Optimization of annealing temperature for amplification of
Ehoscn01a locus in pranajiwa (Euchresta horsfieldii) plant
collected from mountains, urban and coastal areas in Bali

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Abstract. Pranajiwa plant is a medicinal plant that grows wildly and is classified as a rare plant. Currently, its existence is increasingly threatened. Pranajiwa grows around Indonesia and is known with several scientific names and morphological features due to unclear identification. Molecular identification is recommended to clarify its species. DNA Barcoding is considered the suitable method to identify pranajiwa plant molecularly. The purpose of this study was to optimized the PCR annealing temperature of EhcSn01a locus barcoding marker of pranajiwa plants collected from the coastal (Jimbaran), urban (Renon), and mountain (Bedugul) areas, representing three different areas in Bali. Research procedures include total DNA extraction, PCR procedure, and electrophoresis. The primers used in this study were EhoScn01a forward primer and Ehoscn01a reverse primer. Five different temperatures were used for annealing temperature optimization: 51°C, 52°C, 55°C, 57°C, and 60°C. The result showed that all temperatures produced a clear, thick, and single electrophoresis band, indicating that all temperatures were suitable for the annealing temperature and the most optimal temperature is in the Mountains sample (Bedugul) which is 60°C. The Jimbaran, Renon, and Bedugul samples produced 882, 820, and 889 bp, respectively. EhcSn01a locus can be used as the barcoding marker to identify pranajiwa molecularly.

1. Introduction

Pranajiwa, Euchresta horsfieldii plant is a perennial shrub plant spread in China, India, Indonesia, Laos, Nepal, Philippines, Thailand, Bhutan and Vietnam [1]. In Indonesia, the natural habitat of E. horsfieldii is limited to forests with an altitude of 1300-2400 m above sea level. Euchresta horsfieldii is used in traditional herbal medicine in Indonesia, and its pharmacological properties have been evaluated for antitumor, antioxidant, and lipid reducing agents [2]. Despite its potential as an Asian medicinal plant, its existence and endangered habitat, as well as studies on the conservation genetics of these plant species are still under-researched. The purnajiwa plant can be found in several areas, including in Bali it is known as pranajiwa, in Java it is called pronojiwo, while in Indonesia the common name is pranajiwa.

One of the important steps in molecular identification is polymerase chain reaction (PCR). Polymerase Chain Reaction (PCR) is a technique for amplifying DNA sequences in vitro which was developed by Karry Mullis a Biochemist in 1984. The use of PCR techniques has been expanded in
molecular research aimed at amplifying target regions and multiplying template DNA fragments in relatively small volumes with relatively short time. The replication ranges from 30-50 replication cycles, which multiply the target DNA molecule in each cycle [3]. There are several steps in PCR, namely pre-denaturation for 2 minutes at 95°C, denaturation for 30 seconds at 95°C, annealing (primer attachment) for 30 seconds at 50°C, elongation for 50 seconds at 72°C and final DNA polymerization for 1 minutes at 72°C. The cycle was carried out 35 times [4].

The PCR reaction is strongly influenced by the concentration of the reaction components (MgCl2, buffer, DNA polymerase enzymes, DNA templates, primers, nucleotides, and H2O), denaturation temperature, primer attachment temperature on template DNA, primer elongation temperature, number of cycles, and DNA template and purity (Joseph 2010). An important factor of PCR components that can affect the success of PCR is the use of appropriate primers. Primer is a short oligonucleotide sequence used to initiate DNA synthesis in PCR.

In addition to the PCR components, temperature annealing the optimum (Ta) also affects the success of the amplification process. The annealing temperature is the temperature for the attachment of the primer to the template DNA. Melting temperature (Tm) is the basis for determining the optimum temperature by varying the annealing temperature during amplification. At the annealing stage, one of the factors that affect the success of amplification is temperature because the process of attaching the primer to an already open DNA strand requires an optimal temperature. If the temperature is too high it will cause the amplification to fail because there is no attachment to the other side of the genome as a result the DNA formed has low specificity, so it is very important to find the optimum annealing temperature for the amplification process. Because the annealing process is a very important process, it is reasonable to find the optimum temperature, so it is hoped that the maximum amount of DNA can be obtained in the targeted area so that it is quite easy for DNA analysis.

