GENETIC DIVERSITY OF *Dacrycarpus imbricatus* AT BUKIT TAPAK, TABANAN, BALI BASED ON RAPD MARKER**KERAGAMAN GENETIK *Dacrycarpus imbricatus* DI BUKIT TAPAK, TABANAN, BALI BERDASARKAN PENANDA RAPD**Made Pharmawati^{1)*}, I Made Anom Sutrisna Wijaya²⁾

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Authors affiliation:¹⁾ Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Udayana, Kampus Bukit Jimbaran, Bali²⁾ Department of Agricultural and Biosystems Engineering, Faculty of Agricultural Technology, Universitas Udayana, Kampus Bukit Jimbaran, Bali**Correspondence email:**

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How to cite:Pharmawati, M, IMAS Wijaya. 2021. Genetic diversity of *Dacrycarpus imbricatus* at Bukit Tapak, Tabanan, Bali based on RAPD marker. *Journal of Tropical Biology* 9 (2): 157-161.**ABSTRACT**

Dacrycarpus imbricatus Blume is a member of the Podocarpaceae family. In Bali, *D. imbricatus* was found in Bukit Tapak, Tabanan Regency. This species is one of the dominant species in Bukit Tapak. This study aimed to determine the genetic variation of *D. imbricatus* in Bukit Tapak using molecular markers RAPD (Random Amplified Polymorphic DNA). The genetic diversity of *D. imbricatus* needs to be studied to obtain the information used for the conservation of this species. Leaf samples were taken from Bukit Tapak, Candikuning, Baturiti, Tabanan Regency, Bali. DNA was extracted using the CTAB method, followed by extraction using chloroform: isoamyl alcohol. DNA precipitation was carried out using ethanol. RAPD analysis was performed using polymerase chain reaction (PCR) using four primers. PCR products were visualized using agarose gel electrophoresis and ethidium bromide staining. The results showed that the amplified DNA bands ranged from 1 to 5 bands with DNA band sizes ranging from 230 bp - 422 bp. Only OPA4 and UBC106 primers can be used to detect *D. imbricatus* diversity based on the H, D, and R values. The detected genetic variation is low, as indicated by an average polymorphism of 32.5% and similarities between samples ranging from 0.51 to 1.

Keywords: Bukit Tapak, *Dacrycarpus imbricatus*, polymorphism, RAPD**ABSTRAK**

Dacrycarpus imbricatus Blume adalah anggota dari famili Podocarpaceae. Di Bali, *D. imbricatus* ditemukan di Bukit Tapak, Kabupaten Tabanan. Spesies ini merupakan salah satu spesies yang dominan di Bukit Tapak. Penelitian ini bertujuan untuk menentukan variasi genetik *D. imbricatus* di Bukit Tapak menggunakan penanda molekuler RAPD (Random Amplified Polymorphic DNA). Keragaman genetik *D. imbricatus* perlu dipelajari untuk mendapatkan informasi yang digunakan untuk konservasi spesies ini. Sampel daun diambil dari Bukit Tapak, Candikuning, Baturiti, Kabupaten Tabanan, Bali. Ekstraksi DNA menggunakan metode CTAB dilanjutkan dengan menggunakan kloroform: isoamylalkohol. Pengendapan DNA dilakukan menggunakan etanol. Analisis RAPD dilakukan dengan menggunakan polymerase chain reaction menggunakan empat primer. Produk PCR diamati menggunakan elektroforesis gel agarosa dan pewarnaan etidium bromida. Hasil penelitian menunjukkan bahwa pita DNA yang teramplifikasi berkisar 1 – 5 pita dengan ukuran pita DNA berkisar 230 bp – 422 bp. Keragaman *D. imbricatus* hanya terdeteksi bila menggunakan primer OPA4 dan UBC106 berdasarkan nilai H, D dan H. Variasi genetik yang terdeteksi tergolong rendah yang ditunjukkan dengan rata-rata polimorfisme 32,5% dan similarity antar sampel berkisar 0.51 sampai 1.

