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Molecular characterization of red ginger varieties (*Zingiber officinale* Roxb. var. *rubrum*) by DNA markers

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Abstract. *Yunita O, Fadhilah NA, Pramadiyanti S, Jonatan S. 2023. Molecular characterization of red ginger varieties (*Zingiber officinale *Roxb. var.* rubrum*) by DNA markers. Biodiversitas 24: 6905-6913.* Red ginger (*Zingiber officinale* Roxb. var. *rubrum*) rhizomes have been traditionally used in Indonesia to increase human health. Therefore, the assurance of raw material quality holds significant importance in the industrial application of red ginger. The primary step in ensuring the quality, safety, and effectiveness of the final product in the industrial environment is the identification of plants used as raw materials for supplements and herbal medications. DNA-based molecular characterization approaches have been specifically developed to aid in the identification of herbal raw materials prior to their application in production processes. Based on the findings derived from the conducted research, the Random Amplified Polymorphic DNA (RAPD) technique can be employed to amplify the DNA isolates of red ginger rhizome samples. The results obtained from the PCR-RAPD amplification of DNA samples extracted from red ginger plants indicated that 90.48% of the total 147 bands seen exhibited polymorphism. The findings suggest a tendency for DNA banding patterns derived from red ginger samples planted in the same geographic area to display clustering tendencies.

Keywords: DNA marker, RAPD, red ginger, *Zingiber officinale* Roxb. var. *Rubrum*

INTRODUCTION

Zingiber officinale Roxb. var. *rubrum*, often known as red ginger, is a botanical species classified under the Zingiberaceae family. This particular variety of ginger is recognized for its distinct rhizome characteristics, including a more potent fragrance and a heightened level of spiciness in comparison to the other two known ginger variations. Moreover, red ginger is acknowledged for its therapeutic properties. The Indonesian population has historically used red ginger as a remedy for colds and back discomfort (FROTI 2017). This plant exhibits a range of pharmacological properties, including analgesic, wound healing, antibacterial, and antioxidant effects (Agusmawanti 2016; Febriani et al. 2018; Pratoko et al. 2018; Saptiwi et al. 2018).

Data from the Central Statistics Agency (BPS) in 2021 shows that there is a high demand for red ginger in Indonesia with an increase in the price of red ginger to IDR 90,000 per kilogram, higher than ginger. This is also supported by the total ginger exports from Indonesia in 2020 reaching 2,370.47 tons (Ministry of Agriculture Republic of Indonesia 2021). Ensuring the quality of raw materials is crucial for the industrial utilization of red ginger in guaranteeing the quality, safety, and efficacy of the final product within the industrial context. These endeavors can be conducted through the examination of macroscopic and microscopic attributes (sensory testing), analysis of the chemical composition, and use of molecular genetic markers, as demonstrated by previous studies (Liu et al. 2014; Ganie et al. 2015; Upton et al. 2020).

Sensory testing encounters difficulties when dealing with plant varieties that exhibit various kinds and/or possess morphological and anatomical properties that are not distinct when compared to other herbs, such as *Sauropus androgynus* (Yunita and Sulisetiorini 2013) and *Glycine max* (Wiradjaja and Yunita 2021). The process of analyzing herbal chemical ingredients is subject to certain limitations. Furthermore, it is important to note that test results frequently exhibit variability due to the fluctuating chemical composition of herbs, which is influenced by factors such as the season of harvest, the habitat of the plant, and the methods employed for post-harvest drying, among others (Yunita et al. 2019; Balekundri and Mannur 2020; Muyumba et al. 2021).

In the past decade, utilizing molecular characterization techniques has significantly contributed to the authentication of herbal raw materials. The primary goals of characterization are to ascertain the identities of accessions and to determine the genetic relationships between genotypes. One advantage of molecular characterization is its species specificity. Moreover, molecular characterization can be conducted on herbs lacking specificity in terms of morphology, anatomy, and chemical content. It also enables the analysis of smaller quantities of samples compared to chemical tests, including powdered herbal samples (Ichim 2019; Wu and Shaw 2022). The examination of herbal DNA polymorphism can be accomplished using a variety of techniques that can be

classified into three primary categories: hybridization-based methods, Polymerase Chain Reaction-based methods (PCR-based methods), and sequencing-based methods (Pauzi et al. 2022; Wu and Shaw 2022).

Random Amplified Polymorphic DNA (RAPD) approach is a variant of the PCR method. It involves the utilization of a single short random primer, typically consisting of 10 base pairs, to amplify the entirety of genomic DNA. This amplification process occurs at very low annealing temperatures, often ranging from 37 to 40°Celsius. The amplicons obtained from the amplification process were subjected to electrophoresis to observe and analyze their distinct DNA banding pattern. The RAPD method has been employed for various purposes, including the investigation of genetic variation (genotyping) in relation to geographical conditions in plant species such as katuk (*Sauropus androgynus*) (Yunita dan Sulisetiorini 2013), *Aloe* sp. (Ezzat et al. 2016), dan *Strychnos minor* (Arumugam et al. 2019), *Curcuma comosa* (Boonsrangsom 2020) and soybeans (*Glycine max*) (Wiradjaja and Yunita 2021) from diverse geographic regions. This method can also identify plants sold as *Strychnos ligustrina* in traditional markets (Yunita dan Kresnamurti 2005). Utilization of RAPD markers has been documented in many studies for the identification of ginger as a herbal raw material (Istiqomah et al. 2016; Hoque and Zohura 2019), the application of tissue culture techniques in ginger plant cultivation (Gavande et al. 2018), and the examination of ginger mutants (Sharim and Shamsiah 2021).

