Journal of

# Tropical Biodiversity and Biotechnology

VOLUME 7 | ISSUE 1 | APRIL 2022

PUBLISHED BY

UNIVERSITAS GADJAH MADA
FAKULTAS BIOLOGI

IN COLLABORATION WITH

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**Review Article**

Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66552
DOI: 10.22146/jtbb.66552

Short Communications

# Genetic Diversity of *Dicranopteris* and *Sticherus* from Rokan Hulu, Riau Based on ISSR Marker

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**Keywords:**
*Dicranopteris*
Pteridophyte
*Sticherus*.
molecular marker
taxonomy
**Submitted:**
11 June 2021
**Accepted:**
02 September 2021
**Published:**
03 January 2022
**Editor:**
Miftahul Ilmi

**ABSTRACT**
Genetic diversity of eleven taxa consisted of *Dicranoperis speciosa, D. curanii,* and *D. linearis* with seven varieties and *Sticherus truncatus* with two varieties from Rokan Hulu, Riau was analyzed using ISSR markers generated from 10 primers. Nine out of ten ISSR primers produced a high level of polymorphism, with six of them showed 100% polymorphism. The genetic similarity was calculated using Jaccard's similarity coefficient, and cluster analysis using the Unweighted Pair-Group Method with Arithmetic Mean. The result showed that the genetic similarity of the eleven taxa under study ranged from 0.377 to 0.627 indicated a moderate level of genetic diversity and the clusters did not separate *Dicranopteris* from *Sticherus*.



Indonesia is a mega biodiversity country with a high variety of plant taxa including Pteridophytes. The Pteridophytes taxonomical study by Van Steenis (1959) in the Malesian region was the main reference for the fern diversity in Indonesia (Go et al. 2012). There were a number of taxonomic studies on Pteridophytes diversity in Indonesia such as those of Adjie & Lestrari (2011) who reported 54 species of Pteridophytes from Bali, Arini & Kinho (2012) with 41 species from 19 families from North Sulawesi, and Sofiyanti et al. (2020) who found 23 fern species from Meranti Riau.

Gleicheniaceae is an ancient Pteridophyte with the oldest fossil evidence found during the Carboniferous period (Tryon & Tryon 1982). There are six genera of Gleicheniaceae in the world, namely *Dicranopteris* Bernh., *Diplopterygium* (Diels) Nakai, *Gleichenella* Ching, *Gleichenia* Sm., *Sticherus* C. Presl, and *Stromatopteris* Mett. (Smith et al. 2006; PPG I 2016). According to Van Steenis (1959), three genera of Gleicheniaceae found in the tropics and subtropics were *Stromatopteris* (1 species), *Dicranopteris* (10 species), and *Gleichenia* (50 species, which some species were taxonomically revised to *Sticherus*). The diagnostic characters of *Sticherus* are the young organ parts are protected by peltate scales and stellate hairs, sori arranged into two to five sporangia, and simple branching venation. Meanwhile, the distinguishing characters of *Dicranopteris* are the young organs are protected by branched

hairs in various forms, no scales, sori composed of 8-15 sporangia or more, and the venation is branched at least twice. So far, studies on the diversity and taxonomic relationships of Gleicheniaceae in Indonesia were mainly based on morphological characters, and there is no information on the genetic diversity of this taxon based on molecular markers.

Inter Simple Sequence Repeat (ISSR) is a molecular marker commonly used to analyze the genetic variation of closely related species (Zietkiewicz et al. 1994). DNA fingerprinting patterns generated using ISSR markers have been used in taxonomic studies of ferns such as reported by Vidyashree et al. (2019) who evaluated the taxonomy of 19 fern species in India. Other studies on Pteridophyte using ISSR to determine genetic diversity were done by Korpelainen et al. (2005) on *Adiantum*, Wang et al. (2012) on *Alsophila spinulosa*, and Fernando et al. (2015) on *Asplenium scolopendrium* var. *americanum*. In this study, ISSR marker was used to reveal genetic diversity and taxonomic relationships of *Dicranopteris* (three species, one of them with seven varieties) and *Sticherus* (one species, *S. truncatus* with two varieties) as shown in Table 1. Genomic DNA was extracted from 50 mg of fresh leaves using Geneaid Genomic DNA Mini Kit (Plant) following the procedure of the manufacturer. The DNA samples were stored at -20°C before the PCR amplification process. The PCR was performed following Vidyashree et al. (2019) with modifications on the annealing temperature. A total of 10 primers were used in this study. The amplification of ISSR markers was performed in 25 µl volume reaction containing 2 µl DNA (0.5-2.0 ng), 2 µl of primer ISSR, 12.4 µl of DreamTaq™ Hot Start Green PCR Mix (DreamTaq Hot Start DNA polymerase, 2X DreamTaq Green Buffer, 0.4 mM dNTPs and 4 mM $MgCl_2$), and 8,6 µl water free nuclease.

**Table 1.** List of species and varieties used for ISSR analysis.

| No. | Genus | Spesies |
|---|---|---|
| 1 | *Dicranopteris* | *Dicranopteris linearis* (Burm. *f.*) Underw. var. *altissima* Holttum |
| 2 | | *D. linearis* var. *inaequalis* (Rosenst.) Holttum |
| 3 | | *D. linearis* var. *linearis* |
| 4 | | *D. linearis* var. *demota* Holttum |
| 5 | | *D. linearis* var. *alternans* (Mett.) Holttum |
| 6 | | *D. linearis* var. *tertraphylla* (Rosenst.) Nakai |
| 7 | | *D. linearis* var. *subspeciosa* Holttum |
| 8 | | *D. curranii* Copel. |
| 9 | | *D. speciosa* (Presl) Holttum |
| 10 | *Sticherus* | *Sticherus truncatus* (Willd.) Nakai var. *involuta* Holttum |
| 11 | | *S. truncatus* var. *truncata* |

The amplification condition refereed to by Vidyashree et al. (2019) with modification on the annealing temperature after the optimation procedure. The reactions consisted of one cycle of initial denaturation at 94° C for 2 minutes, 35 cycles of amplification reactions consisted of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, extension at 72°C for 30 seconds, and followed by a final extension at 72°C for 10

minutes. The amplification products were detected in 1.5% agarose gel in 1X TBE Buffer stained with ViSafe Green Gel Stain (10,000x in water) for 40 minutes at 100 voltage. The results were photographed under the blue light Transilluminator. The DNA fragments were manually scored as present (1) or absent (0) to generate binary data for cluster analysis. The cluster analysis was done based on Jaccard's similarity coefficients and Unweighted Pair-Group Method with Arithmetic Mean (UPGMA) methods using MultiVariate Statistical Package (MVSP) program Version 3.22 (Kovach 2007). Nine out of ten ISSR primers used in this study produced a high degree of polymorphism ranged from 77.68% to 100%, and only one primer failed to produce amplification products (Table 2). The possibility that primer ISSR 816 failed to produce amplification products might be caused by several factors such as primer incompatibility, non optimum annealing temperature for this particular primer, and different requirements for PCR reaction especially the $Mg^{2+}$ concentration (Ali et al. 2006; Pharmawati 2009; Mariana et al. 2011; Budiani et al. 2016). A total of 96 DNA fragments were generated from nine primers, with the number and size varied from 100 to 1,100 bp. In this study, six primers namely ISSR 845, ISSR 847, ISSR 851, ISSR 855, ISSR 859, and ISSR 888 produced 100 % polymorphisms. Representatives of DNA fingerprinting profiles were shown in Figure 1.

Most of the primers used in this study, with one exception for ISSR 816, showed a higher level of polymorphism than previous studies on ferns genetic diversity using ISSR markers (Vidyashree et al. 2019). A High level of polymorphism indicated the effectiveness of ISSR primers for the purpose of genetic diversity analysis. Other genetic diversity studies using ISSR as molecular markers have found high levels of polymorphism, such as those in *Adiantum* with 82% polymorphic bands (Korpelainen et al. 2005), six ornamental fern species with 71.26% polymorphic bands (Animasaun et al. 2018), and two endemic Lycophyte species, *Isoetes cangae* and *Isoetes serracarajensis* with 87% polymorphic bands (Santos et al. 2020).

The result of cluster analysis on 11 taxa of *Dicranopteris* and *Sticherus* based on ISSR marker was presented as a dendrogram in Figure 2.

**Table 2**. Results of ISSR marker amplification.

| No. | Primer | Sequence | Fragment length (bp) | No. of bands | Polymorphic band (%) |
|---|---|---|---|---|---|
| 1 | ISSR 816 | 5'CACACACACACACACAT3' | 0 | 0 | 0 (0 %) |
| 2 | ISSR 845 | 5'CTCTCTCTCTCTCTCTCTRG3' | 310-900 | 7 | 7 (100 %) |
| 3 | ISSR 847 | 5'CACACACACACACACARC3' | 200-900 | 14 | 14 (100 %) |
| 4 | ISSR 851 | 5'GTGTGTGTGTGTGTGTYG3' | 230-600 | 8 | 8 (100 %) |
| 5 | ISSR 855 | 5'ACACACACACACACACYT3' | 230-500 | 10 | 10 (100 %) |
| 6 | ISSR 857 | 5'ACACACACACACACACYG3' | 100-750 | 13 | 12 (92.31 %) |
| 7 | ISSR 859 | 5'TGTGTGTGTGTGTGTGRTRC3' | 150-550 | 12 | 12 (100 %) |
| 8 | ISSR 861 | 5'ACCACCACCACCACCACC3' | 300-750 | 9 | 7 (77.78 %) |
| 9 | ISSR 862 | 5'ACCACCACCACCACCACC3' | 350-740 | 7 | 6 (85.71 %) |
| 10 | ISSR 888 | 5'BDBCACACACACACACA3' | 150-1100 | 16 | 16 (100 %) |
| | | Total | | 96 | |

