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Optimization of primer and polymerase chain reaction conditions to amplify COI locus for identification of Purnajiwa (*Euchresta horsfieldii* (Lesch.) Benn.) collected from Bedugul, Bali

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Abstract.   The polymerase chain reaction (PCR) has been used for molecular research to amplify DNA fragments, especially from a small amount of genetic material. PCR has been applied for numerous researches, including for plant molecular identification. A good PCR product is dependent on good amplification of the DNA segment in optimum condition. This research was conducted to optimize the primer combination, and the annealing temperature for DNA barcoding application of Balinese rare medicinal plant (*Euchresta horsfieldii* (Lesch.) Benn.) collected from Bedugul. COI is a suitable primer to identify unknown species to higher taxa levels and species with high phenotypic plasticity. The range of annealing temperatures was tested to amplify the combination of five COI primers: GWSF and GWSF5 for forwarding primers and GWSR, GWSR3, and GWSR5 for reverse primers. The annealing temperature was 52, 54, 56, 58, 60°C for 30 seconds. The result showed that GWSF-GWSR, GWSF-GWSR5, GWSF5-GWSR, GWSF5-GWSR5, and GWSF5-GWSR3 produced multiple bands, the double band that cannot be cut, multiple bands, faint amplification band for GWSF5-GWSR5 and GWSF5-GWSR3, respectively. Primer combination of GWSF-GWSR3 with an annealing temperature of 52°Cproduced double bands that were available to have proceeded for sequencing.   In conclusion, the combination of GWSF-GWSR3 that is annealed at 52°C produced the best amplification band. The primer combinations and conditions can be used for further identification of Purnajiwa collected from Bedugul.

1.   Introduction

Pranajiwa or Purnajiwa (Fabaceae) is one of the medicinal plants that grow in the highland forests of Bali. Currently, the existence of these plants is scarce. The Balinese people believe that Purnajiwa plants have various medicinal properties. Our previous research found that fruit extract from Purnajiwa is a potential aphrodisiac and a source of natural antioxidants and can maintain stamina. Different pharmacological properties of retirement have also been evaluated, including an antitumor, antioxidant,

and lipid reducing agent [1]. Various researches also identified the presence of potential phytochemical compounds, especially alkaloids [2,3].

Purnajiwa grows in several areas in Bali and Java. In Bali, it turns out that there are several types of plants that are claimed as Purnajiwa by the Balinese people. Purnajiwa grows in several areas in Bali such as Denpasar, Badung, Jimbaran and Bedugul (Tabanan). However, there are some morphological differences between Purnajiwa growing in the Denpasar area (about 100 m asl) and Bedugul (about 1250 m asl). In terms of morphology, mature plants in the Denpasar area can grow up to 5 meters in height, stem diameter of about 10 centimeters, the color of the bark is gray, the flowers are white like jasmine, the fruit is blue-black, the shape of the fruit is ellipsoid or round with a length of up to 2.2 cm. In comparison, the full-blooded plant from the Bedugul area is an upright shrub with a height of 0.5 m - 1.5 m. The shape of the leaves is similar to the leaves of melinjo (Gnetum gnemon Linn.). Each petiole consists of 3-5 leaves with a shiny green color. The leaves are about 10-15 cm long, with a rounded base and a pointed tip, the arrangement of the leaves alternate with long stalks. The flower stalks sticking out from the leaf axils are arranged in bunches in large numbers; the color of the white flowers is about 1.25 cm. Purnajiwa fruit in the Bedugul area resembles a pod, oval with 1-2 cm length. When young, the pods are brown and turn purplish-black when ripe. In some studies, Purnajiwa plants are referred to in several scientific names, such as Eucresta horsfieldii and Kopsia arborea, indicating the lack of clarity regarding the taxonomy of Purnajiwa.

Understanding natural populations' ecology and genetic structure are essential for determining conservation, breeding, and sustainable management [4]. Genetic studies are also needed for species at risk of extinction, species with limited distribution, and rare frequencies because genetics helps map areas with higher genetic variability as a tool in conservation efforts and species identification. Identification can be obtained using conventional taxonomic methods and molecular techniques [5].

Several markers have been developed to identify a species molecularly, such as using an internal transcribed spacer (ITS) on the citron ribosome, rubisco operon, large subunit of the ribosomal citron, and cytochrome oxidase I (COI). The COI gene is found in the mitochondria. This gene is part of the subunit of the cytochrome oxidase complex, which is part of the electron transport chain [6].

In the molecular identification technique, DNA amplification is performed using PCR. PCR exploits the ability of DNA polymerase to synthesize new complementary DNA strands into available template strands. PCR uses primers in the DNA amplification process. Good primer design is the key to the success of the DNA amplification process with this method [7]. This study identifies Purnajiwa molecularly using DNA Barcoding to determine the relationship of this plant with five COI primers consisting of forward and reverse primers. The amplification temperature was carried out as well to obtain optimal PCR conditions for the following identification process.