The present study conducted molecular characterization of red ginger using RAPD markers in order to establish the authenticity of red ginger raw materials, thereby initiating the process of ensuring the quality, effectiveness, and safety of red ginger products.

MATERIALS AND METHODS

Plant materials

The samples of red ginger included both dry rhizomes (S1 and S3) and fresh rhizomes (S2 and S4). Sample S1 was obtained from the Simalungun region in North Sumatra. Sample S2 was derived from four distinct cultivars, namely Bengkulu (S2-Be), Jahira (S2-Ja), Banggai (S2-Ba), and Medan (S2-Me). On the other hand, sample S3 was obtained through the cultivation of red ginger seeds in vitro. The seeds were subjected to in vitro cultivation for six weeks, after which they were then acclimatized to become whole plants. The S3 samples were categorized into seven distinct sample codes: S3-JaK (Jahira 2 sample control), S3-JaInv (Jahira 2 sample in vitro), S3-JaSK0 (Jahira 2 sample subculture 0), S3-JaSK1 (Jahira 2 sample subculture 1), S3-JaSK2 (Jahira 2 subculture 2 sample), S3-Pa (Jahira 2 accession Palu sample), and S3-Be (Jahira 2 accession Bengkulu sample). Sample S4 was obtained from the seeds of red ginger sourced from Cianjur, located in West Java. The seeds were planted directly in the soil without any prior planting process.

Procedures

Observation of red ginger samples

The identification of red ginger is based on the observation of morphological traits, namely color, texture, and odor. Several sample properties were seen and documented using a digital camera. Specimen identification was conducted to assess compliance with the requirements provided in the Indonesian Herbal Pharmacopoeia Edition II (2017).

DNA extraction

The DNA extraction from red ginger rhizome was performed using a specific set of reagents provided in the Nucleospin Plant II kit (Macherey-Nagel GmbH & Co. Germany) according to the previous research (Yunita et al. 2016). A quantity of 100 milligrams of plant powder was put into a sterile tube. Following the guidelines outlined in the instruction manual, a volume of 400 µL of lysis buffer PL1 and 10 µL of RNase A were introduced to the initial group of samples. Subsequently, the samples were subjected to an incubation period of 10 minutes at a temperature of 65°C. The lysate was subjected to centrifugation at 11,000 x g for 2 min to facilitate filtration using a spin column. Around 450 microliters (µL) of proteinase K (PC) was introduced into the flow-through solution, and afterwards, the resulting mixture was transferred to the DNA binding column. The column was then subjected to centrifugation at 11,000 x g for 1 min. Following this, the DNA that was attached to the substance was subjected to a single wash using 400 µL of PW1, followed by two further washes using 700 and 200 µL of PW2, respectively. The spin columns underwent centrifugation at 11,000 x g for 1 min after each washing stage. The DNA was ultimately eluted using a 50 µL solution of pre-warmed PE (5 mM Tris–HCl, pH 8.5) at a temperature of 65°C. A twofold elution procedure was conducted using the identical column. The quality and quantity of the DNA isolate was measured using the Nanodrop instrument.

DNA amplification

Subsequently, DNA amplification through the Polymerase Chain Reaction (PCR) was carried out using GoTaq® Green Master Mix (Promega) 2x, employing two sets of RAPD primers, namely OPA 01-20 (Sajeev et al. 2011; Ashraf et al. 2014). In the initial stage, primary screening was carried out to select the type of RAPD primer that produced many polymorphic bands. The results of the RAPD primer screening were a selected type of primer that was able to amplify red ginger DNA isolates containing 4-14 distinct and reproducible polymorphic bands in each sample, as in previous research (Yunita and Sulisetiorini 2013). The DNA amplification process with PCR-RAPD requires the following materials: 2.5µL of selected RAPD primer, 12.5µL of PCR Mix, 1µL of isolated DNA, and 9µL of WFN in a PCR tube. The PCR tube containing the mixture of ingredients is vortexed first for 5 seconds. Next, the tube was centrifuged twice for 5

seconds; then, the tube was inserted into the PCR instrument.

The PCR-RAPD amplification process began with an initial denaturation step at 94˚C for 3 minutes, then continued with a denaturation step at 94˚C for 30 seconds. The next stage is the annealing stage at a temperature of 50˚C for 1 minute and the extension stage at a temperature of 72˚C for 2 minutes. All stages were carried out in 40 cycles. Then, the final stage of PCR is a final extension at a temperature of 72˚C for 5 minutes. After that, the PCR samples were stored in a refrigerator at -20ºC before being used for electrophoresis.

Making 1.5% agarose gel is done by adding 0.6 g agarose with 40 mL of 1x a buffer solution containing a mixture of Tris base, acetic acid and EDTA (TAE Buffer). The mixture of TAE buffer and agarose was then heated for 90 seconds in the microwave. After heated, the mixture is allowed to stand until slightly warm then poured into gel apparatus and let cool until it has solidified and turned opaque. The gel in the mold is poured with TAE buffer solution until all the wells are submerged. The DNA solution resulting from PCR and marker is then injected into the well as much as 6 µL. Electrophoresis procedure was conducted with a voltage of 70 Volts. DNA with a negative charge will exhibit an electrostatic attraction towards the positive pole. Following the completion of the electrophoresis procedure, the agarose gel was submerged in a solution containing ethidium bromide at a concentration of 20 µg/mL for a duration of 20 minutes. The gel was subsequently subjected to a 20-minute wash with aquadem, followed by observation and analysis utilizing the GelDoc Analysis Bio-Rad system.

