citrus aurantifolia*	Bali, Indonesia	[29]
		Citrus nobilis, C. amblycarpa, C. reticulata, C. aurantifolia, C. limon and *C. maxima*	Bali, Indonesia	[30]
		Jeruk Siam *Citrus nobilis* var. *microcarpa*	Pontianak, West Kalimantan, Indonesia	[31]
		Mandarin *Citrus reticulata*	Assam, India	[32]
		Citrus maxima, Citrus jambhiri, Citrus macroptera, Citrus medica, and *Citrus reticulata*	Manipur, India	[33]
		Citrus aurantifolia, Citrus sinensis	Uttar Pradesh, India	[34]
		Citrus reticulata	Bhutan	[35]
		Mandarin Orange, Acid lime	Tamil Nadu, India	[36]
		Citrus maxima	Hainan, China	[37]
4.	Tissue print elution(TPE)-qPCR	*Citrus sinensis* *Citrus reticulata*	China	[38]
5.	HLB rapid detection kit	*Citrus nobilis*	Koto Tinggi, West Sumatera, Indonesia	[39]
6.	Multiplex qPCR	Navel orange tree *Citrus sinensis*	Davis, California	[40]
7.	Simple alkaline heat DNA lysis followed by loop-mediated isothermal amplification coupled hydroxynaphthol blue (AL-LAMP-HNB)	*Citrus reticulata*	Chiang Mai, Thailand	[41]
8.	Real-time recombinase polymerase amplification (RPA)	*Citrus sinensis, Citrus hystrix*	Brazil	[42]

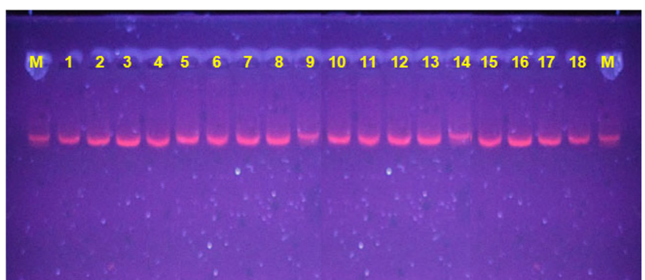

Fig. 4. Total DNA of symptomatic leaves. M = marker; 1–3 = mandarin (cv. selayar); 4–6 = pomelo; 7–9 = mandarin (cv. batu 55); 10–12 = tangerine; 13 = sweet santang orange; 14 = dekopon; 15–16 = Key lime; 17–18 = kaffir lime. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

interaction between pathogenic invasion and plant defensive response facilitates typical leaf symptoms.[44]

Yellow shoot and spots on the leaf are specific symptoms in CVPD-infected citrus plants[45,46] and manifestations of various nutritional disorders.[47] However, it is still unclear as to how nutritional contents and distribution change in response to CLas invasion as the development of the symptoms is yet to be characterized comprehensively. The observation of CVPD disease symptoms in the citrus cultivation areas in five regencies in South Sulawesi generally unveiled symptoms such as yellow shoot, yellow-green blotchy mottle leaf, and asymmetrical blotchy mottle patterns. The differences in symptoms might be attributed to differences in plant age, attack intensity, climatic condition, and the *Candidatus* L. asiaticum strain attacking the plants.[48] They might also be caused by influences from the environment, sapling origins, and variation in disorderly bacterial distribution in the plants.[49,50] Chlorosis symptoms occur due to reduced chlorophyll production, which causes the plant photosynthesis activity to be decreased and infected plants to exhibit necrosis and leaf-fall symptoms.[51–53] There are a variety of interactions between nutrients and CVPD pathogens in citrus plants. The pathogens usually trigger nutritional disorders from starch accumulation, physically block the transport tissue for nutrient mobility, and transfer redistributed nutrients in the phloem[54]. The plant nutrient balance becomes indirectly influenced after the CVPD pathogenic infection.[44]

The research findings of[44] showed that the levels and distribution of various nutrients such as P, Mn, and B vary with the occurrence of symptoms and the subsequent development of the symptoms. The contents and distribution of other nutrients, such as K, Ca, Mg, and Zn, are affected by the development of symptoms and the presence of CVPD pathogens. Carbon metabolism disorder and Zn source imbalance in citrus plants are important determining factors in CVPD pathogenesis.[55,56] Previous studies have reported carbon compound accumulation and starch synthesis that substantially cause physiological disorders such as phloem dysfunction and chloroplast disintegration in CVPD-infected leaves.[57] The observation of disease incidence found that 57–72 % of the citrus plants exhibited CVPD disease symptoms (Table 1), with the average exceeding 50 % (i.e., 66 %). Up to 10 % of the citrus plants were CVPD-positive, as shown by the PCR detection results; 7 of 70 samples were positive, with the presence of 1160-bp-long DNA fragments.