Temperature optimization is carried out to obtain optimal PCR conditions, so that PCR products are produced. One way that can be done to optimize the annealing temperature is to use an annealing temperature that ranges up to 5°C lower than the Tm (Temperature of melting) of the primary pair. The annealing process requires a short time ranging from 30 seconds or less than 30 seconds if the Ta (Temperature of annealing) is close to Tm or unless the primer is not too long as usual [5].

Previous research on pranajiwa (Chao Feng et. al., 2018) which is "Development of 10 single-copy nuclear DNA markers for Euchresta horsfieldii (Fabaceae), a rare medicinal plant" test marker scnDNA 10 primer pairs were obtained for E. horsfieldii and E japonica. Successfully used primers to assess polymorphism of three wild populations of E. horsfieldii from Indonesia and one population of E. tubulosa Dunn from China. Therefore, this sequence of scnDNA markers will assist in the genetic study of populations of E. horsfieldii and related taxa.

This study aimed to obtain temperature annealing the optimal to amplify the locus area EhoScn01a in pranajiwa plants (Euchresta horsfieldii) from Bali.

2. Method

2.1 Total DNA Extraction

Total DNA extraction of the pranajiwa plant (Euchresta horsfieldii) from Bali was carried out using the ZymoBiomics Miniprep Kit (Zymo Research, D4300). Total DNA electrophoresis was carried out at 50 volts for 45 minutes. Visualized electrophoresis results using UV Transilluminator and digital cameras with filter UV. The test with a spectrophotometer or nanodrop in principle is to calculate the difference in UV light absorption where double bands of DNA can absorb UV light at 260 nm, while protein or phenol contaminants can absorb light at 280 nm. The value of DNA purity was calculated by
dividing the absorbance of 260 nm by the absorbance value of 280 (Å260/Å280), and the value of DNA purity ranged from 1.8 to 2.0.

2.2 PCR (Polymerase Chain Reaction)

PCR amplification using MyTaq HS Red Mix (Bioline, BIO-25047). The primer used for the amplification of the locus EhoScn01a is the extracted DNA amplified by PCR using primer pairs for the EhoScn01a loci forward 5’- AAG TTC CGC TTC GAA TC – 3’ and reverse 5’– GTA ATT ACC TTC GCC TGG GG -3’ with a fragment size of 646 bp. The PCR Master Mix Bio 25048 component consisted of 22 L ddO (1x), 25 l MyTaq Red Mix 2x (1x), 1 l 10 M ITSL Primer (0.4 M), 1x 10 M ITS4 Primer (0.4 M), 1 l DNA Template (50 ng/µl).

The PCR program used consisted of pre-PCR with a temperature of 95℃ for 3 minutes, Denaturation at 95 for 15 seconds, primer attachment (annealing) was carried out with several optimization temperatures, namely Tm-1 (51℃), Tm-2 (52 ), Tm-3 (55℃), Tm-4 (57℃), and Tm-5 (60℃) for 30 seconds. Stages of elongation with a temperature of 72℃ for 45 seconds. And the last step is Hold with a temperature of 16℃ until it is removed from the machine.

2.3 Electrophoresis

The PCR products were migrated on 1% agarose using 1x TBE buffer. DNA electrophoresis of PCR results was carried out at a voltage of 50 volts for 45 minutes. The results of the electrophoresis were then visualized by a UV transilluminator and photographed using a UV filtered digital camera.