The repeatability test of the RAPD method was carried out by comparing the DNA banding pattern of red ginger samples on agarose gel, which was carried out seven times using the same sample, isolation procedure, PCR-RAPD amplification, and electrophoresis process and the same operator carried out all processes. The results are then calculated as the standard deviation (SD) and the coefficient of variation (% KV).

Data analysis

The data analysis was performed using the enhanced scoring data. The evaluated molecular data underwent a conversion process to binary data, which was determined by the presence or absence of amplified bands. In the event that a band is amplified, it is assigned a code of 1, and if it is not amplified, it is assigned a value of 0. The distance matrix or genetic dissimilarity was analyzed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) algorithm. This method was employed to depict the clustering of varieties.

RESULTS AND DISCUSSION

Sample characteristics

The findings from the macroscopic analysis of 13 samples have satisfied the criteria outlined in the Indonesian Herbal Pharmacopoeia Edition II (2017). Specifically, the fresh samples were seen to be rhizomes characterized by brown outer skin and a white inner composition. The dried samples exhibited a longitudinal flat shape characterized by a thick, rough outer surface and a fibrous interior surface. Fracture marks were observed, which contained loose fibers. The external layer exhibits a brownish-yellow hue, whereas the internal layer displays a yellowish-white coloration. The aroma commonly associated with ginger and its flavor profile is characterized by spiciness. The physical traits exhibited by the red ginger rhizome samples in Figure 1 present challenges in distinguishing between different cultivars.

The rhizome samples of red ginger were stored in a refrigerator set at a temperature of -20°C, following the methodology employed by Yunita et al. (2016), until the commencement of the sample analysis procedure. The sample is cooled in this manner due to the inactivation of enzymes responsible for the degradation of nucleic acids and plant metabolites at a temperature of -20°C. This ensures preserving the ginger rhizome's freshness and prevents chemical alterations.

Red ginger rhizome DNA extraction

The red ginger rhizome sample underwent particle size reduction prior to the DNA separation method. The methodology employed in this investigation follows the approach outlined in the research conducted by Wiradjaja and Yunita (2021). Their study aimed to decrease the particle size, augmenting the surface area of the sample particles that come into contact with the extraction buffer.

The red ginger simplicia DNA was assessed using Biodrop to determine its quality and quantity. The results revealed that the average red ginger rhizome DNA quality fell within the range of 1.509-1.982, exhibiting a maximum coefficient of variation of 6.11%. The purity of the isolated DNA remains compromised as it does not meet the established criterion for purity, which requires the absorbance ratio of λ 260 nm/λ 280 nm to fall within the range of 1.8 to 2.0. However, subsequent investigations indicate that the isolated DNA can still be utilized for PCR-RAPD analysis.

Additionally, the average quantity of DNA obtained from the 13 samples ranged from 14.47-117.07 ng/µl, with a maximum coefficient of variation of 6.11%. Table 1 displays the outcomes obtained from assessing red ginger DNA's quality and quantity. The PCR-RAPD approach can utilize the number of red ginger DNA isolates for amplification since it adheres to the established RAPD methodology that specifies the inclusion of 5-25 ng of DNA in the PCR process (Mubarak et al. 2020).

The substantial number of DNA isolates can be attributed to the elution phase, when an elution buffer, namely PE, was introduced. The optimization results indicated that PE was incorporated in two elution steps, each consisting of 25 µL. According to Yunita et al. (2016), performing the elution stage twice typically yields a greater quantity of DNA in comparison to conducting a single elution with an equivalent total volume.