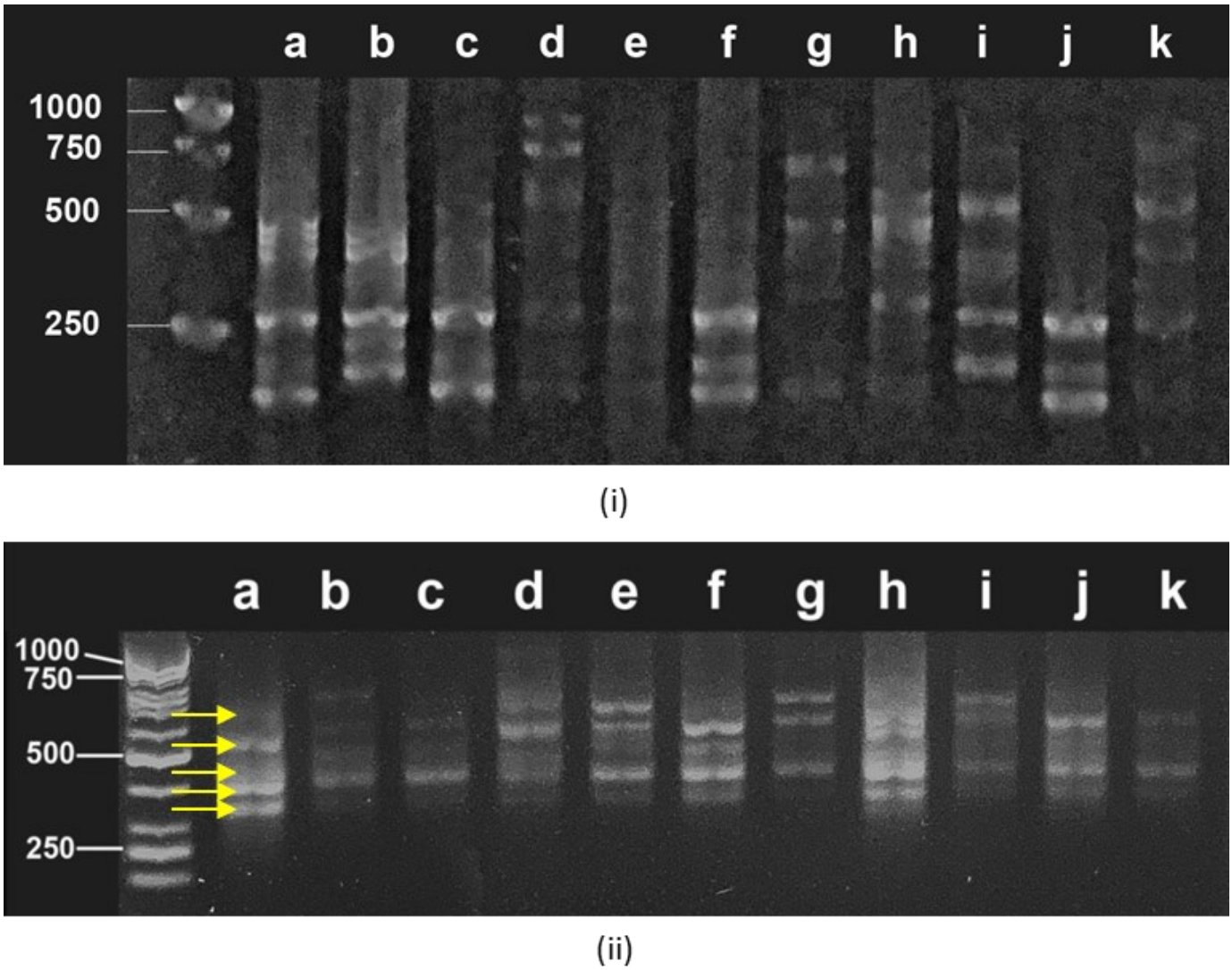


**Figure 1**. DNA fingerprinting profiles of *Dicranopteris* species and *Sticherus truncatus* showing polymorphisms from two primers: (i) primer ISSR 847 shows 100% polymorphic band, (ii) primer 862 shows 85,71% polymorphic band (yellow arrows indicate bands present in all samples with different intensities). **a** *D. linearis* var. *altissima*, **b.** *D. linearis* var. *inaequalis*, **c.** *D. curranii*, **d.** *S. truncatus* var. *involuta,* **e**. *D. linearis* var. *linearis*, **f**. *D. linearis* var. *demota*, **g**. *S. truncatus* var. *truncata*, **h.** *D. linearis* var. *alternans*, **i**. *D. linearis* var. *tertraphylla*, **j**. *D. speciosa*, **k.** *D. linearis* var. *subspeciosa*.

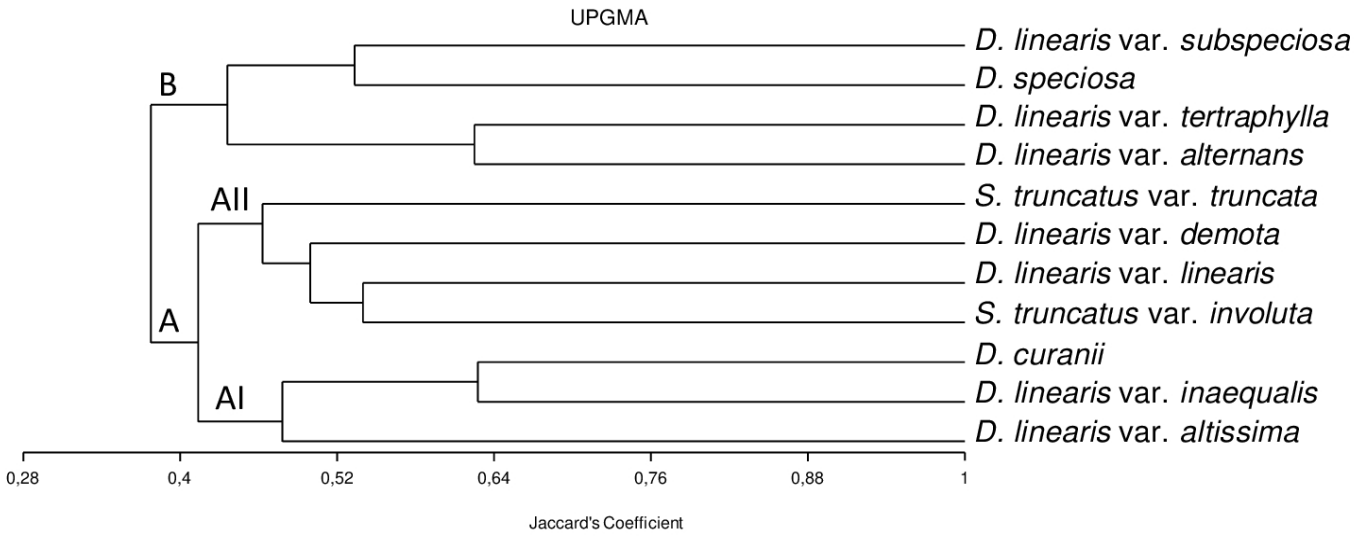


**Figure 2.** Dendrogram showing taxonomic relationships of *Dicranopteris* and *Sticherus* based on ISSR marker.

The dendrogram showed the formation of two main clusters namely clusters A and B, with a genetic similarity of 0.377. Cluster A was made up of seven taxa which were divided into two sub-clusters, whereas Cluster B was made up of four taxa. The genetic similarity of 11 Gleicheniaceae taxa from Rokan Hulu, Riau ranged from 0.377 to 0.627 based on the Jaccard similarity coefficient (Table 3). According to Rahayu et al. (2007), a genetic similarity of 0.29 indicated low diversity whereas 0.889 indicated high diversity. Andayani et al. (2016) also claimed that a value of 0.39 was considered as low, 0.70 was considered as moderate, and 0.92 was considered as high genetic diversity. Based on these previous studies using molecular markers, the genetic diversity of Gleicheniaceae in this study was moderate. The moderate genetic diversity found in the present study might be related to the growing nature of Gleicheniaceae that live in groups (Yatskievych 2018).

Another explanation for the moderate level of genetic diversity was the reproductive system of the taxa under study. Gleicheniaceae are plants that can reproduce sexually by spores and asexually by clonal propagation. Sexual reproduction is essential for dispersal and succession, whereas clonal reproduction plays a role in growth rate and territory coverage (Yang et al. 2020). *Dicranopteris* is known for its rapid clonal proliferation, and according to Russell et al. (1999), the clonal nature of this genus increases the rate of mating among gametophytes produced from the same sporophyte (intergametophytic selfing), which can reduce genetic diversity. A similar result was found in *Monimopetalum chinense* (Xie et al. 2011), *Elodea canadensis*, *Egeria densa* and *Lagarosiphon* (Lambertini et al. 2010), and *Nelumbo nucifera* (Mekbib et al. 2020) as clonal plants species live in groups with high levels of interpopulation interactions tend to have low genetic diversity due to inbreeding between populations. There were many factors causing a low level of genetic diversity in plants such as the inbreeding process in the population

**Table 3.** Similarity coefficient among *Dicranopteris* and *Sticherus*.

| | A | B | C | D | E | F | G | H | I | J | K |
|---|---|---|---|---|---|---|---|---|---|---|---|
| A | 1 | | | | | | | | | | |
| B | 0.446 | 1 | | | | | | | | | |
| C | 0.509 | 0.627 | 1 | | | | | | | | |
| D | 0.438 | 0.484 | 0.492 | 1 | | | | | | | |
| E | 0.482 | 0.458 | 0.441 | 0.54 | 1 | | | | | | |
| F | 0.414 | 0.371 | 0.424 | 0.524 | 0.475 | 1 | | | | | |
| G | 0.269 | 0.375 | 0.318 | 0.478 | 0.475 | 0.435 | 1 | | | | |
| H | 0.421 | 0.424 | 0.383 | 0.484 | 0.458 | 0.393 | 0.419 | 1 | | | |
| I | 0.415 | 0.393 | 0.4 | 0.459 | 0.429 | 0.386 | 0.323 | 0.625 | 1 | | |
| J | 0.4 | 0.333 | 0.386 | 0.444 | 0.367 | 0.421 | 0.333 | 0.429 | 0.423 | 1 | |
| K | 0.321 | 0.237 | 0.286 | 0.333 | 0.316 | 0.321 | 0.283 | 0.404 | 0.489 | 0.533 | 1 |

A=*D. linearis* var. *altisima*; B=*D. linearis* var. *inequalis*, C=*D. curanii*, D=*S. truncatus* var. *involuta*, E=*D. linearis* var. *linearis,* F=*D. linearis* var. *demota*, G=*S. truncatus* var. *truncata*, H=*D. linearis* var. *alternans*, I=*D. linearis* var. *tetraphylla*, J=*D. speciosa*, K=*D. linearis* var. *subspeciosa.*

due to random genetic drift (Ellstrand & Elam 1993), limited gene flow, small population sizes, and fragmented populations also play a role in reducing the genetic variation of a species (Teixeira & Huber 2021).

In this study, seven varieties of *D. linearis* were found in different clusters in the dendrogram, in which four varieties were in the first cluster and the remaining three varieties were in the second cluster. This result indicating that *D. linearis* is a highly variable species. Based on morphological characters, previous research in the Malesiana region found high infraspecific diversity of *D. linearis*, such as Holttum (1957) who described 11 varieties, and Van Steenis (1959) recognized 13 varieties within the species. Plants that have a high level of infraspecific variation based on morphology might also have similar results in their genetic diversity analysis. Korpelainen et al. (2005) in their study on four species of *Adiantum* found differences in the ISSR characteristics on the taxa under study which indicated infraspecific variation. Other molecular genetic diversity studies by Dong et al. (2007) on *Ceratopteris pteridoides*, and Barker & Hauk (2003) on *Sceptridium dissectum* showed that samples from the same species were found in different clusters based on ISSR marker. These studies were, therefore, also indicated the infraspecific variability on ferns.

The result of cluster analysis did not separate Gleicheniaceae taxa based on genera. In this study, *Sticherus* was placed in the same cluster as *D. linearis* in sub-cluster AII. According to Animasaun et al. (2018), species with different phenotypes do not necessarily have different genetic traits. A study by Vidyashree et al. (2019) on 19 species from 13 families of ferns using ISSR marker showed that *Pteris acanthoneura* (Pteridaceae) were found in the same group with Nephrolepidaceae and separated from other Pteridaceae. Fernando et al. (2015) reported similar results on genetic analysis in which *Asplenium longissimum* was found in different clusters from other *Asplenium* species based on ISSR markers.

The genetic diversity of Gleicheniaceae in Rokan Hulu, Riau, was categorized as moderate based on analysis of the ISSR marker. This moderate level of genetic diversity of Gleicheniaceae might be due to this fern's clonal propagation nature in their reproductive system. The results of this study open up opportunities for further research in finding specific molecular markers to identify *Dicranopteris* and *Stricherus*. This study which showed evidence of genetic diversity on infraspecific level of these two genera also provided the basis for the conservation of ferns species as part of biodiversity in this country.

