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Proceeding Paper

Genetic Variability of *Graptophyllum pictum* (L.) Griff Accessions from Western Indonesia by Sequence-Related Amplified Polymorphism †

Dimas Andrianto [1,2,*], Puspa Julistia Puspita [1], Ukhradiya Magharaniq Safira Purwanto [1], Danty Oktiana Prastiwi [1], Suci Hermita [1], Dyah Subositi [3] and Anshary Maruzy [3]

[1] Department of Biochemistry, Faculty of Mathematics and Natural Sciences, IPB University (Bogor Agricultural University), Dramaga 16680, Indonesia; puspajulistia@gmail.com (P.J.P.); irabikg8@apps.ipb.ac.id (U.M.S.P.); danty.islam@gmail.com (D.O.P.); sucihermita@gmail.com (S.H.)
[2] Halal Science Center, IPB University (Bogor Agricultural University), Dramaga 16680, Indonesia
[3] Laboratory of Medicinal Plant Systematics, Medicinal Plant and Traditional Medicine Research and Development Centre (B2P2TOOT), National Institute of Health and Research Development, Ministry of Health, Karanganyar 57792, Indonesia; dyah.subositi@gmail.com (D.S.); anshary.maruzy@kemkes.go.id (A.M.)
* Correspondence: dimasandrianto@apps.ipb.ac.id; Tel.: +62-251-8423-267
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Abstract: *Graptophyllum pictum* (L.) Griff is one of the medicinal plants in Indonesia that has been proved to have antioxidant, antidiabetic, and analgesic properties. However, there is no report about the relationship of its genetic diversity with accession and ethnic aspects. Thus, we carried out polymorphism studies and clusterization of 34 samples of *G. pictum* that were collected from 10 ethnics in Western Indonesia. The research purpose was to determine the polymorphism and kinship relations between ethnic groups and accessions of *G. pictum* in Western Indonesia. DNA was isolated using the GeneJET Plant Genomic DNA purification mini kit. Sequence Related Amplified Polymorphism (SRAP) was used as a molecular marker in PCR amplification. We analyzed the data using NTSYS and PopGene 1.3 software. We found eight combinations of selected primers with a polymorphic percentage average of 72.23% and 45 loci. The ethnicity that has the highest polymorphic percentage is Kutai (East Kalimantan) with 55.56%. The diversity between population and accession is moderate with a Dissimilarity Index (DI) of not more than 0.69. Ribun ethnic in West Kalimantan is the oldest ancestry ethnic that has a locus marker at the 24th and 41st position. In conclusion, we found that *G. pictum* in Western Indonesia is divided into four clusters.

Keywords: accession; *Graptophyllum pictum*; polymorphism

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1. Introduction

Indonesia has 188 million hectares of land area that are spread over more than 10,000 islands, with more than half covered by primary natural forest [1]. However, many of the species are becoming endangered because of the rapidly decreasing vegetation area in Indonesia. Other factors that threaten biodiversity are anthropogenic and growing mining activities, such as logging and land conversion [2,3]. North Sulawesi Province lost up to 12,668.2 hectares forest from deforestation from 2000 to 2013 [4]. Wibisono and Azham (2017) found that there are several medicinal plants in the conservation plot of the Kutai Kertanegara Regency Forest that can be used as traditional medicine [5].

Graptophyllum pictum (L.) Griff is one of the medicinal plants that has not been optimally utilized in Indonesia [6]. This plant has varieties with local names in Indonesia, such as *daun wungu*, *handeuleum*, and *tulak*. It has several properties according to the local ethnic. Most of the people in West Java use *G. pictum* to prevent postpartum infections,

restore stamina, normalize the size of the uterus, stimulate breast milk production, and reduce body weight [6]. *G. pictum* has antidiabetic activity by inhibiting the -glucosidase enzyme in vitro [7]. The extract of *G. pictum* in 70% ethanol can treat diabetes with a dose of 50 mg/kg/bodyweight based on the Intraperitoneal Glucose Tolerance Test (IPGTT) [8]. Andrianto et al. (2015) also found that 70% of *G. pictum* leaves' ethanolic extract was able to protect pancreatic β-cell function in oxidative stress by alloxan injection in rats [9].