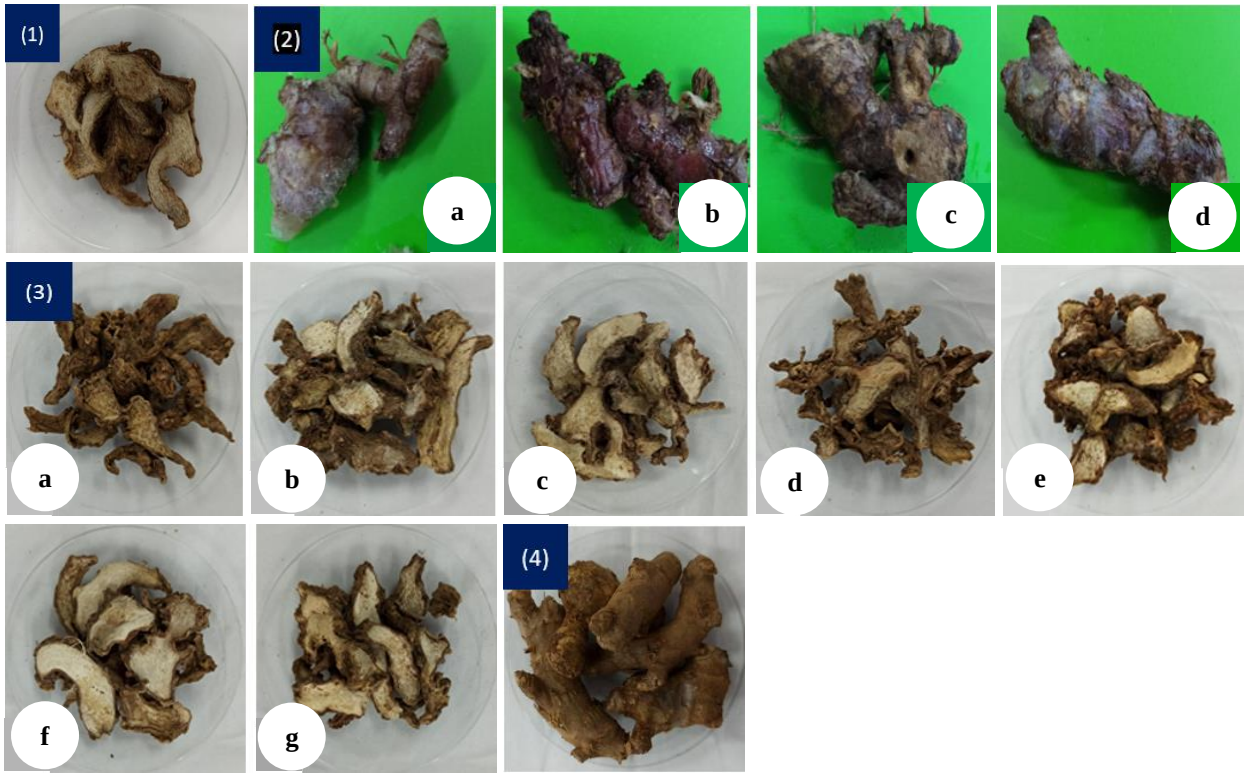

Figure 1. Red ginger cultivar samples from different geographical regions. 1. Dried rhizome samples from Simalungun, North Sumatra (S1); 2. Fresh rhizome samples from (a) Bengkulu, Southwestern Sumatra (S2-Be), (b) Jahira, Sumatra (S2-Ja), (c) Banggai, (S2-Ba), (d) Medan, North Sumatra (S2-Me). 3. Dried rhizome samples obtained through the cultivation of red ginger seeds in vitro (a) Jahira 2 sample control, S3-JaK (b) Jahira 2 sample subculture 0, S3-JaSK0, (c) Jahira 2 sample subculture 1, S3-JaSK1, (d) Jahira 2 sample subculture 2, S3-JaSK2, (e) Jahira 2 in vitro, S3-JaInv, (f) Jahira 2 accession Palu sample, S3-Pa, (g) Jahira 2 accession Bengkulu sample, S3-Be. 4. Fresh rhizome samples from Cianjur, West Java (S4)

Table 1. Qualitative and quantitative estimates of DNA concentration revealed by Nanodrop Spectrophotometer of rhizome samples of red ginger cultivar samples

Sample	DNA quality (A260/A280)		DNA concentration (ng/µl)	
	Mean + SD	CV%	Mean + SD	CV%
S1	1.877 ± 0.0153	0.81	66.77 ± 0.855	1.26
S2-Me	1.982 ± 0.0054	0.27	117.07 ± 2.418	2.08
S2-Ba	1.915 ± 0.0347	1.81	18.15 ± 0.683	3.76
S2-Be	1.947 ± 1.1188	6.11	11.72 ± 0.715	6.11
S2-Ja	1.911 ± 0.0338	1.77	47.15 ± 1.0528	2.25
S3-JaK	1.941 ± 0.0272	1.41	36.24 ± 1.012	2.81
S3-JaInv	1.619 ± 0.0500	3.06	17.79 ± 0.397	2.18
S3-JaSK0	1.663 ± 0.0193	1.16	46.07 ± 0.969	1.95
S3-JaSK1	1.944 ± 0.0097	0.50	110.25 ± 0.772	0.70
S3-JaSK2	1.687 ± 0.0389	2.31	14.47 ± 0.550	3.81
S3-Pa	1.637 ± 0.0091	0.55	20.25 ± 0.931	4.28
S3-Be	1.509 ± 0.0309	2.05	25.65 ± 0.681	2.60
S4	1.884 ± 0.0363	1.88	25.26 ± 0.634	2.66

Note: Mean and Coefficient of Variation, CV (Standard Deviation Divided by Mean) Times 100 (i.e., in Percentage) of Selected Variables. Note: S1: Dried rhizome samples from Simalungun, North Sumatra; S2-Me: Fresh rhizome samples from Medan, North Sumatra; S2-Ba: Fresh rhizome samples from Banggai; S2-Be: Fresh rhizome samples from Bengkulu, Southwestern Sumatra; S2-Ja: Fresh rhizome samples from Jahira, Sumatra; S3-JaK: Dried rhizome samples obtained through the cultivation of red ginger seeds in vitro Jahira 2 sample control; S3-JaInv: Jahira 2 in vitro; S3-JaSK0: Jahira 2 sample subculture 0, S3-JaSK1: Jahira 2 sample subculture 1, S3-JaSK2: Jahira 2 sample subculture 2, S3-Pa: Jahira 2 accession Palu sample, S3-Be: Jahira 2 accession Bengkulu sample, S4: Fresh rhizome samples from Cianjur, West Java