## AUTHORS CONTRIBUTION

AAM collected plant samples, analyzed the data, and wrote the manuscript. RS designed the research, supervised all the processes from the field work to laboratory analysis, and wrote the manuscript.

## ACKNOWLEDGMENTS

The authors would like to thank the Ministry of Finance of the Republic of Indonesia for providing LPDP scholarship as financial support for this study.

## CONFLICT OF INTEREST

The authors state that they do not have any conflicts of interest. The authors are solely responsible for the article's content and writing.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb67129
DOI: 10.22146/jtbb.67129

Short Communications

# Using Feathers for Molecular Sexing of Straw-headed Bulbul (*Pycnonotus zeylanicus*) Offsprings

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**Keywords:**
dPCR
feather
molecular sexing
non-invasive sampling
straw-headed bulbul
**Submitted:**
28 June 2021
**Accepted:**
13 September 2021
**Published:**
21 January 2022
**Editor:**
Ardaning Nuriliani

**ABSTRACT**
Sex determination of of straw-headed bulbul offspring was carried out from 27 offspring's plucked feather samples in a captive breeding program. Using direct PCR, this study provided more evidences that feather samples are reliable as a source of DNA for non-invasive and effective molecular sexing. The study also revealed that the offspring sex ratio of straw-headed bulbul was slightly inclined towards males, but there was no significant difference from the value of 0.5.



Straw-headed bulbul (*Pycnonotus zeylanicus*) was a common bird and widespread from Myanmar, Thailand, Malaysia, Singapore to Sunda Island (BirdLife International 2001), but recently it is rarely found throughout its range, and it seems to be extinct in Southern Myanmar, Southern Thailand, and Java (Atlas Burung Indonesia 2020; Fishpool et al. 2020). The bird is one of the most popular and expensive song pet birds. For that reason, it is mostly trapped and rapidly decreased in numbers (Bergin et al. 2018). In 2018 the species was up-listed as *Critically Endangered Species* in IUCN Red List (BirdLife International 2018).

The breeding program of the straw-headed bulbul has been developed in Indonesia for around 20 years, mainly for commercial purposes (Lestari et al. 2015). One of the most important components of the captive breeding program is the precise assignment of sex to make sure the paired birds are sexually different. Because straw-headed bulbul is a monomorphic bird, which means that male and female birds are difficult to be identified from their external morphological characters (Fishpool et al. 2020).

Various methods have been applied for sex determination of birds, such as behaviour observation, morphometric measurement, cloacal examination, the ratio assay between estrogen and androgen in faces, laparotomy and laparoscopy (Richner 1989; Redelman et al. 1997). However, those techniques are time consuming, invasive, and risky for the birds. Consequently, such techniques for endangered species. Alternatively, non-invasive molecular sexing has been developed (Quintana et al. 2008).

Sex determination in birds mostly applies the standard method based on the amplification by PCR of the chromo-helicase-DNA binding (CHD) genes on the W and Z chromosomes (Fridolfsson & Ellegren 1999). The PCR employs two primers which anneal to conserved exonic regions and amplify across an intron in both CHD-W and CHD-Z. Each length of the intron usually differs between the genes. As a result, the PCR products vary in terms of size. Female birds are heterogametic (ZW), while the males are homogametic (ZZ). Therefore, the amplified products should migrate in electrophoresis as two bands in females and a single band in males.

A large range of primer sets have been developed for molecular sexing (Lee et al. 2010). There are three most commonly used primer pairs for molecular sexing for bird; they are the *CHD1*-linked primers P2/P8 (Griffiths et al. 1998), 1237L/1272H (Kahn et al. 1998), and 2550F/2718R (Fridolfsson & Ellegren 1999). These primer sets have been applied for molecular sexing for Indonesian bird species (Yuda 2008; Wirastika et al. 2015).

The genetic material for bird sexing mostly is blood samples, which consists of nucleated erythrocytes and are rich of nuclear DNA. However, blood sampling is considered invasively and logistically difficult (Horváth et al. 2005). In addition, for endangered species, it can be difficult to obtain research permits for more intrusive sampling methods. As an alternative, biological samples such as feather, buccal cells, faecal matter and post-hatched egg-shell membrane can be collected in the field with minimal disturbance to the species's study (Saputra & Yuda 2020; Wirastika et al. 2015; Yuda & Saputra 2021; Yuda et al. 2020; Ushine et al. 2016).

The type of sample has an effect on the likelihood of effective DNA extraction. For example, large primary, secondary, and tail feathers are preferable for genetic material samples compared to smaller plumulaceous feathers (Vili et al. 2013). Unfortunately, the available samples for this study were down feathers of straw-headed bulbul offspring. Since the number of feather available was limited, direct PCR was applied for molecular sexing of the bird. Direct PCR (dPCR) is a method of DNA amplification that enable PCR amplification without any prior DNA purification from samples due to the enzyme's resistance to inhibitors present in sample components. Comparison among six direct PCR-type DNA polymerases are commercially available, Miura et al (2013) found that KOD DNA polymerases is the most resistant to inhibitory blood components and/or detergent. The KOD also performed well when it applied to molecular sexing of water bird using blood as templates in direct PCR (Pratomo et al. 2021). For that reason, it was assumed that KOD DNA polymerase would also work well on direct PCR using down feather. The objective of this study is to assess the effectiveness of using down feather for direct PCR to identify the sex of straw-headed bulbul's offspring and to measure the sex ratio of hatched offspring in captivity.

Five plugged down feathers per bird were collected from 2-3 weeks nestling of straw-headed bulbul, provided by a commercial bird breeder in Yogyakarta, Indonesia, from September 2017 to February 2018. The feathers

were stored in separated envelope for each bird to minimize the contamination among samples and were kept in freezer (-20 ºC). For direct PCR template, one down feather was cut around 2 mm on the tip calamus using new sterilized razor blade for each sample. The PCR used the primer set of 2561-w (TAC GAG AAC GTG GCA ACA GAG) and 2728-w (CCA GTG CTT GTT TCC TCA ATT C) to amplify CHD-W and CHD-Z genes, with fragment lengths for about 400 and 650 bp respectively. The PCR mix, in a volume of 10 µL, contained DNA template, 0.2 mM each dNTP, 1x PCR Buffer, 1.5 mM $MgCl_2$, 1 U KOD FX Plus Neo DNA polymerase (Toyobo Co, Ltd), 0.3 µM each primer (2561-w /2728-w). The direct PCR was performed on the following cycle conditions: 94ºC for 2 minutes; 40 cycles of 98ºC for 10 seconds, 56ºC for 30 seconds, 68ºC for 30 seconds; and final extension at 68ºC for 7 minutes. PCR products were separated by running on 2% agarose gel and visualized under UV light in KODAK Gel Logic 2200 Imaging System.

The sex ratio was measured based on total samples and per egg clutch. The nestling (29 individuals of 18 clutches) used in this study was the offspring fr five pairs. Total sex ratio measures the proportion of males and females. Meanwhile, sex ratio per clutch was counted as the average of sex ration of the clutches that all eggs in the clutch was hatched. Only in 9 out of 18 clutches that all eggs laid were hatched.

All samples from the plucked down feather of straw-headed bulbul were successfully amplified using direct PCR. Eleven samples showed double band, indicating a female offspring, and the other sixteen samples with single band (males). The sizes of the bands were about 650 bp and 400 bp for the female, and was about 650 bp for the male (Figure 1). The length of CHD1-W gene of straw-headed bulbul was shorter than its CHD-1-Z gene. This result was in accordance with previous studies which using the primer set of 2550F/2718R on different bird species in Indonesia, such as Bali starling (*Leucopsar rothchildi*) (Wirastika et al. 2015), Maleo (*Macrocephalon maleo*) (Yuda

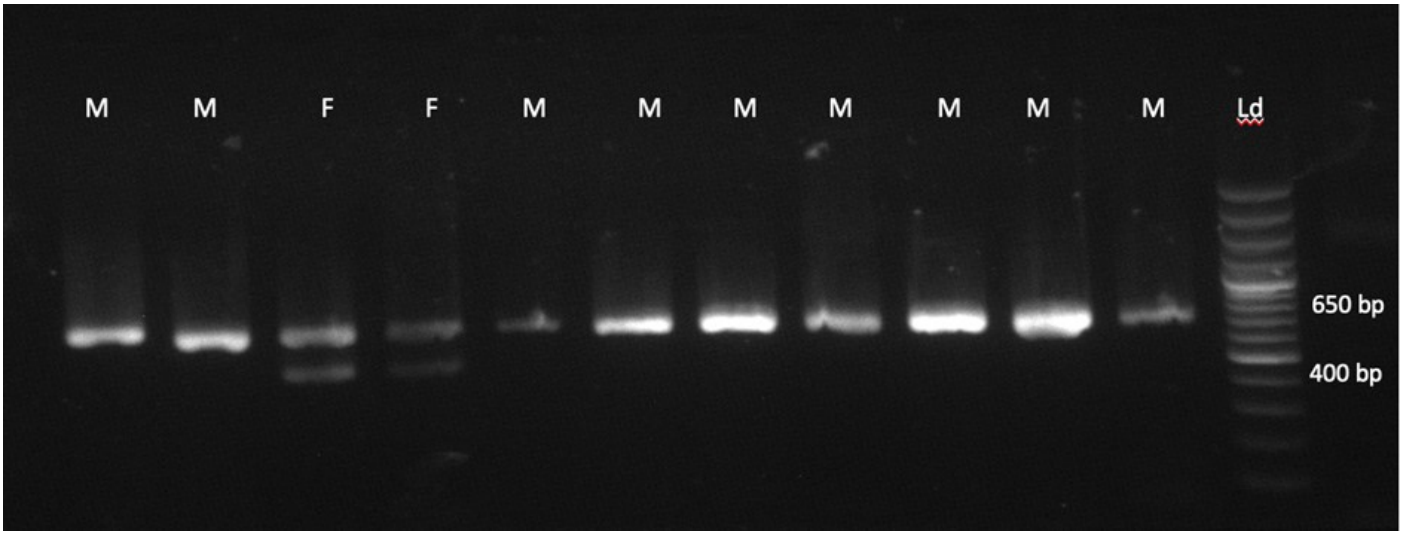


**Figure 1.** The PCR products of the CHD-1 W and CHD-1 Z of straw-headed bulbul (M-male; F –female; Ld – molecular ladder).

& Saputra 2021), 9 bird of prey species (Yuda et al. 2020), and 56 birds species of 13 families (Sulandari & Zein 2012). In contrary, using P2/P8 primer set for molecular sexing for two different species of bulbul (*Pycnonotus* spp), Pamulang & Hanyarto (2021) reported that the length of CHD1-W gene (400 bp) was longer than CHD1-Z (300 bp). The same result was also found in Tanimbar Cockatoo (*Cacatua. goffiniana*) (Hidayat et al. 2021).

Compared to blood samples, feathers provide lower DNA yields. For that reason, using blood as DNA material was preferable and have provided a better result on molecular bird sexing (Hidayat et al. 2021; Pamulang & Haryanto 2021). However, feather sampling is easier, faster, and less invasive. The handling time of the bird is reduced so that it may reduce stress on the captured birds. In addition, feathers have other values such as for isotopes analysis and trace elements as well as age determination. Feather samplings have been applied as alternative DNA sources at migration monitoring stations (Smith et al. 2003). The results of this study supports the previous studies that the plucked feather is a reliable DNA source for sexing wild birds (Harvey et al. 2006; Costantini et al. 2008). Another study which was congruence with this finding was that a single plucked feather is reliable as a source of DNA, not only for sexing but also for other genetic studies (Segelbacher 2002). In addition, the use of direct PCR makes it possible for more rapid determination of sex, as the reaction without isolation or purification of DNA reduces the analysis time.