Kata kunci: Bukit Tapak, *Dacrycarpus imbricatus*, polimorfisme, RAPD**INTRODUCTION**

Dacrycarpus imbricatus Blume or also known as *Podocarpus imbricatus* is a member of Gymnosperms and belongs to the Podocarpaceae family. This plant is a native species of Indonesia and is distributed in Sumatra, Java, Sulawesi, Lesser Sunda, and Papua. This species grows in the highlands from 500 to 2,000 m asl (above sea level) [1].

In Bali, the habitat of *D. imbricatus* is in Bukit Tapak, Tabanan Regency. Bukit Tapak Forest is located at an altitude of 1520-1600 m asl [2]. This plant in Bali is known as the local name *cemara pandak*. *D. imbricatus* is one of the dominant species in Bukit Tapak. Bukit Tapak forest is classified as a secondary forest [3]. *D. imbricatus* is a long-lived pioneer species that grew due to past disturbances in its territory [4]. Conservation of *D. imbricatus* is needed to prevent the loss of this species in the area due to species change.

Information on the genetic diversity of *D. imbricatus* is important for developing conservation strategies. Preservation of genetic diversity is necessary for the survival of a species over a long period. Loss of genetic diversity will decrease the ability to adapt to environmental changes [5]. Genetic diversity studies can identify alleles that can contribute to an organism's ability to survive in its habitat or survive in a varied habitat. Loss of genetic diversity can lead to extinction in small populations due to decreased environmental adaptability [6, 7].

The detection of genetic diversity can be carried out by several methods. The genetic diversity of plants has traditionally been observed based on morphological characters or growth responses. In this method, individuals are differentiated based on their phenotypic character, which can be influenced by the environment [8]. The development of molecular markers provides a sensitive method for detecting diversity at the DNA level. One of the techniques used in DNA fingerprinting in evaluating genetic diversity is RAPD (Random Amplified Polymorphic DNA). The RAPD technique detects DNA polymorphisms using single primers with random sequences [9]. RAPD analysis is useful for detecting nucleotide polymorphisms that show genetic diversity. This study is aimed to analyze the genetic variation of *D. imbricatus* in Bukit Tapak, Tabanan, Bali by using RAPD. The results of this study can be used as a basis for developing a conservation strategy.

METHODS

Sampling of plant materials. *D. imbricatus* leaves were taken from Bukit Tapak, Tabanan Regency, Bali (Figure 1). The leaves used were fully developed leaves. The samples used were 16 individuals' trees of *D. imbricatus* (Figure 2).

Figure 1. Map of the location of Bukit Tapak (arrow) based on Google Earth

Figure 2. *D. imbricatus* tree in Bukit Tapak

DNA extraction. The leaves were washed with soap and running water until clean, then 0.1 g of leaves were crushed using a mortar and pestle. As much as 1 ml of CTAB buffer (2% w / v CTAB, 1.4 M NaCl, 50 mM EDTA, 100 mM Tris-HCl (pH 8), 2% v / v 2- mercaptoethanol) was added [10, 11]. Incubation of the mixture was done at 65°C for 1.5 hours. Following incubation, centrifugation was done at 14,000 rpm for 5 minutes.

The supernatant was collected to a new tube and added 500 µl of chloroform: isoamyl alcohol (24: 1). The mixture was vortexed and centrifuged for 5 minutes at 14,000 rpm. The top layer was removed and transferred to a new tube, and purification using chloroform: isoamyl alcohol (24:1) was repeated. The top layer was transferred to a new tube, and 500 µl of chilled ethanol was added and incubated overnight. After that, the sample was centrifuged at 8,000 rpm for five minutes. The pellets were washed using 70% ethanol and centrifuged for 5 minutes at 8,000 rpm. The pellets were air-dried and resuspended in 100 µl of sterile H₂O [10, 11].