Identification of red ginger cultivar

The RAPD primers used in screening were 40 types of primers from two primer sets, namely OPA1-20 and OPAA1-20, which were selected based on DNA array patterns produced in previous research related to ginger and plants in the Zingiberaceae family by Sajeev et al. (2011) and Ashraf et al. (2014). Sequence of each primer had been shown at Supplementary Material. The two sets of primers were chosen based on several previous publications that amplified ginger DNA using the RAPD method, which produced polymorphic bands that could differentiate between cultivars or varieties. The primary screening of OPA 01-20 and OPAA 01-20 resulted in a diverse range of DNA banding patterns characterized by varying band strengths and the presence of smears in several bands. DNA banding pattern is a DNA band profile consisting of a number of bands of different molecular sizes, characterized by the presence of dark colored bands in the electrophoresis results. Based on the bands' number, intensity and clarity, the primers chosen to amplify the samples were OPA-05, OPA-12, OPAA-03, and OPAA017, with the primer sequence as in Table 2.

For the subsequent step, the utilization of the OPAA 17 primer was employed to conduct repeatability testing (Figure 2), serving as the foundation for the examination of DNA banding patterns in order to ascertain distinct markers and perform cluster analysis. Samples 1-7 that were injected into the well during electrophoresis were the same red ginger PCR samples, originating from Simalungun, North Sumatra, which were injected repeatedly in an effort to ensure the reproducibility of the method. The amplification capability of the OPAA 17 primer extends to all sample types.

Observing the DNA banding pattern (S1) of the red ginger rhizome in Figure 2 revealed twelve (12) DNA bands, indicated by pink lines, with fragment sizes ranging from 210-1500 bp and a maximal coefficient of variation of 4.51%. The repeatability test results indicate that molecular characterization of red ginger rhizomes using the PCR-RAPD method with the OPAA-17 primer generated a consistent DNA banding pattern, as indicated by the consistent number of bands and band positions. The DNA of all red ginger rhizome samples can be amplified using the PCR-RAPD method based on the results of the repeatability test.

Figure 3 shows no DNA band measuring 634-669 bp was detected in the red ginger samples obtained from Batu (Materi Medika Indonesia) and sample S1. This absence suggests that this particular DNA band may serve as a potential specific marker for red ginger samples, particularly in the case of certain samples (S2, S3, S4). However, further investigation is required to confirm its uniqueness compared to other red ginger samples.

Table 2. Four RAPD primers used for the amplification of DNA fragments on red ginger

Primer	Primer Sequence (5' - 3')
OPA-05	AGGGGTCTTG
OPA-12	TCGGCGATAG
OPAA-03	TTAGCGCCCC
OPAA-17	GAGCCCGACT

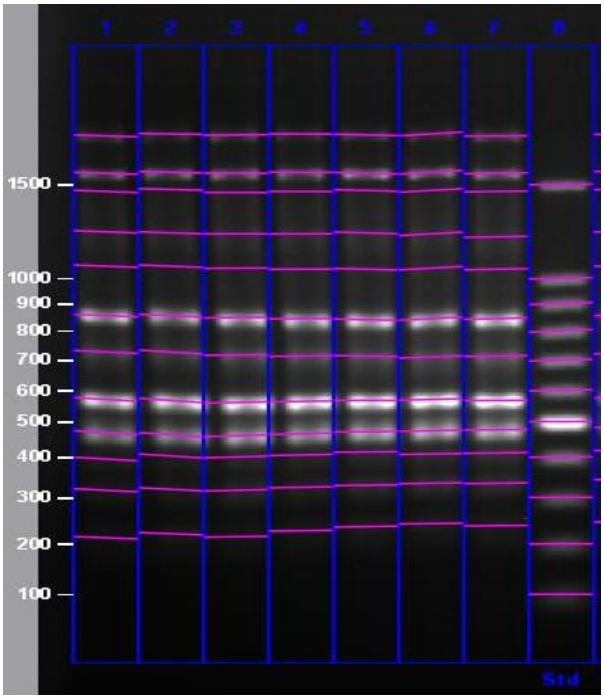

Figure 2. Results of a PCR-RAPD method repeatability test. ote: 1-7: Sample S1 (Dried rhizome samples from Simalungun, North Sumatra), replication 1-7; 8: Marker BenchTop 100bp DNA Ladder