There are several reports on the bioactivity of *G. pictum* [7–9], but the relationship of its genetic diversity with accession and ethnic aspects is currently unconfirmed. Thus, polymorphism studies and clusterization of *G. pictum* from 34 samples of 10 ethnics in Western Indonesia is important. We chose Sequence Related Amplified Polymorphism (SRAP) for the molecular markers, because it provides a good level of polymorphism in plant species. This study aimed to determine the polymorphism and kinship relations between ethnic groups and accessions of 34 samples of *G. pictum* from 10 ethnics in Western Indonesia. This research will provide a better understanding of conservation support, breeding strategies, and the basis for developing *G. pictum* as an ethnopharmacological product in Indonesia.

2. Experiments

2.1. Study Area

The samples of this research were taken from South Sumatera, West Java, Central Java, East Java, West Kalimantan, North Kalimantan, South Kalimantan, and East Kalimantan Provinces in the Western part of Indonesia (GMT+7) (Table 1). South Sumatera samples consisted of four accessions that were taken from the Pegagan Ethnic, in Ogan Ilir Regency, which is located about 35 km west of Palembang City. West Java samples were taken from the Cirebon and Tasik Ethnics. Four accessions were taken from the Cirebon Ethnic in Cirebon City, which is located 130 km northwest of Bandung City. Three accessions were taken from the Tasik Ethnic in Tasikmalaya Regency, which is located 100 km southwest of Bandung City.

Table 1. *Graptophyllum pictum* (L.) Griff leaves from 10 ethnics in Western Indonesia. Location based on WGS84 geographic coordinate system.

Accession Code	Ethnic	Province	East Longitude	Latitude
1–4	Pegagan	South Sumatera	104°40′4″	3°20′00″ S
5, 9, 32, 33	Cirebon	West Java	108°33′00″	6°20′00″ S
7, 10, 34	Tasik	West Java	108°13′30″	7°19′56″ S
8, 19, 20	Putak	North Kalimantan	116°35′20.5″	03°32′15.8″ N
11–16	Kutai	East Kalimantan	116°31′36″	00°10′08″ N
6, 17, 18	Ribun	West Kalimantan	110°26′3.9″	00°11′54.5″ N
21–24	Sambas	West Kalimantan	108°59′50.1″	01°03′00″ N
29–31	Pagatan	South Kalimantan	115°20′00″	03°15′00″ S
25–27	Majapahit	East Java	112°26′00″	07°33′00″ S
28	Jawa	Central Java	110°50′00″	07°35′00″ S

Central Java samples were taken from the Java Ethnic that consisted of one accession located in Surakarta Regency, 65 km northeast of Yogyakarta City. East Java samples consisted of three accessions that were taken from the Majapahit Ethnic, located in Kediri Regency, 129 km west of Surabaya City. Samples of West Kalimantan were obtained from the Ribun and Sambas Ethnics. The Ribun Ethnic consisted of three accessions taken from Sanggau Regency, 186 km north of Pontianak City. The Sambas Ethnic was located in Bengkayang Regency, 227 km south of Pontianak City. We collected four accessions from the Sambas Ethnic. The North Kalimantan samples were taken from the Putak Ethnic that consisted of three accessions, located in Malinau Regency, in the west part of North Kalimantan Province. The South Kalimantan samples consisted of three accessions that were taken from the Pagatan Ethnic, located in Tanah Bumbu Regency, 183 km north of

Banjarmasin City. Samples of East Kalimantan were taken from the Kutai Ethnic that consisted of seven accessions. This ethnic was located in Kutai Kertanegara Regency.