3. Results and Discussion

The total DNA molecule obtained and the total DNA extraction process of the pranajiwa plant (Euchresta horsfieldii) from Bali. Pranajiwa (Euchresta horsfieldii) belongs to the Fabaceae family and is one of the forest plants that has the potential as a source of traditional medicine. This plant lives in forests with lots of shade and thick litter as forest humus. Total DNA isolation in Pranajiwa plants was carried out using the kit method Zymo Research. Pranajiwa plant DNA was amplified using ZR Plasmid MiniPrep with the target area being the locus EhoScn01a. The samples used are 3, which are located in the coastal area, namely Jimbaran, the Urban area, namely Renon, and the mountainous area, namely Bedugul.

The results of electrophoresis of PCR products describe the number and size of bands (band patterns) that differ from each temperature. This difference is influenced by the purity and concentration of total DNA. If the total DNA used contains phenolic, polysaccharide compounds, and the DNA concentration is low, it will produce an unclear or faint band [6]

3.1 The Assessment of DNA Quality and Quantity

Quality of each extracted pranajiwa leaf genome DNA sample was verified spectrophotometrically using the instrument NanoDrop and agarose gel electrophoresis. The visualization of the extraction of genomic DNA from local Balinese pranajiwa leaves consisting of three samples from various regions including plants from the Coast (Jimbaran), Urban (Renon) and Mountains (Bedugul) showed different results, as seen from the strong luminescence and integrity of the genomic DNA band. on samples from the Mountains. The absorbance value of NanoDrop is useful for detecting contaminants such as proteins, salts, and polysaccharides that can inhibit DNA amplification.

In a DNA isolation technique, a step is still needed to minimize contaminant compounds that can interfere with the PCR reaction such as polysaccharides and secondary metabolites. This is because the presence of polysaccharides and secondary metabolites in plant cells often makes it difficult to isolate nucleic acids [7].
The content of secondary compounds in plant cells varies, so each plant requires an optimum isolation procedure in order to obtain genomic DNA that can be used as material in molecular analysis. Optimization of the procedure can be carried out on the composition of the lysis buffer solution or physical handling techniques in the separation of genomic DNA from other compounds. In principle, this optimization procedure aims to protect genomic DNA from degradation due to secondary compounds released when cells are destroyed or damaged due to physical handling [8].

The thick and clumped (non-spooling) DNA bands showed high concentrations and the total extracted DNA was intact. Meanwhile, the visible DNA bands showed that the bonds between DNA molecules were broken during the extraction process, so that the DNA genome was cut into smaller parts. The breaking of the bonds between the molecules can be caused by excessive physical movement that can occur in the pipetting process, when being turned over in an eppendorf, centrifuged, or even because the temperature is too high and due to the activity of certain chemicals.

**Table 1. Value of genomic DNA quality of pranajiwa plants isolated using a spectrophotometer (NanoDrop).**

| No | Sample Name                        | Sample Code | Conc. (ng/µl) | A<sub>260/280</sub> | Volume (µl) |
|----|-----------------------------------|-------------|---------------|----------------------|-------------|
| 1  | Tanaman dari Pesisir (Jimbaran)   | D1          | 54.0          | 1.87                 | 30          |
| 2  | Tanaman dari Urban (Renon)        | D2          | 50.6          | 1.80                 | 30          |
| 3  | Tanaman dari Pegunungan (Bedugul) | D3          | 39.7          | 1.90                 | 30          |

The purity of DNA obtained in this study ranged from 1.80 to 1.90. The ratio at 260/280 nm wavelength reading of 1.8 indicates that the extracted DNA was of high purity in the absence of protein and phenol [9]. From the three samples of pranajiwa plant DNA, the purity values were Coastal (1.87), Urban (1.80) and Mountain (1.90). According to Pervaiz et al. (2011), the A260/280 ratio with a range from 1.8 to 2.0 did not show significant levels of contaminants [10].