Based on the method to detect CVPD disease, Table 2 shows the detection technique applied to each citrus variety sampled for identifying the presence of *Candidatus* bacteria. The detection of bacterial presence in the symptomatic citrus leaf samples in this study was carried out with the polymerase chain reaction (PCR) technique to amplify the 16S rDNA of *Candidatus* L. asiaticus. PCR is a sensitive, rapid, accurate detection technique. It uses the specific primer pair of 011 forward primer and 012c reverse primer to amplify the 16S rDNA of *Candidatus* L. asiaticus that measures approximately 1160 bp.[17–19] The results of the PCR detection with the specific primer pair showed 1160-bp-long DNA fragments in 7 citrus leaf samples, which means that the samples were positive for *Candidatus* L. asiaticus infection. The samples were of the Key lime, tangerine, Mandarin (cv. Batu 55), and Mandarin (cv. Selayar) varieties from Sidrap, Malangke Barat, and Bantaeng. The other two regencies, Pangkep and Selayar, were negative as no specific 1160-bp-long DNA fragments were found. Previously, the CVPD disease was reported in several citrus production centers in South Sulawesi, including Sidrap, Bantaeng, and Luwu Utara Regencies (Asaad, 2001, 2006). The three regencies were with their

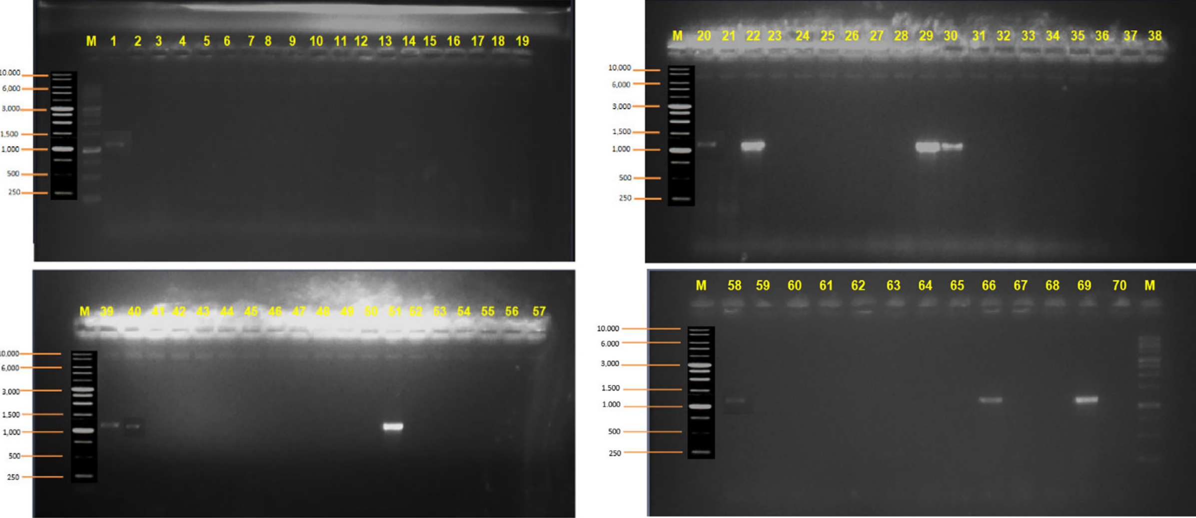

Fig. 5. DNA visualization from the CVPD disease detection in symptomatic leaf samples in five citrus cultivation locations. M = marker; 1 = positive control; 2–7 = Mandarin (cv. Selayar); 8–13 = pomelo; 14–19 = dekopon; 20 = positive control; 21–30 = Mandarin (cv. Batu 55); 31–38 = kaffir lime; 39 = positive control; 40–49 = Key lime; 50–57 = positive control; 59–64 = sweet santang orange; 65–70 = Mandarin (cv. Selayar). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

