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, OPN-08, OPN-20, OPW-16, 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.
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 Bontoaharu, 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.

| 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     | Bontoaharu | PP4         |
| 10               | Selayar Biji   | Bontoaharu | PS1         |
| 10               | Selayar-Selayar| Bontoaharu | PS6         |
| 10               | JS-Selayar     | Bontoaharu | PP8         |
| 10               | Selayar Biji   | Bontoaharu | 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 RNAs were 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 ddH2O. 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 72ºC for 60 seconds, final elongation 72ºC 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 band |
| 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 band |
| 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 clear band |
## Primer Sequences

| Primer | Primer Sequences 5'-3' | Tm (ºC) | Ta (ºC) | Total bands | Polymorphic band | Monomorphic band | Quality of produced band |
|--------|------------------------|---------|---------|-------------|------------------|------------------|-------------------------|
| OPR-20 | TCG GCA CGC A          | 44.5    | 45.1    | 12          | 3                | 1                | 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].

Primers 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 Bontomate; 2=selayar oranges from Bontomate; 3=selayar oranges from Bontomate; 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 30.4-45.1°C. The selected primers will be used in the analysis of citrus genetic diversity.

References
[1] El-Mouei R, Choumane W and Dway F 2011 Molecular characterization and genetic diversity in the genus Citrus Syria Int. J. Agric. Biol. 13 151–356
[2] A T, A D C, A M and Sundari 2020 Genetic diversity of lemon(Citrus spp.) from Ternate Island (Indonesia) based on morphological and molecular characterization Biodiversitas 21 1908–13
[3] Al-Janabi A S A 2016 Molecular characterization and genetic diversity analysis of Sweet orange (Citrus sinensis L. Osbeck) cultivars in Iraq using RAPD markers Eur. J. Mol. Biotechnol. 11 4–12
[4] Ahmed S, Rattanpal H S, Kumari P and Singh J 2017 Study of Genetic Variability in Citrus Fruit Crop by Molecular Markers-A Review Int. J. pure Appl. Biosci. 5 111–28
[5] Abobatta W F 2019 Nutritional benefits of citrus fruits Am. J. Biomed. Sci. Res. 303–6
[6] Susanto S 2004 Perubahan kualitas jeruk besar (Citrus grandis (L) osbeck) yang disimpan dan diibirkan di pohon Hayati 11 25–9
[7] Mahardika I B K, Rai I N, Mahendra M S and Dwiyani R 2017 Genetic diversity and fruit qualityof several pomelo “jeruk bali”(Citrus grandis L. Osbeck) cultivars in Bali Int. J. Biosci. Biotechnol. 5 43–59
[8] Shahzadi K, Nasz S and Ilyas S 2016 Genetic diversity of citrus germplasm in pakistan based on random amplified polymorphic DNA (RAPD) markers J. Anim. plant Sci. 26 1094–100
[9] Larekeng S H, Dermawan R, Iswoyo H and Mustari K 2019 RAPD primer screening for
amplification on Katokkon pepper from Toraja, South Sulawesi, Indonesia IOP Conf. Series: Earth and Environmental Science vol 270 pp 1–7

[10] Hadi S N and Nurchasanah S 2020 Genetic diversity of potato based on random amplified polymorphic dna and simple sequence repeat marker J. of Agro Sci. 8 54–62

[11] Larekeng S H, Paelogan R, Cahyaningsih Y F, Nurhidayatullah and Restu M 2020 Primer screening and genetic diversity analysis of jabon putih Anthocephalus cadamba (Roxb) Miq.) based on Random Amplified Polymorphic DNA (RAPD) markers Int. J. Curr. Res. Rev. 12 173–8

[12] Gusmiaty, Sari N A, Safira T N, Budiman A and Larekeng S H 2021 Polimorfisme penanda RAPD untuk analisisisi keragaman genetic kemiri Aleurites mollucana di Kabupaten Maros Bioma J. Biol. Makassar 6 22–30

[13] Jumsari, Darussalam, Syahlena R, Syahputra M, Darnetty R and Putri N E 2007 Seleksi primer RAPD dan studi kekerabatan Capsicum sp. koleksi dari Sumatera Barat J. Akta Agrosis 11 172–81

[14] Salamena F, Hiariej A and Seumahu C A 2018 Genetic characterization of galoba durian Amonum spp. in Ambon Island based on Random Aplified Polymorphic DNA (RAPD) Agrotech J. 3 27–33

[15] Mustafa H, Widayati A N, Gunawan and Rachmawaty 2020 Variasi genetik (polimorfisme) Anopheles barbirostris dengan menggunakan metode RAPD_PCR Prosiding Seminar Nasional Biologi FMIPA UNM pp 97–104

[16] Wen G Q, Li, Liu, Zhang and Wen 2014 Extraction of total DNA and optimization of the RAPD reaction system in Dioscorea opposita Thunb Genet. Mol. Res. 13 1339–47

[17] William J G K, Kubelik A R, Livak K J, Rafalski J A and Tingey S V 1990 DNA polymorphism amplified by arbitrary primers are useful as genetic markers Nucleic Acids Res. 18 6531–5