2.2. Materials

The equipment used in this research included water baths (Memmert, Schwabach, Germany), electrophoretic device sets (Biorad, CA, USA), a Nano Pro spectrophotometer (Maestrogen, Hsinchu, Taiwan), a Gel Documentation System (Maestrogen, Hsinchu, Taiwan), a PCR (Biorad, Hercules, CA, USA), and a refrigerated centrifuge (Tomy, Tokyo, Japan). The material used included 34 samples of Graptophyllum pictum (L) leaves from 10 ethnic groups in western Indonesia, liquid nitrogen, a GeneJET Plant Genomic DNA Purification Mini Kit (Thermo Fischer Scientific, Waltham, MA, USA), Invitrogen proteinase-K (Thermo Fischer Scientific, Waltham, MA, USA), water, agarose (Thermo Fischer Scientific, Waltham, MA, USA), Safe DNA dye (Peqlab, Radnor, PA, USA), Tris Boric-acid EDTA (TBE) (Sigma, Darmstadt, Germany), DreamTaq Green PCR Master Mix (2X) (Thermo Scientific, Waltham, MA, USA), Invitrogen SRAP primer (Thermo Fischer, Waltham, MA, USA), Free Nuclease Water (Thermo Fischer, USA), and a Generuler ladder 100 and 1000 base pair (Thermo Fischer Scientific, Waltham, MA, USA). This study also used NTSYSpc (Numerical Taxonomy and Multivariate Analysis System) 2.1 and Pop Gene (Population Genetic Analysis) 1.3 software.

2.3. Primary Selection and Annealing Temperature Optimization

The research began with the isolation of genomic DNA, including sample preparation, DNA extraction, electrophoresis, and quantification of the isolate. Then the SRAP primer selection that matched the printed DNA and its temperature was optimized. There were 16 SRAP primer combinations tested (forward Me 1–4 and reverse Em 1–4 combination) as well as 8 annealing temperature combinations (between 33.0 and 58.0 °C). Subsequently, amplification of 34 DNA isolates using the 8 selected primers through PCR was conducted; then, the amplicon was visualized via electrophoresis (data not shown).

2.4. Amplification of Genomic DNA Using SRAP Markers

DNA amplification was carried out twice during primary selection (16 primary combinations of 1 sample) and the polymorphism test (8 primary combinations of 34 samples) [10]. Both used the PCR cocktail formula and the PCR cycle. The PCR reaction started with a 1 initial denaturation cycle for 5 min at 94 °C. Then denaturation and annealing were 5 cycles, with 1 min for each cycle. This denaturation used a temperature of 94 °C. The first annealing used different temperatures according to the optimum temperature of the primer used. Next, there were 35 denaturation cycles (94 °C), second annealing (temperature was according to the primer), and elongation (72 °C), each for 1 min. Furthermore, the final extension was finished once at 72 °C for 8 min. The reaction was complete when the sample was allowed to stand for approximately 10 min in PCR at 4 °C as holding temperature before visualization. The sample was visualized using the Gel Documentation System.

2.5. Data Analysis

2.5.1. Allele Scoring

The base DNA fragments' length from each accession from each primer was calculated. The presence of DNA fragments in each accession was given a different score; the score was 1 if it showed there was a fragment, and a score of 0 meant there was no DNA fragment. Furthermore, monomorphic, polymorphic, and total alleles of each primer were calculated to show that the primer met the requirements to test the polymorphism of a species.

2.5.2. Analysis of Diversity through Software NTSYS 2.1 and Pop Gene 1.3

The Similarity Index was calculated using the DICE similarity index formula. We also obtained the polymorphic percent for each population of *G. pictum*. The dendrogram construction was carried out using the Unweighted Pair Group Method Using Arithmetic

Method (UPGMA). Then, Principal Component Analysis was performed to obtain the eigenvalues and cumulative variations of the main alleles in diversity. Subsequently, the ethnics were clustered based on the results of the NTSYS phylogenetic dendrograms. Data were interpreted and supported by gene migration data and allele frequencies. Furthermore, the data were visualized as a map of the distribution and classification of *G. pictum* from western Indonesia.

3. Results

3.1. Polymorphic-Monomorphic Determination

The eight selected primer combinations were applied to 34 isolated DNA genomes of *G. pictum*. Electrophoresis result of 34 PCR samples at 2.5% agarose showed the presence of polymorphism and monomorphism bands. The monomorphism in this research was demonstrated by the presence of identical or similar allele patterns in 28 samples. This meant that the appearance of the ribbon in 28 samples was categorized as monomorphic. The polymorphic was shown by the variety of the patterns of band appearance with the same size in the sample being tested. The results were scanned into binary data and processed using NTSYS software (data not shown). Subsequently, there were 45 selected fragments (alleles) as variables and 34 individuals representing 10 different ethnicities (Table 2).

Table 2. Total of DNA fragments from amplification of each locus in eight primer combinations.