2.   Material and Methods

2.1   Sample collection and total DNA Extraction

Samples of Purnajiwa leaves were taken from the Pancasari Mountains area (Bedugul) Tabanan Bali in June 2021 - July 2021. The research was carried out at the PT. Genetica Science, Indonesia and the Laboratory of Genetic and Molecular Resources, Udayana University. Total DNA extraction was carried out using the Zymo Biomics Miniprep Kit (Zymo Research, D6020) following the manufacturer's procedure. The method for the whole process was adapted from Wirawan et al. (2021) [8].

2.2 PCR Optimization

PCR amplification using KOD FX Neo (Toyobo, FX-NEO-201). Optimization of DNA amplification refers to Saunders, 2009 [9]. Five primers (Table 1) were used, designed from the COI region, consisting of two forward primers and three reverse primers.

Table 1. COI primer and its sequences used in the identification of Purnajiwa Sp.

Primer	Sequence
	Forward Primer
GWSF	5′ TCCCAGTCACGACGTCGT TCAACAAAYCAYAA AGATATYGG 3′
GWSF5	5′ AC AAAY C AY AAI G AT AT YGG 3′
	Reverse Primer
GWSR	5′ GGAAACAGCTATGACCATG GGRTGTCCRAARAAYCARAA3′
GWSR3	5′ GGAAACAGCTATGACCATGGGRTGTCCAAA IAAYCARAA 3′
GWSR5	5′ T CAGGRT GNC CI AAR AAY C A 3′

Optimization of DNA amplification was carried out on the five primers to obtain six combinations of PCR primers: GWSF and GWSR; GWSF and GWSR3; GWSF and GWSR5; GWSF5 and GWSR; GWSF5 and GWSR3; and GWSF5 and GWSR5. The PCR conditions used are shown in Table 2, using 50μl DNA template with a concentration of 100ng/μl DNA template, 250μl 2nM dNTPs with a concentration of 4 M, and each 75μl primer with a concentration of 0.3μM.

Table 2. PCR conditions.

Phase	Step	Temperature	Time
1	Denature	94°C	2 min
2	Denature	98°C	15 s
3	Anneal	52, 54, 56, 58, 60 °C	30 s
4	Elongate	6 8 °C	45 s
Repeat step 2-4 (35 times)			
Termination	Step	Temperature	Time
5	Elongate	68 °C	3 min
6	Hold	1 6 °C	Until moving from machine re

2.3. Electrophoresis of PCR products

A total of 1μl of PCR product (added with 2μl of loading dye) was electrophoresed on 1% TBE agarose gel. Electrophoresis was carried out for 30 minutes at a voltage of 100 voltages. Electrophoretic DNA was visualized using a UV translator.

3.   Results and Discussion

Purnajiwa (*Euchresta horsfieldii* (Lesch.) Benn.) fruit has been traditionally used in Indonesia to improve male sexuality. Purnajiwa (*Euchresta horsfieldii* (Lesch.) Benn) is considered one of the most popular plants providing aphrodisiac activity. Purnajiwa is known in several names around Indonesia. This plant grows in several areas in Indonesia, especially in Bali and Java. Balinese people traditionally use Purnajiwa to ameliorate males' sexual function [10]. Chemical investigation revealed alkaloids, phenolics, tannins, flavonoids, saponins, steroids, and terpenoid compounds. Significant compounds in root, seed, stem, and leaves of *E. horsfieldii* are mome-inositol, sophoridane, fatty acids such as palmitic acid, and stearic acid [11,12]. Empirically, Balinese people use the fruit decoction or infusion or add the fresh fruit to traditional alcoholic beverages.

Purnajiwa lives in the highland forests of Bali, many of which are found in the mountainous areas of Tabanan Regency. Currently, the existence of this plant is scarce. In Bali itself, it turns out that there are several types of Purnajiwa. Purnajiwa grows in several areas in Bali such as Denpasar, Badung, Jimbaran and Bedugul (Tabanan). However, there are some morphological characteristics between Purnajiwa Sp. that Sp grow Sp. in the Denpasar and Bedugul areas.