Most bird captive breeders believe that the sex of the nestling from the same clutch is always male and female. On the other hand, this study revealed that only 5 of 9 complete hatched clutches have both sexes for two nestlings in the same clutch (Table 1). The sex ratio of all nestling is slightly biased to male (60:40), but there is no significant difference from 0.5 ($p=0.33$). Meanwhile, the offspring sex ratio for the complete hatched offspring in the same clutch were 11 males and 7 females, and there was no significant difference from 0.5 ($p=0.34$).

This finding revealed that in captive breeding the offspring sex ratio of straw-headed bulbul is a typical sex ratio to most birds (0.5). Based on the estimation of the sex ratio of 140 offspring from 114 species, mostly they were 0.5, and only 11 inclined towards males (Donald 2007).

This study provided more evidences that a plucked feather sample is reliable as a source of DNA for molecular sexing. The use of the down feathers of straw-headed bulbul as DNA template on direct PCR requires less time and it is non-invasive, as this may apply for other endangered species. The study also revealed that the offspring sex ratio of straw-headed bulbul was slightly inclined towards males, but there was no significant difference from 0.5 value.

**Table 1.** The sex of straw-headed chicks in the captive breeding program based on molecular sexing.

| Parent | Clutch | Number of eggs laid | Number of hatched eggs | Sex | |
|---|---|---|---|---|---|
| | | | | Male | Female |
| I | 1 | 2 | 1 | - | 1 |
| | 2 | 2 | 2 | 1 | 1 |
| | 3 | 2 | 1 | - | 1 |
| | 4 | 2 | 1 | - | 1 |
| II | 1 | 2 | 1 | 1 | - |
| | 2 | 2 | 2 | 2 | - |
| | 3 | 2 | 2 | - | 2 |
| III | 1 | 2 | 1 | 1 | - |
| | 2 | 2 | 1 | 1 | - |
| | 3 | 2 | 2 | 1 | 1 |
| IV | 1 | 2 | 2 | 1 | 1 |
| | 2 | 2 | 2 | 1 | 1 |
| | 3 | 2 | 2 | 2 | - |
| | 4 | 2 | 2 | 1 | 1 |
| | 5 | 2 | 2 | 2 | - |
| | 6 | 2 | 2 | - | 2 |
| | 7 | 2 | 2 | 2 | - |
| V | 1 | 2 | 1 | 1 | - |
| Total | | | 29 | 17 | 12 |

## AUTHORS CONTRIBUTION

PY designed and carried out the laboratory works and wrote the manuscript. WW designed the research. All authors contributed to this research and approved the final manuscript.

## ACKNOWLEDGMENTS

The authors would like to thank Mr. Unggul Simanjuntak, who provided the feathers for this study.

## CONFLICT OF INTEREST

The authors declare that there are no competing interests.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb67494
DOI: 10.22146/jtbb.67494

Short Communications

# Biodiversity of Freshwater Fish in Kelekar Floodplain Ogan Ilir Regency in Indonesia

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**Keywords:**
aquatic biodiversity
Kelekar river
lebak lebung
swamp
**Submitted:**
08 July 2021
**Accepted:**
22 October 2021
**Published:**
31 January 2022
**Editor:**
Ardaning Nuriliani

**ABSTRACT**
The purpose of this study is to investigate fish biodiversity in the Kelekar floodplain. The study is explorative, with the determination of observation stations and with purposive sampling methods. Fishes were captured approximately 1.509 individuals consisting of 17 families and 24 species. The Shannon-Weiner diversity index was 2.394; 2.691; and 2.183 for station 1, 2, and 3, respectively. The Evenness index was 0.764; 0.871; and 0.806 for station 1, 2, and 3, respectively, meanwhile the highest value of Simpson's dominance index was 0.045. The biodiversity index of the three stations was in the medium category.



Floodplains are major, seasonal wetlands habitat that is formed by the overspill of flooding from the rivers with which they are connected. The biodiversity in these systems is very high and riverine faunas depend on the intimate linkage between the flowing water (riverine) component and the static water (floodplain) (Welcomme 2000). One of the floodplain areas is called "lebak lebung", known only in South Sumatra, a habitat of various fish species, which are feeding, growing, spawning, and nursery ground (Muslim 2012). In South Sumatra Province, the area of open waters is approximately 2.5 million ha, where 43% is "lebak lebung" (Muslim 2013, 2012), consists of swamps, oxbow lakes, and rivers. This area is highly fertile because it contains a lot of nutrients and also natural feed, especially from the decomposition process of flooded forest vegetation (Ajai et al. 2020). The ecological function of these waters is as a feeding ground, spawning, nursery (Ammar et al. 2014; Haryono 2007; Nurdawati & Prasetyo 2007). However, Welcomme (2000) stated that living aquatic resources in floodplain are extremely intense in their response to natural climatic variability and flood strength variations.

The "lebak lebung" distribution area is in the districts of Ogan Ilir, Ogan Komering Ilir, Musi Banyuasin, Banyuasin, Muara Enim, Penukal Abab Lematang Ilir, and Palembang. One of the "lebak lebung" areas in the Ogan Ilir regency is the Kelekar floodplain area, which is located on the riverbank of the Kelekar river. The upper stream of the river is in the Prabumulih and

Muara Enim districts, meanwhile the middle stream, and downstream are in the Ogan Ilir districts. The river is a source of clean water, transportation, food (fish), as well as the daily activities of the people who live on the banks. The purpose of this study is to make an inventory of the diversity of fish species captured in the Kelekar floodplain. The results of this study are beneficial for the government and other stakeholders to design management strategies of aquatic resources in Ogan Ilir Regency and selecting candidates for aquaculture local species.

Fish samples were collected as many as 1.509 individuals (representing 24 species) from six local fishermen in the Kelekar floodplain, Ogan Ilir regency, South Sumatra, Indonesia (Figure 1). The specimens were collected from three Tanjung sampling stations: (S1) Tanjung Pring (3°14'36.2" S 104°38'58.8" E), (S2) Raya (3°14'41.0" S 104°39'28.4" E), and (S3) Indralaya Mulya (3°23'89.8" S 104°64'94.8" E). The fish samples were periodically collected from January to December 2020 (January, April and June represented the dry season, while September, October, and December represented the rainy season).

Fishes were caught with traditional fishing gears such as square lift net (*jaring angkat*), monofilament fixed gill net (*jaring insang*), cast net (*jala*), fish barrier (*empang*), and seine net (*arat waring*). Samples were collected, photographed and refrigerated, then they were transferred to the laboratory for taxonomic identification. The specimens were identified using the keys of Kottelat et al. (1993), Kottelat & Whitten (1996), and Saanin (1984). Water

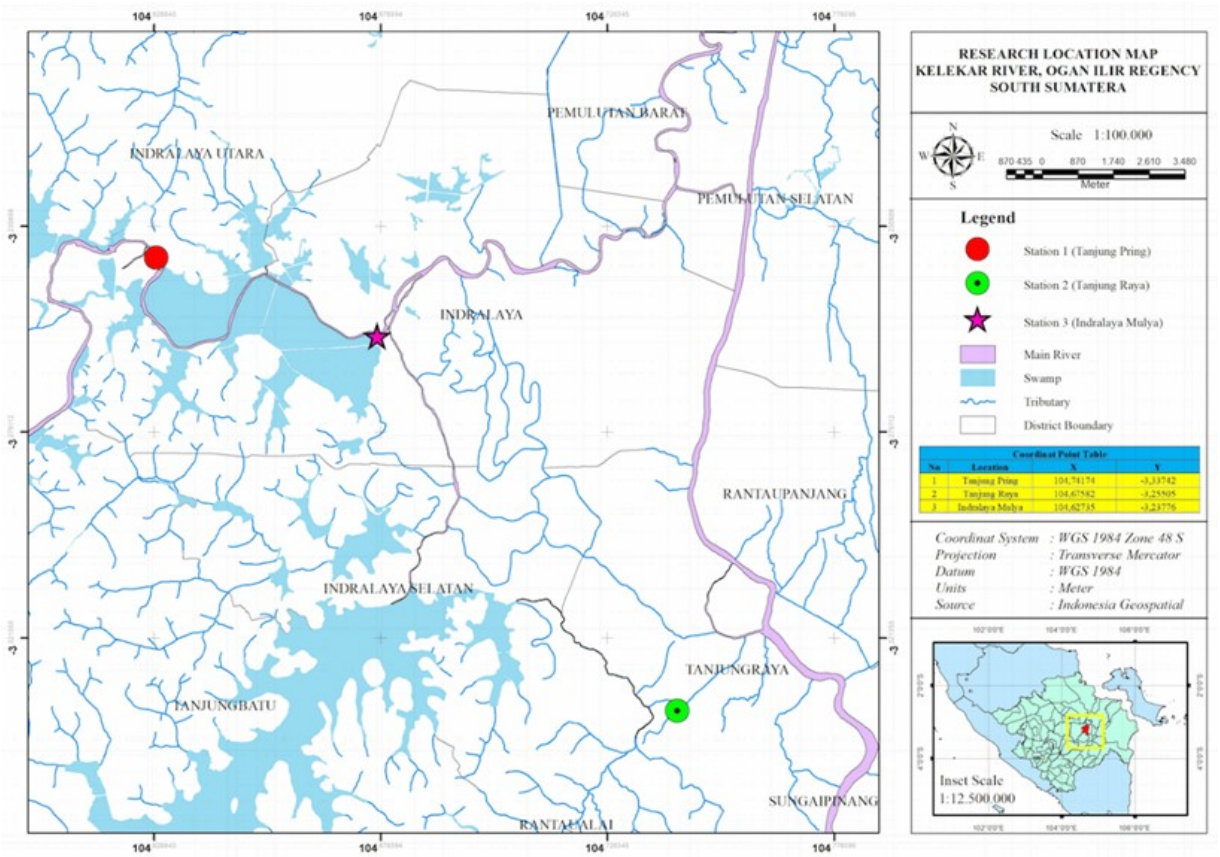


**Figure 1.** Map of sampling site in the Kelekar River. (S1), Tanjung Pring, (S2) Tanjung Raya, (S3) Indralaya Mulya of Ogan Ilir Regency, South Sumatra Province, Indonesia.

quality observed were water temperature, dissolved oxygen, and water acidity (pH), carried out in situ.

Data on fish number and species were tabulated and computed in the Microsoft Excel. The diversity for fish species was calculated using the Shannon-Wiener diversity index (Sweke et al. 2013):

$$H' = \sum_{i=1}^{S} \text{Pi. ln Pi}$$

Where *S* is the number of species in the sample, and *Pi* is the relative importance values obtained as the squared ratio of the important values of *S* individual value for all species to N the total importance. Determination of criteria: H' < 1.0 (low diversity); H' = 1.0 – 3.0 (medium); H' > 3.0 (high)

The evenness index is calculated by a formula Magurran (1988):

$$E = \frac{H'}{H'max}$$

Where, H' is Shannon-Wiener diversity index, E (Evennes index (value 0-1), H' maks (Maximum diversity index), S (Number of species). Determination of criteria: E < 0.4 (low); E = 0.4-0.6 (medium); E > 0.6 (high).

The dominant fish species is determined using the following formula:

$$C = \sum_{i=1}^{S} (Pi)^2$$

Where, C is Simpson's dominance index, $Pi$ is the relative importance values obtained as the squared ratio of the important value, $S$ is the individual value for all species.