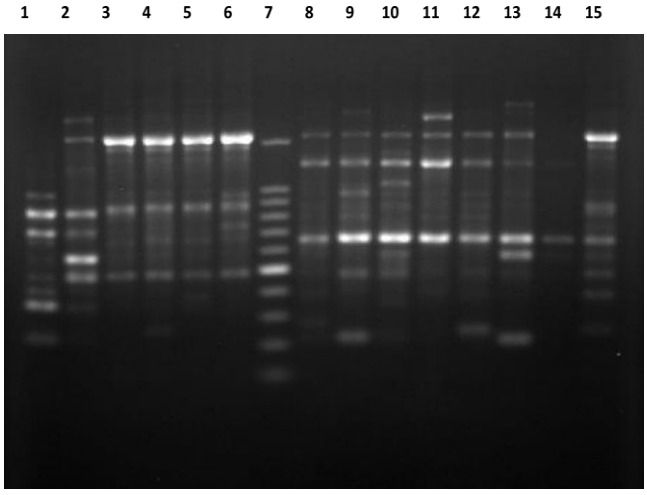

Figure 3. Visualization Results of RAPD PCR with OPAA-17 Primers in All Samples. Note: Note: 1. MMI: Dried rhizome samples from Materia Medica Batu, East Java; 2. S1: Dried rhizome samples from Simalungun, North Sumatra; 3. S2-Me: Fresh rhizome samples from Medan, North Sumatra; 4. S2-Ba: Fresh rhizome samples from Banggai; 5. S2-Be: Fresh rhizome samples from Bengkulu, Southwestern Sumatra; 6. S2-Ja: Fresh rhizome samples from Jahira, Sumatra; 7. marker BenchTop 100bp DNA ladder; 8. S3-JaK: Dried rhizome samples obtained through the cultivation of red ginger seeds in vitro Jahira 2 sample control; 9. S3-JaInv: Jahira 2 in vitro; 10. S3-JaSK0: Jahira 2 sample subculture 0; 11. S3-JaSK1: Jahira 2 sample subculture 1; 12. S3-JaSK2: Jahira 2 sample subculture 2; 13. S3-Pa: Jahira 2 accession Palu sample; 14. S3-Be: Jahira 2 accession Bengkulu sample; 15. S4: Fresh rhizome samples from Cianjur, West Java

Based on the amplification of red ginger DNA isolates with the primer OPAA 17 in the PCR-RAPD method, the monomorphic bands found in all red ginger samples were DNA bands measuring 461-491 bp. A DNA band of 1500 base pairs (bp) was detected in all red ginger samples (S1, S2, S3, S4). Additionally, most samples (S2, S3, and S4) exhibited a DNA band ranging in size from 634 to 669 bp.

Polymorphic bands that exhibit differential characteristics across red ginger samples (S1, S2, S3, S4) may be observed in the DNA band ranging from 1409 to 1443 base pairs (bp), exclusively present in samples S2 and S4. Additionally, the DNA band measuring 1178 to 1261 bp is exclusively present in samples S1 and S3. Furthermore, a multitude of polymorphic bands exist that exhibit variation among different samples of red ginger. The proportion of polymorphic bands observed in the PCR-RAPD amplification outcomes utilizing the OPAA 17 primer was found to be 90.48% out of a total of 147 bands. The findings presented exhibit a resemblance to the outcomes obtained from the DNA amplification of *Curcuma longa*, often known as turmeric, originating from India. This particular plant belongs to the Zingiberaceae family. The PCR-RAPD method, employing primer OPAA 17, was employed in both studies, resulting in the generation of 90% polymorphic bands. This parallel is highlighted in the research conducted by Ashraf et al. in 2017.

The present study involves an examination of the outcomes obtained from the amplification of DNA isolates derived from red ginger samples (S1, S2, S3, S4) using the OPAA 17 primer in the PCR-RAPD technique. The experimental procedure led to the construction of a dendrogram, as depicted in Figure 4. In this figure, the abbreviation MMI represents the code assigned to dry red ginger simplicia samples obtained from Materia Medika Indonesia, Batu, which were included in the analysis for comparative purposes.

The dendrogram illustrates that samples cultivated in the same geographical area, such as S2 or S3, exhibit a tendency to cluster together. However, it is worth noting that the dry sample Jahira sub culture 0 (S3-JaSK0) deviates from this pattern, as it demonstrates greater similarity to clusters S2 and S4 rather than cluster S3. Based on an analysis of the DNA banding patterns observed in the S3 samples derived from in vitro cultivation, it was observed that the dry samples of Jahira subculture 0 (S3-JaSK0) and Jahira subculture 1 (S3-JaSK1) exhibited several distinct polymorphic bands. Notably, the DNA bands measuring 1054 bp and 1362 bp were observed in S3-JaSK0, whereas a DNA band measuring 1139.2 bp was present in S3-JaSK1. Additionally, DNA bands measuring 1243-1261 bp were consistently detected in all S3 samples that were initially cultivated using in vitro techniques. The in vitro cultivation process utilizing plant tissue culture techniques has the potential to induce genetic variations, also known as somaclonal variations. This phenomenon is attributed to the influence of multiple factors, such as the composition of growth regulators, temperature, light conditions, osmolarity, and agitation rate within the culture medium. These factors have been observed to impact the regulation of the plant cell cycle during in vitro cultivation (Krishna et al. 2016).