DNA was visualized with agarose gel (1%) electrophoresis in TAE buffer for 45 minutes at 100 volts. DNA staining was done with ethidium bromide. Visualization was performed using UV-transilluminator [12]. The DNA concentration was estimated by comparing with 50 ng and 100 ng of lambda (λ) DNA.

RAPD. PCR-RAPD was conducted in 20 µl volume with 25 ng DNA, 1 x PCR buffer, 200 µM dNTP, 3 mM MgCl₂, 1 mM primer, and 1 unit taq polymerase. Eight primers from operon primers (OP) and the University of British Columbia (UBC) were screened (Table 1).

Table 1. Name of primers screened and their sequences

Primer Name	Sequence (5'-3')
OPA2	TGCCGAGCTG
OPA4	AATCGGGCTG
OPB8	GTCCACACGG
OPD11	AGCGCCATTG
OPD14	CTTCCCCAAG
OPH1	GGTCGGAGAA
UBC106	CGTCTGCCCG
UBC250	CGACAGTCCC

The PCR cycle was 1x initial denaturation at 95°C for 5 minutes, 40 x denaturation at 95°C for 1 minute, primer annealing at 37°C for 1 minute, extension at 72°C for 1 minute. The final extension was carried out for 10 minutes at 72°C. PCR products were visualized using 1.8% agarose gel electrophoresis with ethidium bromide staining.

Data analysis. Existing DNA bands were score 1, whereas the absence of DNA band was scored 0. Primary efficiency analysis was performed using iMEC [13]. The kinship between individuals was analyzed using NTSYS 2.1 [14], with the UPGMA method with a simple matrix similarity coefficient.

RESULTS AND DISCUSSION

DNA extraction. DNA with concentrations between 80 ng/μl to 175 ng/μl was obtained. The resulting DNA has a large size indicated by the position of the DNA band at the top of the gel. In addition to these DNA bands, there is DNA that appears smeared (Figure 3). The smeared DNA indicated that the DNA had been degraded into fragments of various sizes. DNA smearing is caused by nuclease activity during extraction [15, 16].

Figure 3. Results of *D. imbricatus* DNA electrophoresis. Numbers 1 – 16 are the individual sample numbers. The DNA was compared with λ DNA at 50 ng and 100 ng

In the early stages of DNA extraction, the crushed leaves were incubated in a CTAB buffer at a temperature of 65°C for 1.5 hours. This incubation period was much longer than the plant DNA extraction method by Doyle & Doyle [10]. The long incubation time in the CTAB buffer was done because the leaves of *D. imbricatus* were quite hard. By prolonging the incubation time, the

cells are exposed to lysis buffer (CTAB) longer so that a better DNA lysis process occurred [17].

The EDTA concentration in the CTAB lysis buffer used in this study was also higher than that of Doyle & Doyle [10]. Modifications have been made previously using 50 mM EDTA [11]. In the lysis buffer, EDTA functions as a chelator of Mg^{2+} , where Mg^{2+} is a cofactor of the enzyme nuclease [18]. By increasing the EDTA concentration, it is expected that the amount of Mg^{2+} was chelated more, thereby inhibiting nuclease activity.

Diversity analysis with RAPD. DNA amplification by PCR-RAPD was successfully performed using four primers (OPA4, OPD14, UBC106, and UBC250) of the eight primers tested. Only the four primers mentioned above produced clear PCR products, while the other four primers (OPA2, OPB8, OPD11, and OPH1) produced products with a very strong background smear so that the DNA bands were not clearly observed. Figure 4. shows the PCR-RAPD results using UBC 106 primers with a 1kb ladder as a size marker.

Primer selection in diversity analysis plays an important role. The analysis of the RAPD primer efficiency is shown in Table 2, while the number of amplified DNA bands, the size range of DNA bands, and the degree of polymorphism was shown in Table 3.