This study indicated a wide distribution of fishes in the Kelekar river floodplain. A total of 1509 individuals that were identified can be classified into 12 families and 17 genera. Five hundred and twenty-nine (529) individuals were dominated by members of Cyprinidae, followed by Osphronemidae (276), Channidae (196), Helostomatidae (163), Pristolepidae (145), Anabantidae (100), Bagridae (42), Notopteridae (26), Claridae (17), Tetraodontidae (9), Pangasidae (5), and Mastocembelidae (2) (Table 1). The five most species of total individuals found were *R. agryrataenia* (185), followed by *H. temmincki* (165), *T. pectoralis* (146), *P. johorensis* (112), *Anabas testudineus* (100), and the least number of individuals were *M. maculatus* (2), *N. chitala* (3), *O. schlegeli* (5), *P. polyuranodon* (5), and *T. palembangensis* (7). During the dry season, the dominant fish obtained were from groups of black fishes, which included *T. tricopterus*, *H. temmincki*, *P. pectoralis*, *C. striata*, and *A. testudineus*, while in the rainy season dominated by groups of white fishes, namely *R. agriratаenia*, *P. johorensis*, and *C. apogan*. The six species with the largest number of individuals found at each station were presented in Figure 2.

The dominance index indicated various dominant species. At station 1 (S1), the most dominant species, in order from highest to lowest are as follows: *R. agryrataenia* with a dominance index (C) of 0.045, *P. johorensis* (0.029), *C. apogan* (0.019), *P. grootii* (0.009), *M. nemurus* (0.006), and *B. schwanenfeldii*

**Table 1.** Fish diversity of Kelekar floodplain in Ogan Ilir regency.

| Family | Genus | Species | Station | | | Total | Body weight (g) | Total length (cm) |
|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | | | |
| Anabantidae | Anabas | *Anabas testudineus* | 2 | 30 | 68 | 100 | 10-90 | 3-12 |
| Bagridae | Hemibagrus | *Hemibagrus nemurus* | 37 | 6 | - | 42 | 100-250 | 20-30 |
| Channidae | Channa | *Channa pleuropthalma* | - | 10 | 30 | 40 | 37-150 | 15-26 |
| | | *Channa striata* | 3 | 20 | 70 | 93 | 50-250 | 10-28 |
| | | *Channa lucius* | 5 | 45 | 13 | 63 | 20-150 | 8-20 |
| Claridae | Clarias | *Clarias batrachus* | 1 | 4 | 12 | 17 | 30-160 | 20-30 |
| Cyprinidae | Puntius | *Puntius johorensis* | 80 | 30 | 2 | 112 | 0.2-0.4 | 3-6 |
| | | *Puntioplites bulu* | 30 | 12 | - | 42 | 10-20 | 8-11 |
| | Osteochilus | *Osteochilus hasselti* | 25 | 15 | - | 40 | 10-25 | 7-12 |
| | | *Osteochilus schlegelii* | 5 | - | - | 5 | 10-30 | 7-14 |
| | Cycloheilichthys | *Cycloheilichthys apogan* | 65 | 10 | 2 | 77 | 15-20 | 8-13 |
| | Hampala | *Hampala macrolepidota* | 2 | 15 | 1 | 18 | 20-200 | 14-20 |
| | Barbonymus | *Barbonymus schwanenfeldii* | 30 | 10 | - | 40 | 100-200 | 15-20 |
| | Rasbora | *Rasbora argyrotaenia* | 100 | 70 | 15 | 185 | 0.2-5 | 5-7 |
| Helostomatidae | Helostoma | *Helostoma temmincki* | 5 | 60 | 98 | 163 | 20-120 | 10-18 |
| Mastocembelidae | Mastocembelus | *Mastocembelus maculatus* | 2 | - | - | 2 | 100-250 | 20-30 |
| Notopteridae | Notopterus | *Notopterus chitala* | 2 | 1 | - | 3 | 200-500 | 20-30 |
| | | *Notopterus notopterus* | 7 | 12 | 4 | 23 | 50-150 | 10-18 |
| Osphronemidae | Trichogaster | *Trichogaster trichopterus* | 6 | 65 | 60 | 130 | 6-12 | 3-9 |
| | | *Trichogaster pectoralis* | 12 | 48 | 86 | 146 | 10-25 | 8-12 |
| Pangasidae | Pangasius | *Pangasius pangasius* | 2 | 5 | - | 9 | 200-800 | 25-35 |
| | | *Pangasius polyuranodon* | 2 | 3 | - | 5 | 130-300 | 15-30 |
| Pristolepidae | Pristolepis | *Pristolepis grootii* | 45 | 65 | 35 | 145 | 20-80 | 5-12 |
| Tetraodontidae | Tetraodon | *Tetraodon palembangensis* | 1 | 7 | 1 | 7 | 10-30 | 7-10 |
| Total | | | 469 | 543 | 497 | 1509 | | |
| Diversity index (H') | | | 2. 394 | 2.691 | 2.183 | | | |
| Evennes index (E) | | | 0.764 | 0.871 | 0.806 | | | |

(0.004). At S2, the most dominant species are: *R. agryrataenia* (C= 0.016), *P. grootii* (0.014), *T. tricopterus* (0.014), *H. temmincki* (0.012), *P. pectoralis* (0.008), and C. *lucius* (0.007). Meanwhile, at S3, the dominant species are *H. temmincki* (C= 0.039), *T. pectoralis* (0.030), *C. striata* (0.020), *A. testudineus* (0.019), *T. tricopterus* (0.015), and *P. grootii* (0.005). The six species that were most dominant at each station are presented in Figure 3.