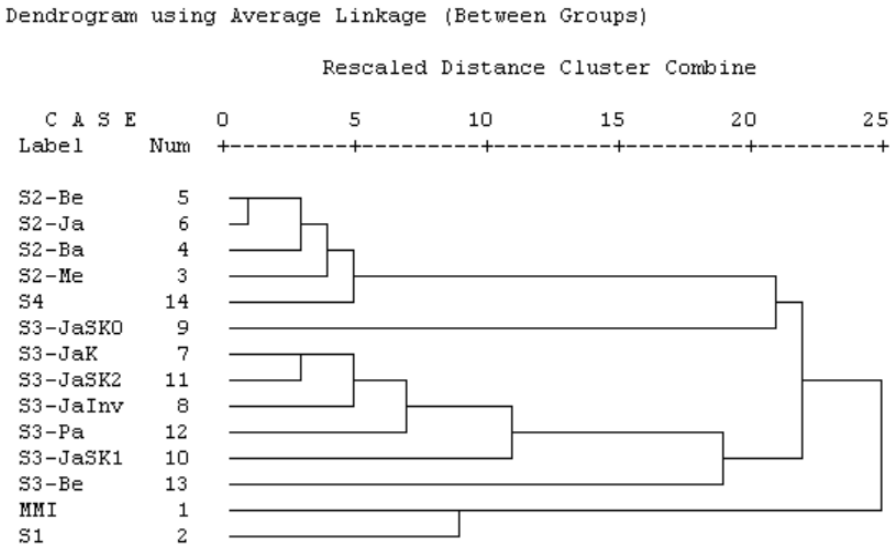

Figure 4. Dendrogram of 13 samples of red ginger from various locations in Indonesia. Note: 1. MMI: Dried rhizome samples from Materia Medica Batu, East Java; 2. S1: Dried rhizome samples from Simalungun, North Sumatra; 3. S2-Me: Fresh rhizome samples from Medan, North Sumatra; 4. S2-Ba: Fresh rhizome samples from Banggai; 5. S2-Be: Fresh rhizome samples from Bengkulu, Southwestern Sumatra; 6. S2-Ja: Fresh rhizome samples from Jahira, Sumatra; 7. S3-JaK: Dried rhizome samples obtained through the cultivation of red ginger seeds in vitro Jahira 2 sample control; 8. S3-JaInv: Jahira 2 in vitro; 9. S3-JaSK0: Jahira 2 sample subculture 0; 10. S3-JaSK1: Jahira 2 sample subculture 1; 11. S3-JaSK2: Jahira 2 sample subculture 2; 12. S3-Pa: Jahira 2 accession Palu sample; 13. S3-Be: Jahira 2 accession Bengkulu sample; 14. S4: Fresh rhizome samples from Cianjur, West Java

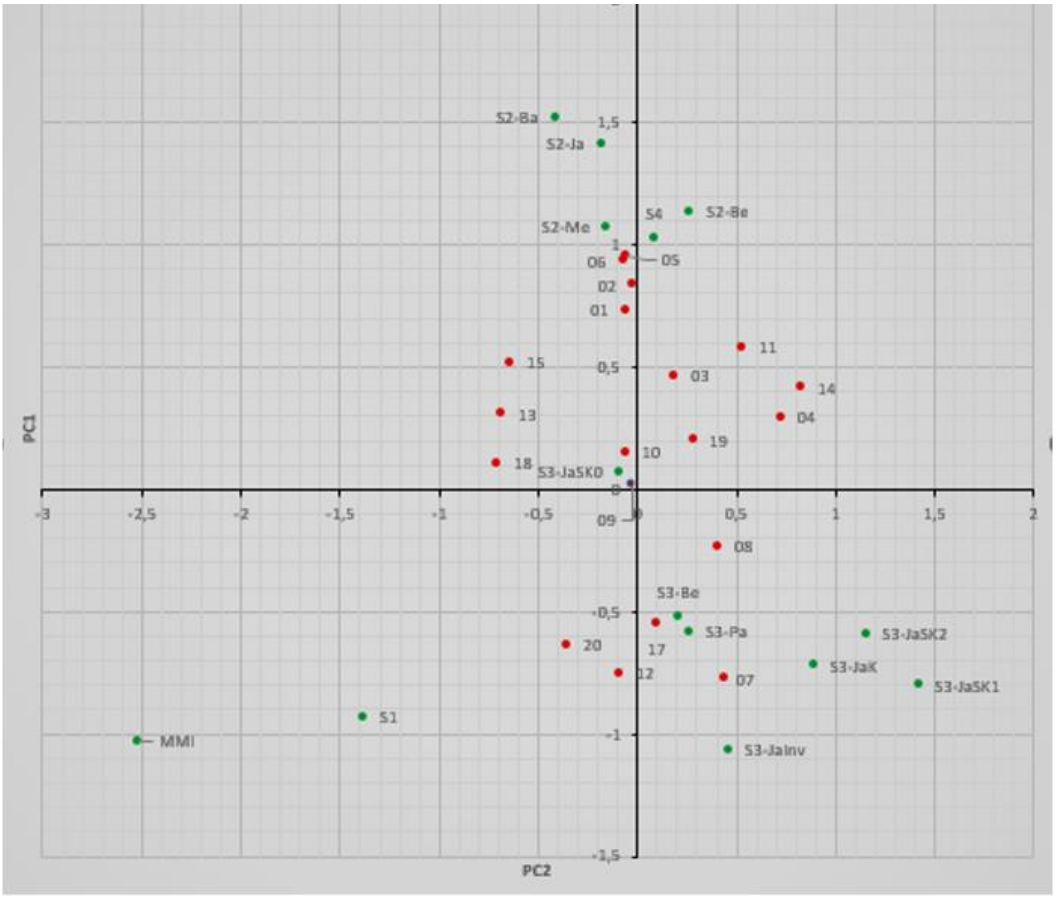

Figure 5. Bi-plot of DNA of red ginger samples based on the DNA banding pattern resulting from amplification with OPAA 17 primer using the PCR-RAPD method. Note: MMI: Dried rhizome samples from Materia Medica Batu, East Java; S1: Dried rhizome samples from Simalungun, North Sumatra; S2-Me: Fresh rhizome samples from Medan, North Sumatra; S2-Ba: Fresh rhizome samples from Banggai; S2-Be: Fresh rhizome samples from Bengkulu, Southwestern Sumatra; S2-Ja: Fresh rhizome samples from Jahira, Sumatra; S3-JaK: Dried rhizome samples obtained through the cultivation of red ginger seeds in vitro Jahira 2 sample control; S3-JaInv: Jahira 2 in vitro; S3-JaSK0: Jahira 2 sample subculture 0, S3-JaSK1: Jahira 2 sample subculture 1, S3-JaSK2: Jahira 2 sample subculture 2, S3-Pa: Jahira 2 accession Palu sample, S3-Be: Jahira 2 accession Bengkulu sample, S4: Fresh rhizome samples from Cianjur, West Java