Molecular identification of Purnajiwa plants was carried out using DNA Barcoding with COI primers. The optimization of the primer and annealing temperature was carried out first. The results of the optimization of the annealing temperature for amplifying the CO1 locus in Pranajiwa plants can be seen in Figure 1.
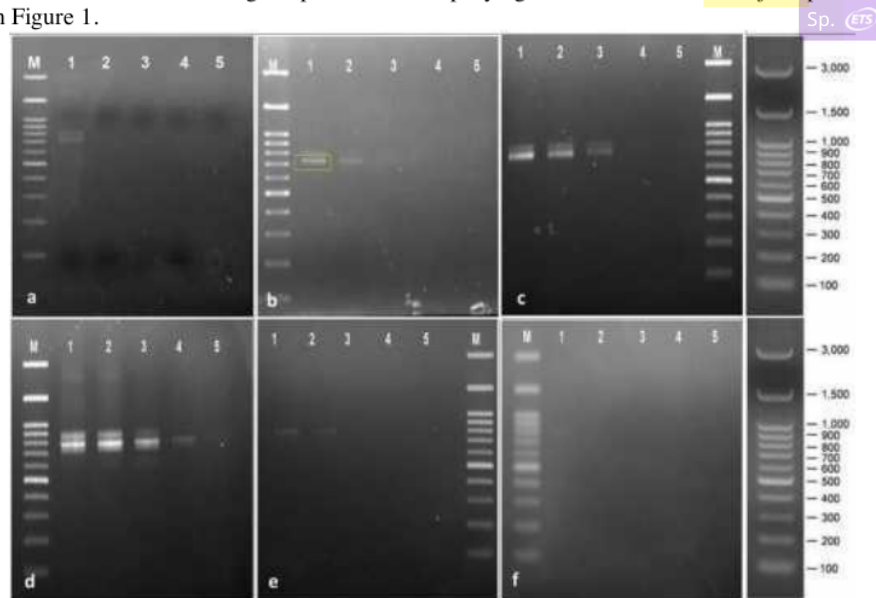

Figure 1. Results of PCR amplification electrophoresis on the six primer combinations: a) GWSF-GWSR; b) GWSF-GWSR3; c) GWSF – GWSR5; d) GWSF5 – GWSR; e) GWSF – GWSR5; f) GWSF5- GESR3; M (markers). Figures 1 to 5 show the resulting bands with annealing temperatures of 52,54,56,58 and 60°C, respectively.

The combination of GWSF and GWSR primers showed a double band and was unclear at all annealing temperatures. The combination of GWSF-GWSR3 primers, at an annealing temperature of 52°C, produced double bands. The GWSF – GWSR5 primer combination showed double bands and could not be cut, the GWSF5 - GWSR combination showed multiple bands and could not be cut, the GWSF5 - GWSR5 primer combination showed a faint amplification band, while the GWSF5 - GWSR3 combination also had a faint amplification band, similar to the combination the previous primer combination. Based on the electrophoresis results, the primer combination of GWSF-GWSR3 at the annealing temperature of 52°C produced a double band. However, the resulting band can be cut (marked with a yellow mark) and used for further identification.

Polymerase chain reaction (PCR) is a common technique used in molecular biology analysis. PCR consists of several phases in which annealing is considered necessary in PCR. In the annealing phase, the primer attaches to the template and then elongates in the next phase. Without this phase, the PCR process cannot be continued. Several studies on optimizing annealing conditions have been carried out and show that optimizing these conditions is essential to produce a good PCR amplification product.

In general, optimization of the PCR process can be done by varying the conditions used in the PCR process. Optimization conditions are closely related to factors such as the type of DNA polymerase; temperature; concentration, in this case, related to dNTPs, MgCl2, and DNA polymerase; PCR buffer, and time. The choice of temperature in the PCR process is crucial because the temperature is one factor that determines the success of a PCR. In general, the annealing temperature used ranges from 37 - 60°C. The choice of annealing temperature is related to the Tm primer used for the PCR process. The annealing temperature can be calculated using an addition and subtraction range of 5 Celsius degrees from the

melting temperature. In determining the annealing temperature used, it is necessary to pay attention to mispriming in the target and non-target areas. The success of a PCR process will be determined by experiment [13–15].

In this study, COI primer was used to apply DNA Barcoding in identifying purnajiwa. COI primer is one of the markers used in DNA Barcoding. Hebert et al. (2003) reported that using this marker in animals can identify unknown species to higher taxa levels. Hebert et al. (2003) confirmed that COI could be used as a marker to identify species with high phenotypic plasticity. The COI gene has two advantages as markers in its application to animals: universality in primers to amplify the 5' end in various animal species and general phylogenetic levels. The COI gene has a relatively fast divergence rate, can be designed as a universal primer on certain gene sections, has a low prevalence of indels so that it can well facilitate cross-phylum alignment [16]. In intraspecific divergence, COI markers can identify 0 – 2 base pairs, while interspecific divergences show more than 30 base pairs. Universal primers for the COI region are currently being developed [17].

4. Conclusion

Primer amplification of GWSF – GWSR3 with an annealing temperature of 52°C resulted in the best amplification band that could be used for further analysis. This analysis used COI primers, which were considered conservation sites and suitable as barcodes. The cytochrome c oxidase I (COI) gene is commonly used for this DNA barcoding technique, and extensive reference sequences are readily available in online databases. Therefore, this COI gene has been widely used in DNA Barcoding methods.

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