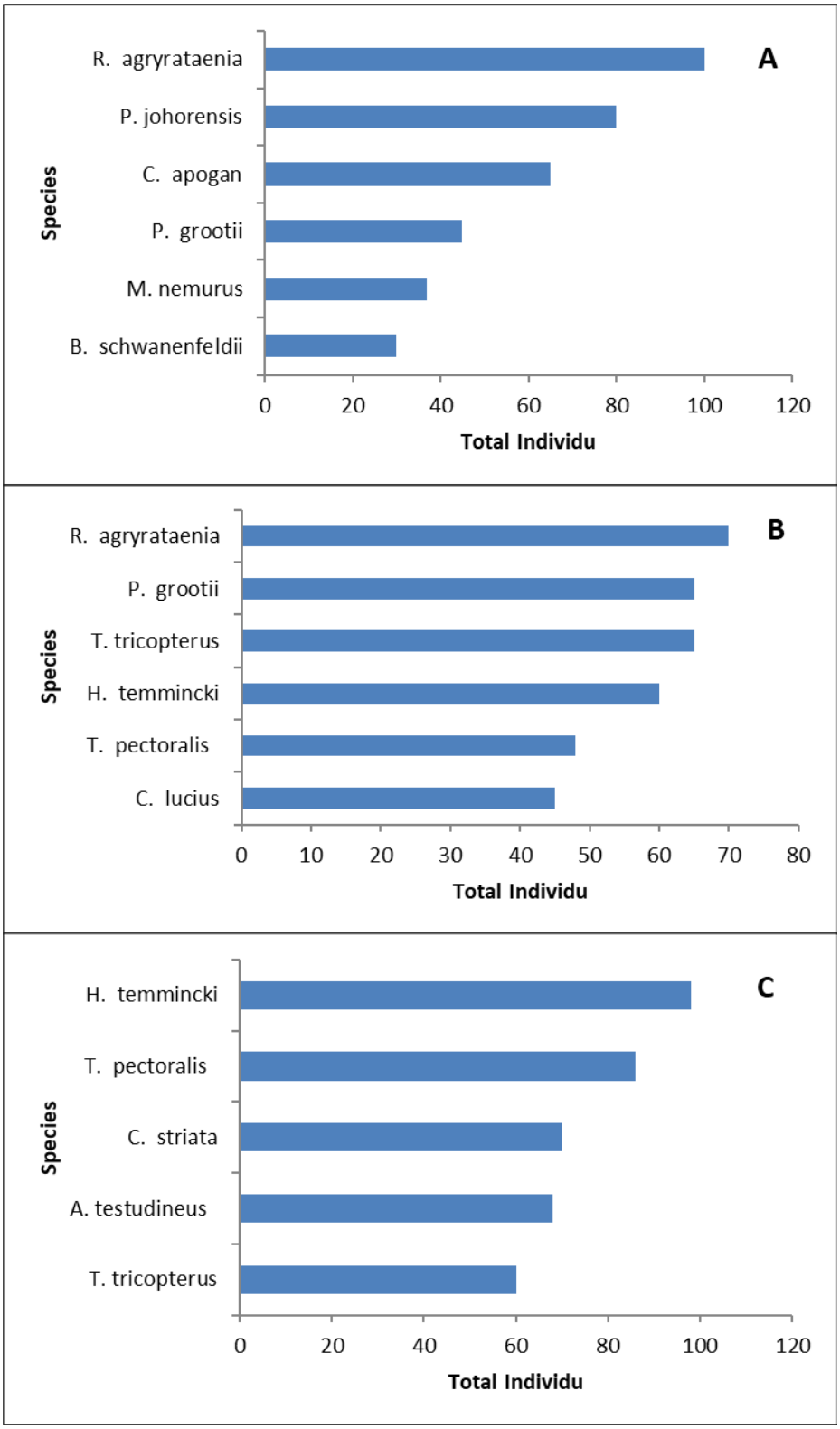


**Figure 2.** The six largest species found at each station. (A = station 1), (B = station 2), (C = station 3).

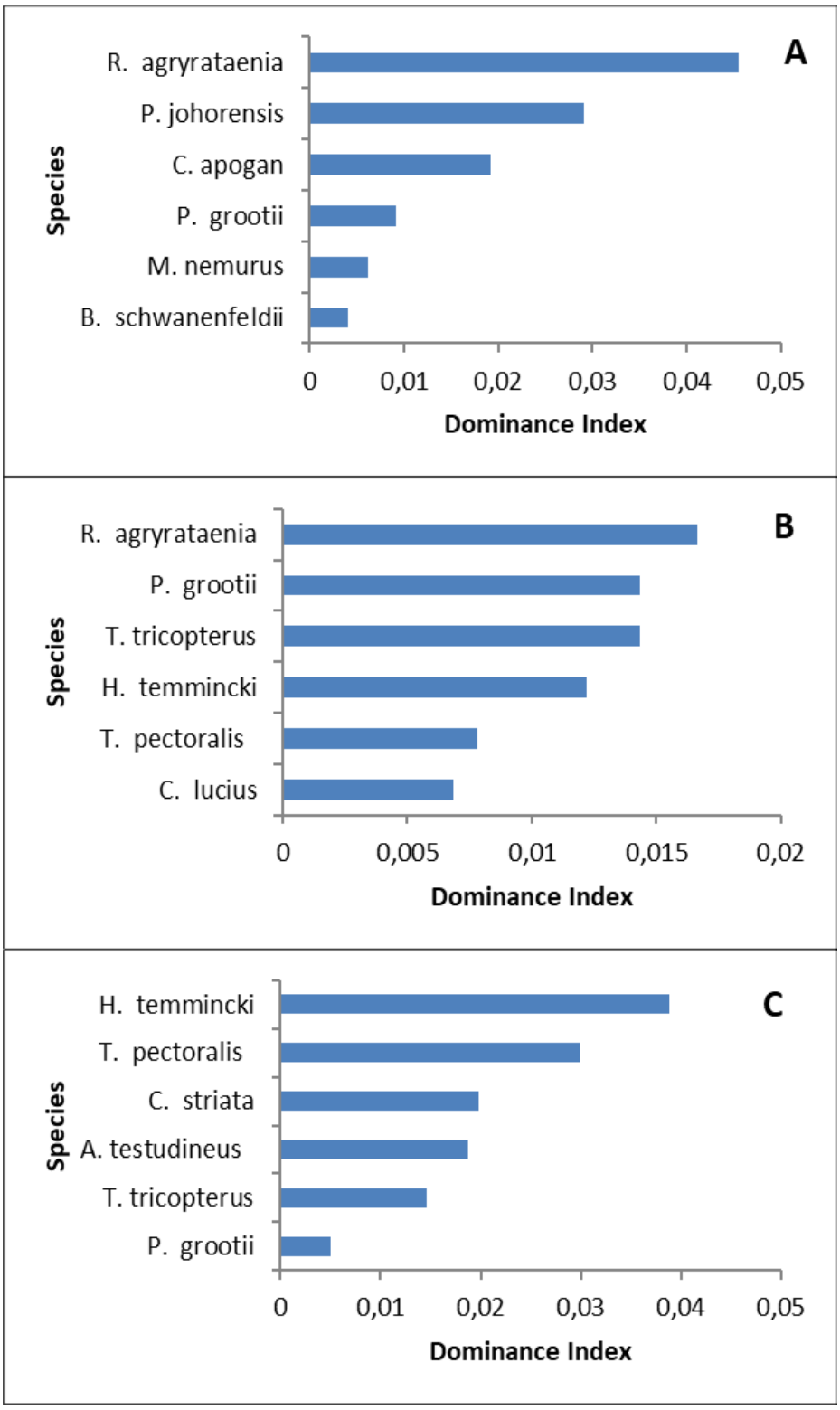


**Figure 3.** Six dominant species at each station. (A = station 1), (B = station 2), (C = station 3).

Water quality affects fish species abundance. Water quality at station 1 indicated that water temperature (24-28 $^{o}$C), dissolved oxygen (6.56-7.61 mg.L$^{-1}$), water acidity (5.6-7.0), while in station 2, water temperature (25-30 $^{o}$C), dissolved oxygen (5.67-6.41 mg.L$^{-1}$), water acidity (4.5-6.3). Station 3 denoted the value of water temperature (25-31 $^{o}$C), dissolved oxygen (4.32-5.21 mg.L$^{-1}$), water acidity (4.0-5.6).

The floodplain of Kelekar river indicated high diversity of freshwater species as it was showed in Table 1. However, the number of species found is inadequate. There are still more species that are not captured during this study, due to the limited ability of fishermen and existing fishing gear, chosen fishing grounds, and time constraints of fish collection. Nevertheless, the total number of families in this study was higher than the previous study (Patriono & Junaidi 2001; Muslim & Lestari 2005). The presence of species affects the number of species, individuals, families, and also affects the diversity, evenness, and dominance values (Magurran 1988). Furthermore, fish species composition is affected by habitat heterogeneity, environmental gradients, and human activity (Cheng et al. 2019). Natural river structures and varying habitat conditions can establish geographic barriers that constrain the dispersal potential of fish species (Fu et al. 2004). The fast population growth and economic development in the riverbank in recent decades could lead the fish diversity and aquatic resources to confront serious threats (Li et al. 2019).

The Shannon-Wiener diversity index represented the richness and proportion of each species, whereas the evenness and dominance indicated the relative number of individuals in the sample and the fraction of common species, respectively (Hossain et al. 2014). The highest Shannon-Wiener index was at the S2, while the lowest was at the S3 site. The diversity of fish species describes the entire scope of ecological adaptation, as well as the evolution of species to the environmental condition. Therefore, the diversity of fish can differ from a location to another (Syafei 2017). The index of species diversity in the Kelekar floodplain was relatively moderate. According to Magurran (1988), diversity is high if the diversity index value (H ') > 3; moderate 1 < H '< 3. At the S3 station, the water quality tends to be poor in comparison to S1 and S2, where the dissolved oxygen and the water acidity were quite low. The flooded swamp which are overgrown by high-amount of aquatic plants cause low dissolved oxygen levels so that only certain fish species can survive. The fish which has additional air-breathing organs, for instance, the labyrinth, can survive in waters with low dissolved oxygen levels (Zaccone et al. 2018).

The uniformity of individual distribution of a species at all stations was high. Based on evenness index values (Heip 1974), Cypriniformes was the dominant species at S1, Cypriniformes and Anabantiformes at S2, and Anabantiformes at S3. At S1, the dominant fish are whitefishes, however, at S3, blackfishes were dominant. One of the species *P. grootii* indicated the six-dominant species at three stations. This species lives in the headwaters (main river), tributaries, and floodplain. Several freshwater fish in South Sumatra waters have been barcoded their DNA, especially an endemic species of this region. There was a high similarity (%) of gen COI DNA mitochondria (95-100%) of stripped snakehead (*C. striata*), ocellated snakehead (*C. pleuropthalma*), Asian redtail catfish (*H. nemurus*), Pangasidae (*P. macronema*), *T. trichopterus,* and & *T. pectoralis* against the same species in the NCBI GenBank, except

in bagridae (*Mystus singaringan*) which showed a lower percentage (89%) in comparison to the same species (Syaifudin et al. 2020).

There are differences in water quality between the main river habitats, tributaries, and flooded swamps. The water quality in floodplains tends to be more acidic than the other two habitats. Dissolved oxygen content in main river habitats tends to be higher than in tributaries and floodplains. In the riverine, the water flows so that the oxygen content is higher and the water temperature tends to be lower than the other two habitats. In Figure 3 (A), the most dominant species at station 1 was the Cyprinidae family. Fish from this family distribute in a wide area including main rivers and tributaries and are even slightly found in flooded swamps.

The study found that seven species have important economic value because of their high selling price and demand, i.e *N. chitala* (IDR 80,000-120,000.kg$^{-1}$), *H. nemurus* (IDR 80,000-90,000.kg$^{-1}$), *C. striata* (IDR 60,000-70,000.kg$^{-1}$), *A. testudineus* (IDR 40,000-50,000.kg$^{-1}$) and *H. temmincki* (IDR 25,000-30,000.kg$^{-1}$). All these species were native fish that were cultured prospectively. Fish of high economic value are potential candidates for cultured species. Environmentally, these native species are well adapted, so that their entire life cycle can take place perfectly. The Kelekar floodplain could become a pivotal source of aquaculture for *N. chitala*, *H. nemurus, C. striata, A. testudineus, H. temmincki.* Further research and attempts should be made to improve local people's ability to conserve and culture the fish.

## AUTHORS CONTRIBUTION

Design the research: MM; collect and analyse the data: MSF, MM; Funding Acquisition: MM, writing-original draft: MM; writing-review and editing: MSF, MM.

## ACKNOWLEDGMENTS

We would like to thank the Fisheries Services of Ogan Ilir Regency, South Sumatra Indonesia and the local fisherman: Mr. Lukman, Mr. Nur Rohman and Mr. Ali for their assistance and facilities during the research.

## CONFLICT OF INTEREST

The authors declare no competing interests regarding the research or the research funding.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66061
DOI: 10.22146/jtbb.66061

**Short Communications**

# Essential Oils Composition of Kaffir Lime (*Citrus hystrix* DC.) Collection of Bogor Botanic Gardens from Central Java and East Sumba

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**Keywords:**
aromatic constituents
fruit oil
hydro-distillation method
leaf oil
kaffir lime
**Submitted:**
24 May 2021
**Accepted:**
10 July 2021
**Published:**
04 February 2022
**Editor:**
Miftahul Ilmi

**ABSTRACT**
The Bogor Botanical Gardens' *Citrus hystrix* collections from East Sumba and Central Java differ in morphology and fruit aroma compared to the common *C. hystrix*. Hence, this study aimed to determine the essential oils' compositions of *C. hystrix* originated from Central Java and East Sumba to further clarify these differences. Extraction of essential oils were done using hydro-distillation, and the chemical compositions were investigated using GC-MS. The main compound of the leaf oil from East Sumba and Central Java was Linalool and Citronellal, respectively. Meanwhile, the main constituents were almost identical for the fruit oils, namely L-β-Pinene, D-Limonene, and L-α-Terpineol.



*Citrus hystrix* DC. (kaffir lime) or "jeruk purut" in Indonesia, "limau purut" in Malaysia, and "som makrut" in Thailand, belongs to the Rutaceae family, which is cultivated mainly in Asian countries (Omar 1999; Ngan et al. 2019). This plant has been widely known and used by the community as a flavoring agent and to cure various diseases (Mustafa 2015). According to previous studies, *C. hystrix* has antioxidant, antibacterial, antimicrobial activity (Warsito et al. 2017; Ngan et al. 2019; Tunjung et al. 2018), anticancer (Ampasavate 2010; Tunjung et al. 2014), and antiinflammatory effects (Kidarn et al. 2018). Furthermore, this plant is also used as a natural insecticide (botanical insecticide) (Ikawati et al. 2017; Wikandari & Surati 2018) and also as antidandruff (Tanzil et al. 2017).

The Bogor Botanical Gardens (BBG) has several *C. hystrix* that originated from Central Java and East Sumba. Astuti (2011) stated that both *C. hystrix* plants in the BBG collection need to be restudied due to the differences in morphology and also in aromas produced by the fruits of these BBG collections in comparison to the common *C. hystrix*. Compared to com-

mon *C. hystrix*, *C. hystrix* fruits from Central Java are more oval, smaller in size, and have smoother peels. On the other hand, *C. hystrix* from East Sumba has thorns that are stronger and longer than the thorns of the common *C. hystrix* and also the *C. Hystrix* from Central Java (Astuti 2011). Common *C. hystrix* is kaffir lime which is commonly known, used, and cultivated by people in Asian countries. Generally, the fruit of *C.hystrix* are globose, ovoid to elliptic, green and turns yellowish-green when ripe, 5-7 cm in diameter, the pulp is yellowish, the rind is thick, and taste very acid and bitter (Lim 2012).

According to Astuti and Ajiningrum's (2019) observations on the leaves, fruits, flowers, and seed morphological characters, *C. hystrix* from Central Java differed from *C. hystrix* from East Sumba and common *C. hystrix*. However, the results from anatomical structure observations of leaves and petioles of *C. hystrix* from Central Java, common *C. hystrix,* and *C. hystrix* from East Sumba showed a quantitative similarity. Therefore, Astuti and Ajiningrum (2019) proposed that *C. hystrix* from Central Java and *C. hystrix* from East Sumba are only varieties from common *C. hystrix*. However, the study of the morphology and anatomy cannot be used to reveal the species diversity of *C. hystrix* from Central Java and East Sumba. Further study is still needed as supporting data. One of them is the study of the essential oil composition of each *C. hystrix*, considering that *C. hystrix* is a member of the *Citrus* genus that contains many essential oils (Mustafa 2015; Ngan et al. 2019).

*Citrus* plants are one of the primary essential oil sources (Mustafa 2015). Research Amaral et al. (2012) stated that the essential oil of *Neomiranthes obscura* fruit with different fruit morphology produced various types of compounds. Ebrahimi et al. (2010) stated that the difference in accessions of *Coriandum sativum* indicated varieties of the essential oil. Moreover, both *C. hystrix* collections from BBG have differences in the aroma of their fruits compared to the *C. hystrix* commonly known by the public. Based on Omar (1999), each volatile compound has a distinctive aroma. Therefore, the different scents in *C. hystrix* collections of BBG can be distinguished by differences in the essential oil composition. The characterization of essential oil composition of each *C. hystrix* provides information not only about the metabolism-related research but also for chemotaxonomy and aromatic compound diversity (Baccati et al. 2021). Hence, this study aimed to determine the composition of the essential oils of *C. hystrix* leaf and fruit from Central Java and East Sumba collection of BBG.