3.2 Locus Amplification Fragment EhoScn01a on Multiple Temperature Annealing

Temperature optimization results annealing for amplification of loci EhoScn01a on pranajiwa plants can be seen in Figures 1, 2 and 3.
Figure 1. Profile DNA temperature optimization results annealing PCR using EhoScn01a the sample D1 pranajiwa plant (Euchresta horsfieldii) from Coastal (Jimbaran) from Bali at Tm-1 (51°C), Tm-2 (52°C), Tm-3 (55°C), Tm-4 (57°C), Tm-5 (60°C). M= 1000 bp DNA ladder.

Figure 2. Profile of DNA bands from optimization of temperature annealing PCR using primer EhoScn01a on D2 sample of pranajiwa plant (Euchresta horsfieldii) from Urban (Renon) from Bali at Tm-1 (51°C), Tm-2 (52°C), Tm-3 (55°C), Tm-4 (57°C), Tm-5 (60°C). M= 1000 bp DNA ladder.

Figure 3. Profile of DNA bands from optimization of temperature annealing PCR using primer EhoScn01a on D3 sample of pranajiwa plant (Euchresta horsfieldii) from Mountains (Bedugul) from Bali at Tm-1 (51°C), Tm-2 (52°C), Tm-3 (55°C), Tm-4 (57°C), Tm-5 (60°C). M= 1000 bp DNA ladder.

The results of the amplification of the locus in EhoScn01apranajiwa plants obtained DNA bands with temperatures annealing at Tm-1 (51°C), Tm-2 (52°C), Tm-3 (55°C), Tm-4 (57°C), Tm-5 (60°C). Based on the results of the study to obtain the locus fragment EhoScn01a from the D1 sample of pranajiwa plants from the Coastal (Jimbaran) and D2 samples of pranajiwa plants from Urban (Renon) can be amplified at Tm-1 with the temperature annealing optimum is at a temperature of 51°C (Fig. 1). the gel is thin and sharper than Tm-2 which is very thin, while Tm-3, Tm-4, Tm-5 have not yet obtained a specific and sharp amplification band using a primer, it is suspected that the sample used cannot attach to the DNA template. According to Haris et al. (2003), the concentration of DNA will have an impact on the quality of the
amplified fragments. A DNA concentration that is too low will result in very thin fragments on the gel or even invisible visually, on the other hand, a DNA concentration that is too high will cause the fragments to appear thick so that it is difficult to distinguish one fragment from another. [6]

Visualization results of agarose gel from D3 samples of pranajiwa plants (Euchresta horsfieldii) from the Mountains (Bedugul), at Tm-1 (51°C), Tm-2 (52°C), Tm-3 (55°C), Tm-4 (57°C), Tm-5 (60°C) gives a clear, single and thick band image (Fig. 3). The Temperature of annealing 60°C is the optimal temperature for the primer amplification reaction EhoScn01a, because the DNA band of pranajiwa plants looks the thickest compared to the PCR reaction with temperature annealing other. It is known that DNA amplification works optimally to produce amplification products with high specificity.

The temperature is annealing determined based on the primary temperature (Tm), generally plus minus 5°C from the Tm temperature. Primer EhoScn01a forward has a temperature (Tm) of 54°C and EhoScn01a reverse has a temperature (Tm) of 55°C. So the temperature annealing is plus minus 5°C from 54°C -55°C is 49°C -60°C, so 5 temperatures are formed from 50°C -60°C.

Temperature annealing Too low of the optimum temperature causes false priming, while if the temperature annealing is too high then the primer will not stick to the template DNA so that the PCR process did not succeed. Thick or thin ribbons of DNA obtained in the PCR process will also affect the process of nucleotide sequencing (sequencing)[11]. The success of PCR is influenced by the use of appropriate primers and temperature annealing (Tm). Therefore, optimization of the annealing temperature is one of the important steps to determine the success of PCR.

4. Conclusion

The Temperature annealing optimal for amplifying the local area EhoScn01a was at 60°C which was found in the D3 sample of pranajiwa plant (Euchresta horsfieldii) from the Mountains (Bedugul). The PCR product obtained DNA sequences measuring 889 bp. The locus EhoScn01a can be used as a barcode marker for molecular identification of souls.

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