Annealing I and II Temperature	Primer Combination	Total Alleles	Monomorphic Alleles	Polymorphic Percent (%)	Fragment Sizes (bp)
35.2–47.5	Me 1–Em 4	3	2	33.34	200–800
44.0–57.0	Me 2–Em 2	4	0	100.00	90–300
44.0–57.0	Me 2–Em 3	12	3	75.00	90–1200
35.2–47.5	Me 2–Em 4	5	2	60.00	90–1000
44.0–57.0	Me 3–Em 1	7	4	42.86	90–1000
44.0–57.0	Me 3–Em 3	8	0	100.00	90–1500
44.0–57.0	Me 3–Em 4	3	0	100.00	90–250
35.2–47.5	Me 4–Em 3	3	1	66.67	90–200
Total		45	4	577.87	-
Average		5.63	0.50	72.23	-

In addition to the diversity shown by the allele frequencies, PopGene can also indicate the value of gene flow or migration. Two loci specifically gave rise to a migration value of 0 and Genetic diversity (Gst) of 1, namely the alleles 24 and 41. So, these two alleles have the potential to become specific marker alleles for *G. pictum* from the Ribun ethnic origin.

3.2. Diversity of Intra-Population Based on Ethnicity

We can see the genetic diversity in a population according to ethnicity (Table 3). The Shannon Index (I) in each ethnic corresponded to a low level of diversity due to I1. The populations of G. pictum with high percent polymorphic loci were Kutai (55.56%), Pegagan (48.89%), and Sambas (48.89%). The ethnicities with the lowest polymorphism were Pagatan (8.89%), Tasik (15.56%), and Putak (15.56%). The number of observed alleles (Na) was relatively low due to the limited sample size of each ethnic group. The value of effective allele (Ne) was proportional to the number of heterozygous individuals in the population.

Table 3. Genetic variety of each population based on ethnic.

Population	Total Samples	Na [1]	Ne [2]	H [3]	I [4]	Polymorphism Locus	Polymorphic Percent (%)
Cirebon	4	1.4000	1.2756	0.1611	0.2366	18	40.00
Tasik	3	1.1556	1.1244	0.0691	0.0990	7	15.56
Majapahit	3	1.2889	1.2311	0.1284	0.1839	13	28.89
Jawa	1	1.0000	1.0000	0.0000	0.0000	0	0.00
Sambas	4	1.4889	1.3111	0.1889	0.2807	22	48.89
Ribun	3	1.4222	1.3378	0.1877	0.2688	19	42.22
Pagatan	3	1.0889	1.0711	0.0395	0.0566	4	8.89
Kutai	6	1.5556	1.4297	0.2383	0.3434	25	55.56
Putak	3	1.1556	1.1244	0.0691	0.0990	7	15.56
Pegagan	4	1.4889	1.4089	0.2194	0.3127	22	48.89
Average	3.40	13.04	12.31	0.1302	0.1881	13.70	30.45

[1] Na = total observed alleles, [2] Ne = total effective alleles, [3] H = Shannon index, [4] I = heterozygosity.

3.3. Diversity and Phylogenetics between Accessions

Phylogenetic trees were obtained from 28 *G. pictum* accessions of 10 ethnics in Western Indonesian through the Numerical Taxonomy System (NTSYS) software (Figure 1). The Dissimilarity Index (DI) between 34 accessions reached a maximum value of 0.6444 obtained from accessions 6 and 30 (Ribun and Pagatan). The second highest DI value was 0.6222 representing the genetic distance between accession 6 and 31 (Ribun and Pagatan). The maximum DI value in this research was classified as moderate but higher than the DI between ethnicity with a maximum DI value of 0.4359 (Figure 1). Individual diversity ranged from 5 to 80% based on the dendrogram.