The leaves and fruits of *C. hystrix* from Central Java and East Sumba used in this study were gathered from the BBG collections. *C. hystrix* from Central Java was planted in area XXIV.A.49 in 1975 (46 years) and *C. hystrix* from East Sumba was planted in area XXIV.A.183 in 2002 (19 years). Both plants are planted in the same area with a spacing of about 5 meters without any other plant barriers (Figure 1). Fresh leaves and fruits with the same level

of maturity were collected from the plants, and the freshness was maintained for the essential oil isolation process.


**Figure 1.** *Citrus hystrix* (A) Central Java (B) East Sumba.

The collected leaves and fruits were cleaned from visible dust and other contaminants before extraction. Essential oils were isolated from 300 grams of clean, fresh leaves and 500 grams of whole fruits. Extraction was done using the hydro-distillation method using laboratory-scale hydro-distillation apparatus (Sibata, Japan) for approximately 5 hours for each sample. In the distillation result, two phases were observed, namely the aqueous phase and the organic phase or the essential oil phase. The essential oil was then separated from the aqueous phase, and the collected essential oil was then dried further using $NaSO_4$ anhydrite. The essential oil was stored at 4$^{o}$C in sealed vials until further analysis.

The chemical compositions of essential oils were evaluated using gas chromatography coupled with mass spectrometry (GS-MS) (Shimadzu GCMS-QP Ultra, Japan). The GC-MS analysis was performed using the Rtx-5MS column (30 m x 0.25 mm) from Restek, US. Ultrahigh purity helium was used as carrier gas with the pressure set at 30.6 KPa. The injector and interface temperature were set at 150$^{o}$C and 230$^{o}$C, respectively, while the split ratio was programmed at 1:75. The column temperature was programmed at 35$^{o}$C for 1 minute, then raised to 200$^{o}$C at 10$^{o}$C per minute, and maintained at 200$^{o}$C for 10 minutes.

The identification of the different compounds was defined by comparing the mass spectra of the compounds within the sample with the data in the NIST library. The results of leaf and fruit essential oils composition analysis of *C. hystrix* from East Sumba and Central Java using GC-MS

indicated a variation of detected volatile compounds. Figure 2 and Table 1 provide the results for the volatile composition of the two leaf EOs from BBG as well as from a reference by Warsito et al. (2017) that used common kaffir lime leaves and fruit from East Java using the same extraction method as this study. A total of 26 compounds was identified in the leaf EO of *C. hystrix* from East Sumba whereas, 21 compounds were identified in the leaf EO of *C. hystrix* from Central Java. The major component in the leaf EO from East Sumba was Linalool, with a high percentage reaching up to 86.06%. In comparison, the primary compound in the leaf EO from Central Java and in the common *C. hystrix* was Citronellal (56.99% and 85.07%, respectively). Linalool belongs to the monoterpene group which is known to have biological activities such as antimicrobial, anti-inflammatory, anticancer, antioxidant properties, and several in vivo studies. Linalool also has a role as a key compound for the industrial production of fragrance chemicals and also as a lead compound in the synthesis of vitamins A and E (Kamatou & Viljoen 2008).

At the same time, Citronellal is a monoterpene present in the oil of several plants, such as *Cymbopogon winterianus* Jowitt (Poaceae) (Java citronella) and *C. citrates* (Lemongrass). Moreover, it has biological properties including antinociceptive and antiinflammatory effects (De Santana et al. 2013; Sudiyarmanto et al. 2017). Besides Citronellal, Citronellol was also detected in the leaf EO from Central Java with a quite high amount (11.66%). In contrast, in the leaf EOs from East Sumba and the reference, this compound was not detected.

The observation by Omar (1999) revealed that *C. hystrix* leaf oil is dominated by Citronellal and Citronellol. Based on Loh et al. (2011), kaffir lime leaf oil from Selangor, Malaysia was dominated with β-Citronellal (66.85%), followed by β-Citronellol (6.59%) and Linalool(3.90%). In addition, differences were also exhibited from the minor compounds detected

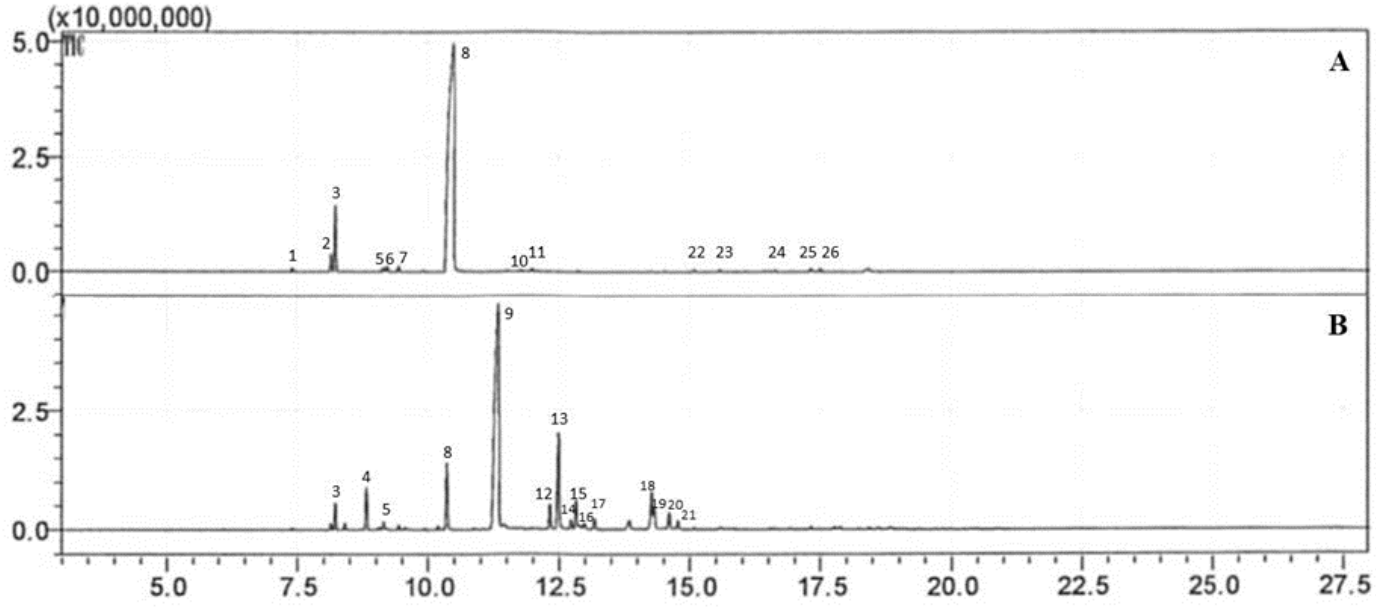


**Figure 2.** Gas chromatography chromatogram of *C. hystrix* leaf essential oils. (A) East Sumba, (B) Central Java.

**Table 1**. Composition of *C. hystrix* leaf essential oils from East Sumba and Central Java.

| No. | Compound | | Area component (%) | | |
|---|---|---|---|---|---|
| | | | East Sumba | Central Java | Common* |
| 1 | α-Pinene | Monoterpene hydrocarbon | 0.38 | - | - |
| 2 | α-Sabinene | Monoterpene hydrocarbon | 1.79 | - | 2,79 |
| 3 | β-Pinene | Monoterpene hydrocarbon | 7.02 | 2.44 | 0,33 |
| 4 | 3-Carene | Monoterpene hydrocarbon | - | 3.98 | - |
| 5 | D-limonene | Monoterpene hydrocarbon | 0.58 | 0.79 | 0,13 |
| 6 | Eucalyptol | Oxygenated monoterpene | 0.72 | - | - |
| 7 | β-Ocimene | Monoterpene hydrocarbon | 0.65 | - | 0.44 |
| 8 | Linalool | Oxygenated monoterpene | **86.06** | 6.85 | 3.46 |
| 9 | Citronellal | Oxygenated monoterpene | - | **56.99** | **85.07** |
| 10 | L-4-Terpineol | Oxygenated monoterpene | 0.16 | - | - |
| 11 | L-α-Terpineol | Oxygenated monoterpene | 0.35 | - | - |
| 12 | Cyclohexane, 2,4-diethyl-1-methyl | Non-terpene hydrocarbon | - | 2.68 | - |
| 13 | Citronellol | Oxygenated monoterpene | - | **11.66** | - |
| 14 | β-Citral | Oxygenated monoterpene | - | 0.86 | - |
| 15 | (R)-Citronellol | Oxygenated monoterpene | - | 2.75 | - |
| 16 | Octane,1-ethoxy | Oxygenated non-terpene | - | 0.60 | - |
| 17 | α-Citral | Oxygenated monoterpene | - | 1.09 | - |
| 18 | Cyclohexanol, 2-(2-hydroxy-2-propyl0-5-methyl- | Oxygenated non-terpene | - | 6.75 | - |
| 19 | Citronellol acetate | Oxygenated monoterpene | - | 1.74 | 2.77 |
| 20 | Geranyl acetate | Oxygenated monoterpene | - | 0.82 | 0.61 |
| 21 | β-Elemene | Sesquiterpene hydrocarbon | 0.31 | - | - |
| 22 | Caryophyllene | Sesquiterpene hydrocarbon | 0.26 | - | 1.77 |
| 23 | γ-Gurjunene | Sesquiterpene hydrocarbon | 0.23 | - | - |
| 24 | E-Nerolidol | Oxygenated sesquiterpene | 0.39 | - | - |
| 25 | β-Germacrene | Sesquiterpene hydrocarbon | 0.34 | - | - |
| 26 | β-Myrcene | Monoterpene hydrocarbon | - | - | 1.04 |
| 27 | Linalool epoxide | Oxygenated monoterpene | - | - | 0.70 |
| 28 | Linalool oxide | Oxygenated monoterpene | - | - | 0.33 |
| 29 | Cyclo-Germacrene | Monoterpene hydrocarbon | - | - | 0.30 |
| 30 | Cadinene | Sesquiterpene hydrocarbon | - | - | 0.22 |

from the three different leaf EOs. Some minor compounds were detected in the leaf EO from East Sumba but not in the leaf EO from Central Java as well in the common leaf EO from the reference and the other way around. These differences were displayed as different small peaks appear in Figure 1 and also from the Venn diagram (Figure 3).