Figure 4. PCR-RAPD products using UBC 106 primer. Numbers 1 – 16 are the individual sample numbers. 1 kb ladder was used as a size marker

Of the four primers used in PCR-RAPD, only two primers (OPA4 and UBC106) produced polymorphic DNA bands, while the other two primers (OPD14 and UBC250, produced monomorphic DNA bands.

Based on the primary efficiency analysis, OPD14 primer and UBC250 primer did not have the ability to differentiate between individuals. This could be seen from the value of H, D, and R, which are 0 (Table 2). The value of H was the probability of an individual being heterozygous for a particular locus in the population, the value of D is the probability that two individuals randomly have different patterns, while the value of R is the primary ability to distinguish a number of large genotypes [13]. Primer UBC106 had the highest H, D, and R values indicating that UPB106 is the best primer in differentiating individuals.

Table 2. The efficiency of each primer in the analysis of the diversity of *D. imbricatus* in Bukit Tapak

Primer Name	H	D	R
OPA4	0.219	0.238	0.5
OPD14	0	0	0
UBC106	0.447	0.564	1.875
UBC250	0	0	0
Average			32.5

H=heterozygosity index; D=discriminating power; R=resolving power

Table 3. The number and size range of DNA bands and the percentage of polymorphisms from PCR products

Primer Name	No. of DNA Bands	Band Size (bp)	Polymorphism (%)
OPA4	2	422-250	50
OPD14	1	415	0
UBC106	5	408-230	80
UBC250	2	410-230	0
Average			32.5

RAPD analysis of *D. imbricatus* showed an average polymorphism of 32.5%. The low percentage of polymorphic DNA bands indicates low genetic diversity and vice versa [19]. Table 3 supported the analysis shown in Table 2. Primer UBC106 showed the highest percentage of polymorphism. Based on Table 2 and Table 3, primer OPA4 and UBC106 were suitable for detecting the genetic variation of *D. imbricatus*.

The kinship between individuals of the 16 samples tested is shown in Figure 5. It can be seen that the similarity coefficient ranges from 0.51 to 1. This showed a fairly high level of similarity between individuals. Some individuals have a similarity coefficient of 1, namely between individuals 1, 2, 4, and 5, between individuals 3, 7, and 13, between individuals 10 and 11, also between individuals 14, 15, and 16. A high value of the similarity coefficient means the population did not have enough diversity [20]. Based on the average polymorphism and the tree of kinship, the results of this study indicate that the genetic diversity of *D. imbricatus* in Bukit Tapak was low and needs to be improved.

Figure 5. Genetic relationship between individuals *D. imbricatus*. Number 1 - 16 are the individual sample number. The coefficient showed in the figure was the coefficient of similarity

CONCLUSION

The population of *D. imbricatus* in Bukit Tapak, Tabanan, Bali showed a low level of diversity, which has an average polymorphism of 32.5% and a similarity index between samples between 0.51 to 1. Further research is needed by increasing the number of samples, the number of primers, and testing for the occurrence of inbreeding pressure.

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REFERENCES

- [1] Thomas P (2013) *Dacrycarpus imbricatus*. The IUCN Red List of Threatened Species. e.T42445A2980614. <http://dx.doi.org/10.2305/IUCN.UK.2013-1.RLTS.T42445A2980614.en>.
- [2] Fardila D, Sutomo (2011) Efek tepi koridor jalan di Hutan Bukit Pohen, Cagar Alam Batukahu, Bali. *Berkala Penelitian.Hayati* 17: 9–13.
- [3] Sutomo, Erosi NK, Undaharta, Bangun TM, Lugrayana IN (2012) Studi awal komposisi dan dinamika vegetasi pohon, hutan Gunung Pohen Cagar Alam Batukahu Bali. *Bumi Lestari* 12(2): 366-381.
- [4] van Steenis CGGJ (1972) The mountain flora of Java. E. J. Brill, Leiden.
- [5] van Zonneveld M, Scheldeman X, Escibano P, Viruel MA, van-Damme P, Garcia W, Tapia C, Romero J, Siguen M, Hormaza JJ (2012) Mapping Genetic Diversity of Cherimoya (*Annona cherimola* Mill.): Application of spatial analysis for