respective mainstay citrus commodities. Sidrap Regency, particularly Pitu Riawa and Pitu Riase Districts, is a center for Key lime production. Bantaeng is a new production center; in 2011, it was appointed to be a basis for Mandarin (cv. Batu 55) production. Malangke and Malangke Barat Districts in Luwu Utara Regency had the tangerine as their signature commodity until the 1990s when they were still declared CVPD-free. However, in 1999 farmers discovered greening symptoms, which were assumed to be signs of CVPD. As of 2004, 75–100 % of citrus plants in a number of cultivation locations died. Finally, in 2002–2005 the Malangke tangerine was declared extinct. At the end of the year 2009, citrus planting at the *demplot* (a plot of land for fruit-planting) in Waeto Village, Malangke Barat District, used saplings certified by the Research Institute for Citrus and Sub-tropical Fruits (Balitjestro) of Batu, and the plants started to bear fruits in mid-2013. However, in 2015, 700 citrus plants in the *demplot* died from a flood that inundated the plants for over one month. The farmers in Luwu Utara Regency remained highly enthusiastic about the tangerine commodity as proven by the tangerine cultivation that started in 2017 by farmers in Pembuniang Village, Malangke Barat District, being able to yield 10 tons of citrus in a harvest, which happened twice a year.

Based on the PCR detection results, CVPD was identified in three locations, Sidrap, Malangke Barat, and Bantaeng. Most of the citrus leaf samples which visually exhibited typical CVPD symptoms turned out to show no signs of the presence of DNA fragments after PCR visualization result amplification, which means that the samples did not contain the pathogenic bacterium *Candidatus* L. asiaticum. This suggests that the presence of CVPD symptoms in citrus plants does not necessarily indicate that the plants are CVPD-infected. Transmission of the CVPD disease may occur through the insect vector *Diaphorina citri* Kuwayama (Homoptera: Psyllidae)[58] or through infected samplings that are propagated by grafting or bud shielding. Grafting and bud shielding are highly effective in transmitting the bacterium *Candidatus* L. asiaticus. It is challenging to monitor the mobility or traffic of planting materials from one area to another area, opening up the chance of CVPD infections. The samples from three locations were found to be positive for CVPD infection, which probably was caused by disease transmission during propagation by bud shielding/grafting, in which case the shoots were probably extracted from infected parent plants and carried from infected places,[59–61] given that no vector insects were found in the field. This is supported by the finding of CVPD-positive Mandarin (cv. Selayar) in Bantaeng Regency based on PCR detection. The priority citrus plants developed in the regency, i.e., Mandarin (cv. Batu 55), were also detected as CVPD-positive based on PCR detection. Thus, there was a high likelihood that infection occurred during propagation by bud shielding/grafting, or it was feared that the saplings distributed by local farmers were already CVPD-infected. Similarly,[30] discovered that five of six citrus varieties in Taro Village, Gianyar, Bali, Indonesia were CVPD-infected. This location is a new citrus cultivation area which previously was reported to be CVPD-free. It is assumed that the CVPD infection of the citrus plants in this location originated from propagation in another region that had already been infected. In addition, in the five research locations, a number of drawbacks that might threaten the sustainability of the citrus agribusiness were found: persistent bad-quality sapling traffics took place between production centers, the distribution of propagation materials from disease-free parent trees all the way to farmers did not follow the national standard flow, there was a difficulty in accessing rootstocks and scions which led to procurement from other areas and overdependence on sapling producers, and the saplings procured were at times short of the standard. The CVPD control program has grown in complexity as it requires integrated implementation due to the complex interaction between host plants, pathogenic bacteria, insect vector behavior, and farmer habit or behavior in citrus cultivation.[62]

5. Conclusion

The citrus plants in the five regencies under study exhibited varied chlorosis symptoms with a mean incidence rate of 66.56 %. However, the insect vector *Diaphorina citri* was nowhere to be found in the five citrus cultivation areas in South Sulawesi. PCR amplification detected 7 of 70 samples as positive for CVPD as marked by 1160-bp-long DNA bands. The 7 samples were of the varieties of Key lime, tangerine, Mandarin (cv. Batu 55), and Mandarin (cv. Selayar) from Sidrap, Luwu Utara, and Bantaeng.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The author declares no competing interests.

Funding

Not applicable.

Authors contribution

MT designed the research, conducted the experiments, analyzed the data, and writing-original draft. TK conceptualization, supervised experiments and interpreted the data, AN supervised, review and editing, ET supervised and analyzed the data.

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