The observed resemblance between samples S2 and S4, despite originating from planting regions with varying elevations, may be attributed to the genetic affinity among the red ginger plants from which the samples were obtained. The findings of a study conducted by Yunita and Sulisetiorini (2013) align with the present observations. They employed RAPD primers to amplify the Internal Transcribed Spacer (ITS) region of *S. androgynus* DNA. Their results indicated the existence of genetic resemblances among *S. androgynus* plants cultivated in diverse geographical locations, thus corroborating the current findings. The DNA sequence of each sample is influenced not only by the geographic proximity but also by the genetic characteristics of the plant from which it originates.

The bi-plot graph derived from the results of Principle Component Analysis (PCA) reveals the distinct DNA bands responsible for defining the unique characteristics of the red ginger sample's DNA banding pattern (Figure 5). Additionally, the DNA bands were amplified through PCR-RAPD using OPAA 17 primers. Principal Component Analysis (PCA) results are typically represented through the use of score and loading charts, also used by Hoque and Zohura (2019). The score plot indicates the proximity between samples and the potential for distinguishing them into distinct groups. In the interim, the loading plot illustrates the variable (DNA band) that exerts the most influence on the composition and structure of the created group. The examination of both plots facilitates the elucidation of the relationship between factors and the categorization of samples.

The bi-plot graph obtained from principal component analysis (PCA) reveals that the DNA bands play a prominent role in defining the clustering patterns observed in the red ginger samples. The predominant DNA bands seen in red ginger samples S2 and S4 were identified as band no. 6, measuring 1285-1329 bp, and band no. 5, measuring 1409-1443 bp, as indicated in Figure 5. The DNA bands that influence the categorization of S3 red ginger samples include bands measuring 217-225 bp (band no. 20), 380-417 bp (band no. 17), 796-804 bp (band no. 12), and 1242-1261 bp (band no. 7).

Based on the findings derived from conducted research, several conclusions may be drawn about the molecular characterization of red ginger (*Z. officinale* var. *rubrum*) by the utilization of the Random Amplified Polymorphic DNA (RAPD) technique. The PCR-RAPD method can be employed to amplify the DNA isolates of red ginger rhizome samples. This suitability is supported by the consistent results observed in the method's repeatability test, which demonstrated uniformity in the number and size of DNA bands. The maximum coefficient of variation recorded was 4.51%.

The PCR-RAPD primer screening findings utilizing the OPA1-20 and OPAA 1-20 kits show the existence of four primers, specifically OPA 5, OPA 12, OPAA 3, and OPAA 17. These primers provide discrete DNA banding patterns with no smearing and generate an adequate number of DNA bands for identifying DNA polymorphisms in samples. The OPAA-17 primer is a Random Amplified Polymorphic DNA (RAPD) primer that can amplify all red ginger samples, including the S1, S2, S3, and S4 samples. The DNA band observed in all red ginger samples is monomorphic and measures 461-491 base pairs (bp). However, in the majority of red ginger samples (S2, S3, and S4), a distinct DNA band spanning 634-669 bp is present. This particular DNA band exhibits the potential to serve as a specific marker candidate. The findings from PCR-RAPD amplification of red ginger DNA samples revealed that 90.48% of the total 147 bands were polymorphic. Moreover, the results indicate that DNA banding patterns obtained from red ginger samples cultivated in the same geographical location, such as S2 or S3, tend to exhibit clustering tendencies. However, it is worth noting that the dry sample Jahira sub culture 0 (S3-JaSK0) deviates from this pattern. The clustering of red ginger samples may be attributed to the existence of distinct DNA bands of a particular size, indicating that these DNA bands could serve as specific attributes for particular clusters of red ginger samples.

Molecular characterization of red ginger varieties (*Z.officinale* var. *rubrum*) by RAPD markers are not only useful for controlling the quality of raw materials but also for ensuring the genetic stability of herbal raw material plants cultivated using the plant tissue culture system as has been carried out in our previous research using RAPD as a preliminary technique to evaluate the genetic similarity of the shoot cultures of *S. androgynus* and their corresponding mother plants (Yunita et al. 2021). Plant tissue culture can induce genetic and epigenetic modifications, leading to heritable alterations in phenotypes. This occurrence is commonly referred to as somaclonal variation. The presence of somaclonal variations during the process of in vitro propagation and industrial production of phytochemicals can have significant economic implications and poses a substantial challenge in the practical application of plant tissue culture methods for the synthesis of bioactive compounds. Hence, to evaluate somaclonal variation in a cell culture, it is imperative to systematically observe and evaluate the genetic composition and constancy of the plants regenerated in vitro (Wang et al. 2013; Espinosa-Leal et al. 2018).

In future research, it is necessary to validate further analysis so that the results of the molecular characterization of red ginger raw materials can be used as a comparative analysis, not only for the analysis of new sample raw materials, but also for subsequent research purposes, both for molecular characterization of red ginger cultures and herbal products containing red ginger.

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