- conservation and use of plant genetic resources. PLoS ONE 7(1):e29845. doi:10.1371/journal.pone.0029845.
- [6] Martin MA, Herrera MA, Martín LM (2012) In situ conservation and landscape genetics in forest species. Journal of Natural Resources and Development 2: 1-5.
- [7] Nonić M., Šijačić-Nikolić M. (2019) Genetic diversity: Sources, threats, and conservation. In: Leal Filho W., Azul A., Brandli L., Özuyar P., Wall T. (Eds) Life on land. Encyclopedia of the UN Sustainable Development Goals. Springer Cham, Switzerland. doi.org/10.1007/978-3-319-71065-5_53-1.
- [8] Choudhary N, Ahuja U, Chawla V, Jain RK, Kumari P, Batan KR. (2011) Morphological and molecular variability in weedy rices of Haryana. Asian Journal of Agricultural Research. 5: 250-259.
- [9] Welsh J, M. McClelland M (1990) Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18(24):7213-7218.
- [10] Doyle JJ, Doyle JL (1990) A rapid total DNA preparation procedure for fresh plant tissue. Focus 12: 13-15.
- [11] Pharmawati M, Yan G. McFarlane I. J. (2004) Application of RAPD and ISSR markers to analyse molecular relationships in *Grevillea* (Proteaceae). Australian Systematic Botany 17: 49-61. doi.org/10.1071/SB03016.
- [12] Sambrook J. Fritsch EF, Maniatis T (1989). Molecular Cloning. A Laboratory Manual.: Cold Spring Harbor, Lab.Press,USA.
- [13] Amiryousefi A, Hyvönen J, Poczai P. (2018) iMEC: Online marker efficiency calculator. Application in Plant Science 6(6):e01159. doi: 10.1002/aps3.1159.
- [14] Rohlf F.J (2000). NTSYSpc. Numerical taxonomy and multivariate analysis system. Version 2.1. Exeter Publishing Setauket, New York.
- [15] Sahu SK, Thangaraj M, Kathiresan K (2012) DNA extraction protocol for plants with high levels of secondary metabolites and polysaccharides without using liquid nitrogen and phenol. ISRN Molecular Biology 2012: 205049,. doi:10.5402/2012/205049.
- [16] Aboul-Maaty NAF, Oraby HAS (2019). Extraction of high-quality genomic DNA from different plant orders applying a modified CTAB-based method. Bulletin of the National Research Center 43: 25 doi.org/10.1186/s42269-019-0066-1.
- [17] Jain SA, de Jesus FT, Marchioro GM, de Araújo, ED (2013). Extraction of DNA from honey and its amplification by PCR for botanical identification. Food Science and Technology 33(4): 753-756. doi.org/10.1590/S0101-20612013000400022.
- [18] El-Ashram S, Al Nasr I, Suo, X. (2016). Nucleic acid protocols: Extraction and optimization. Biotechnology reports (Amsterdam, Netherlands) 12: 33–39. doi.org/10.1016/j.btre.2016.10.001.
- [19] Probojati RT, Wahyudi D, Hapsari L (2019). Clustering analysis and genome inference of pisang raja local cultivars (*Musa* spp.) from Java Island by Random Amplified Polymorphic DNA (RAPD) Marker. Journal of Tropical Biodiversity and Biotechnology 4(2): 42- 53.
- [20] atyawan D, Tasma IM (2011) Genetic diversity analysis of *Jatropha curcas* provenances using Randomly Amplified Polymorphic DNA Markers Jurnal AgroBiogen 7(1):47-55.