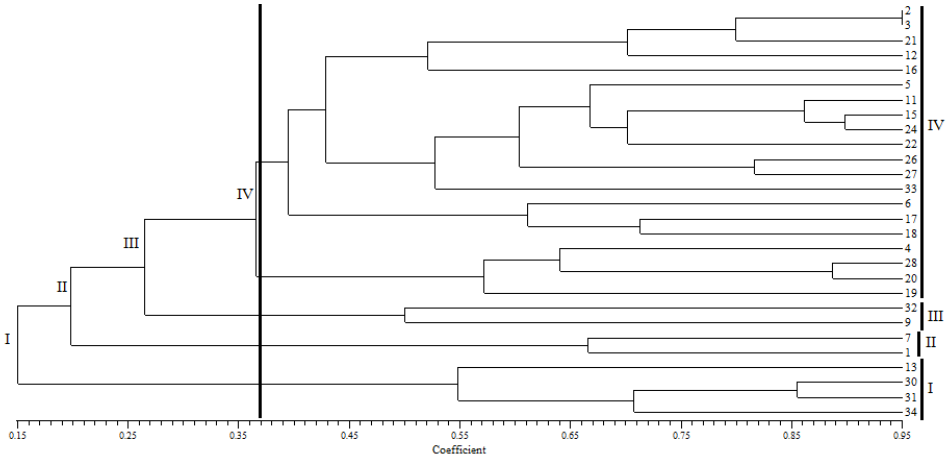


Figure 1. Phylogenetic tree of 34 accessions of *G. pictum* from 10 ethnics in Western Indonesia using the UPGMA method. Clusterization coefficient = 0.37 (Dissimilarity Index 0.63).

4. Discussion

The numbers at the end of the phylogenetic tree showed the accession clusterization of *G. pictum* in Western Indonesia (Figure 1). We obtained four clusters based on the coefficient value of 0.37 or a diversity index of 0.63. The accessions that had the closest diversity based on DI were the accessions of 28 and 20 (Jawa–Putak) and 30 and 31 (Pagatan–Pagatan) with a DI of 0.02222. After that, the closest diversity were the accessions of 2 and 3 (Pegagan–Pegagan) and 15 and 24 (Kutai–Sambas) with a DI of 0.04444. Clusterization was expected to be able to inform genetic similarity patterns, making it easy to make cross maps for the development of superior varieties of *G. pictum*.

Figure 2 shows the clusterization of *G. pictum* in Western Indonesia was random, it did not follow the pattern of geographical proximity. Cluster I consisted of all accessions

from the Pagatan Ethnic and several accessions from the Tasik and Kutai Ethnics. Cluster II consisted of several accessions from the Tasik and Pegagan Ethnics. Cluster III consisted of several accessions from the Cirebon Ethnic. Cluster IV consisted of all accessions from all ethnics, except the Pagatan and Tasik Ethnics. The pattern of the genetic closeness of *G. pictum* populations is thought to be the role of humans in mixing genes between populations such as the *Anthocephalus cadamba* tree in Indonesia [11]. Many studies report that humans play a large role in shaping the genetic structure of a population, through domestication and mixing origins [12]. Thus, ethnic migration is one of the important factors that determined the kinship of *G. pictum*.

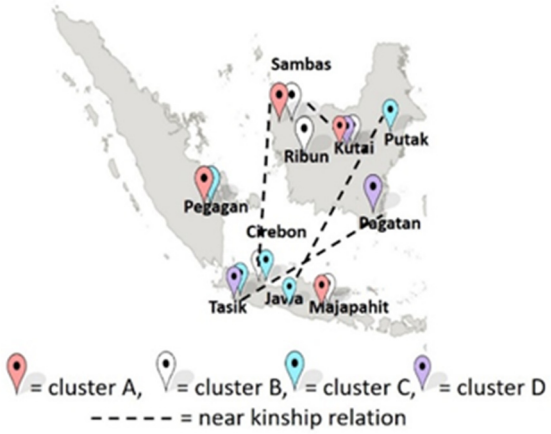


Figure 2. Clusterization map of Graptophyllum pictum in Western Indonesia.

5. Conclusions

The mean polymorphic percent of 16 primary SRAP combinations was 72.23%. *G. pictum* with the closest genetic distance came from the ethnics Tasik (West Java) and Pagatan (South Kalimantan) (ID = 0.0094). The accessions with the furthest genetic distance were the accessions 6 and 30 (ID = 0.6444) of the Ribun (West Kalimantan) and Pagatan ethnic groups. *G. pictum* of Ribun ethnic origin was the oldest ancestry. The high polymorphism *G. pictum* population came from Kutai (East Kalimantan) (polymorphic percent = 55.56%). In general, *G. pictum* polymorphisms in Western Indonesia were classified as moderate (ID < 0.69). *G. pictum* clusterization from Western Indonesia did not show a pattern that matched its ethnicity or geography.

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