The results of fruit EOs from East Sumba and Central Java displayed a more similar composition profile than the leaf EOs (Figure 4 and Table 2). Three major compounds were detected in each of *C. hystrix* fruit EO. Two compounds, L-β-Pinene dan D-Limonene, were present as the same major

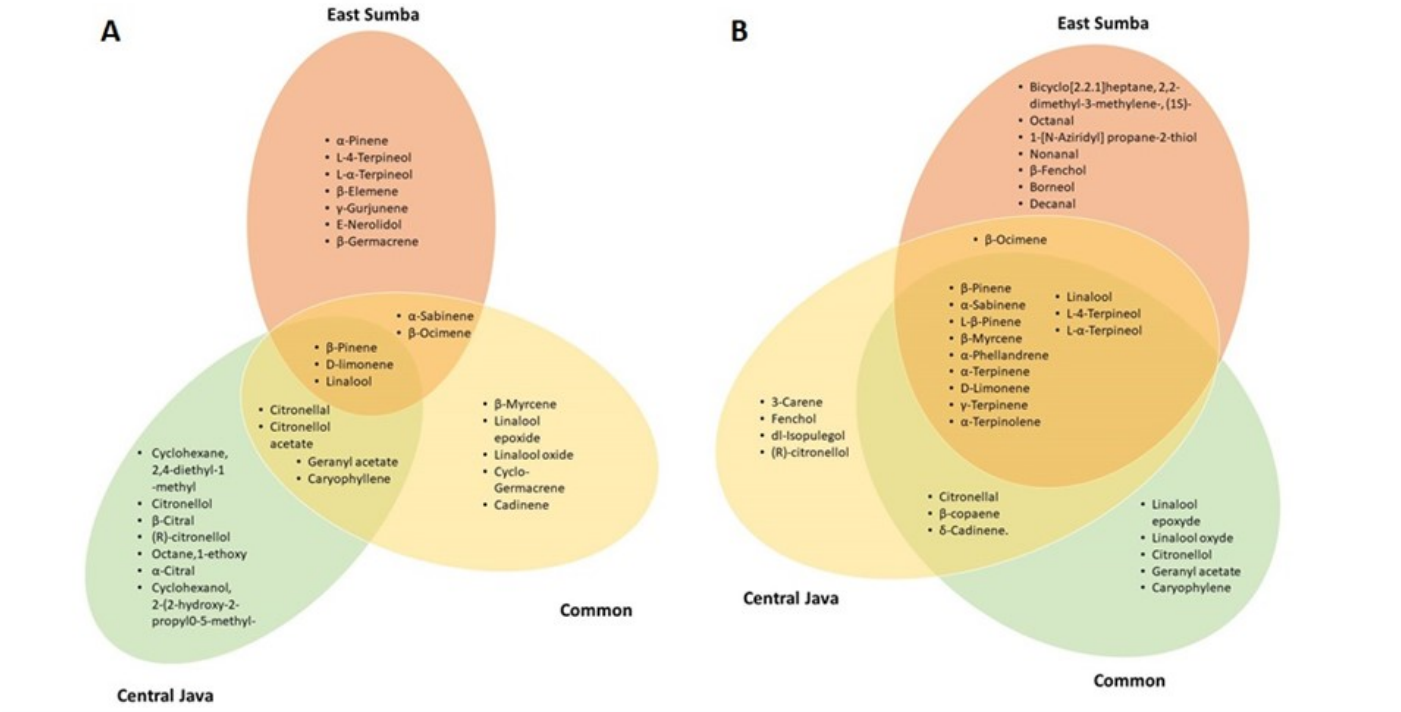


**Figure 3.** The Venn diagram of essential oils' chemical constituents from the (A) leaf and (B) fruit of *C. hystrix* originated from East Sumba, Central Java, as well as from common *C. hystrix*.

components in the three different fruit EOs. In the fruit EO from East Sumba and common *C. hystrix*, L-β-Pinene was more dominant, while in the fruit EO from Central Java, the result indicated D-Limonene as the more dominant compound. β-pinene belongs to the monoterpenes group and is found in many plants EOs. A wide range of biological properties have been reported, such as antibiotic, anticoagulant, antitumor, antimicrobial, antimalarial, antioxidant, anti-inflammatory, and analgesic effects (Salehi et al. 2019). Limonene is one of the major compositions of citrus peel, which contributes to the smell of citrus peel. It has many biological activities,

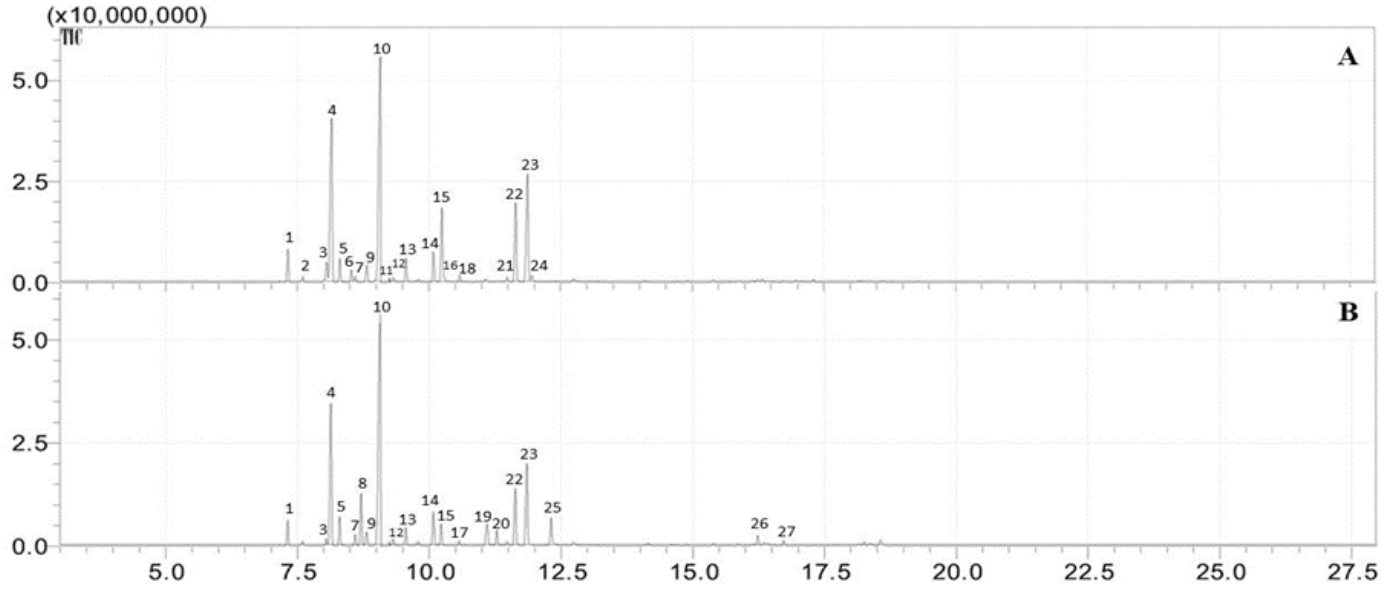


**Figure 4.** Gas chromatography chromatogram of *C. hystrix* fruit essential oils. (A) East Sumba, (B) Central Java.

**Table 2.** Composition of *C. hystrix* fruit essential oils from East Sumba and Central Java.

| No. | Compound | | Area component (%) | | |
|---|---|---|---|---|---|
| | | | East Sumba | Central Java | Common* |
| 1 | α-Pinene | Monoterpene hydrocarbon | 3.5 | 2.5 | 1.26 |
| 2 | Bicyclo[2.2.1]heptane, 2,2-dimethyl-3-methylene-, (1S)- | Monoterpene hydrocarbon | 0.4 | - | - |
| 3 | α-Sabinene | Monoterpene hydrocarbon | 1.91 | 0.75 | 9.21 |
| 4 | L-β-Pinene | Monoterpene hydrocarbon | **21.55** | **17.6** | **21.44** |
| 5 | β-Myrcene | Monoterpene hydrocarbon | 2.2 | 2.78 | 1.98 |
| 6 | Octanal | Oxygenated non-terpene | 1.31 | - | - |
| 7 | α-Phellandrene | Monoterpene hydrocarbon | 0.64 | 1.22 | 0.10 |
| 8 | 3-Carene | Monoterpene hydrocarbon | - | 5.58 | - |
| 9 | α-Terpinene | Monoterpene hydrocarbon | 1.89 | 1.63 | 1.23 |
| 10 | D-Limonene | Monoterpene hydrocarbon | **28.14** | **32.4** | **12.59** |
| 11 | 1-[N-Aziridyl] propane-2-thiol | Non-terpene hydrocarbon | 0.15 | - | - |
| 12 | β-Ocimene | Monoterpene hydrocarbon | 0.51 | 0.74 | - |
| 13 | γ-Terpinene | Monoterpene hydrocarbon | 2.28 | 1.73 | 2.29 |
| 14 | α-Terpinolene | Oxygenated monoterpene | 3.03 | 3.65 | 0.62 |
| 15 | Linalool | Oxygenated monoterpene | 8.09 | 2.2 | 4.23 |
| 16 | Nonanal | Oxygenated non-terpene | 0.26 | - | - |
| 17 | Fenchol | Oxygenated monoterpene | - | 0.53 | - |
| 18 | β-Fenchol | Oxygenated monoterpene | 0.68 | - | - |
| 19 | Citronellal | Oxygenated monoterpene | - | 3.11 | **20.91** |
| 20 | dl-Isopulegol | Oxygenated monoterpene | - | 1.62 | - |
| 21 | Borneol | Oxygenated monoterpene | 0.52 | - | - |
| 22 | L-4-Terpineol | Oxygenated monoterpene | 9.04 | 6.61 | 11.93 |
| 23 | L-α-Terpineol | Oxygenated monoterpene | **13.77** | **10.5** | 5.16 |
| 24 | Decanal | Oxygenated non-terpene | 0.58 | - | - |
| 25 | (R)-Citronellol | Oxygenated monoterpene | - | 3.23 | - |
| 26 | β-copaene | Sesquiterpene hydrocarbon | - | 1.1 | 0.18 |
| 27 | δ-Cadinene. | Sesquiterpene hydrocarbon | - | 0.57 | 0.23 |
| 28 | Linalool epoxide | Oxygenated monoterpene | - | - | 3.29 |
| 29 | Linalool oxide | Oxygenated monoterpene | - | - | 1.57 |
| 30 | Citronellol | Oxygenated monoterpene | - | - | 0.46 |
| 31 | Geranyl acetate | Oxygenated monoterpene | - | - | 0.43 |
| 32 | Caryophyllene | Sesquiterpene hydrocarbon | - | - | 0.24 |

including antioxidant, anti-inflammatory, anticancer, analgesic, antidiabetic activity, and some effects on the gastrointestinal and respiratory tract (Soulimani et al. 2019). The third major component in the fruit EOs from East Sumba and Central Java was L-α-Terpineol, while in the common *C. hystrix,* Citronellal was one of the major compositions (20.19%). These results have some degree of similarities with a previous study. Research conducted by Ngan et al. (2019) reported that kaffir lime essential oil from Vietnam was rich in β-Pinene (35.54%), sabinene (23.64%), and D-Limonene (19.08), while the kaffir lime essential oil from Thailand rich in Citronellal

(23.85), and the kaffir lime essential oil from Malaysia rich in Sabinene (35.2%), β-Pinene (16.8%), and D-Limonene (19.8%).

Based on Palazzolo et al. (2013), the major chemical component of most of the types of fruit citrus oils is Limonene. The Limonene content ranges are about 32 to 98%. In particular, the Limonene content ranges from 68 to 98% in sweet orange, from 45 to 76% in lemon, and from 32 to 45% in bergamot. These values are more significant than the Limonene content of *C. hystrix* from East Sumba (28.14%) and common *C. hystrix* (12,59%), while *C. hystrix* from Central Java had a value that fell in the range (32.4%). In addition, some minor constituents were detected in the fruit EO from East Sumba but not in the fruit EO from Central Java as well in the common leaf EO from the reference, and the other way around (Figure 3).

Oxygenated monoterpene is the dominant group of composition in the leaf EOs from East Sumba, Central Java, and in the common leaf EO from reference (Table 3). However, in the leaf EO from East Sumba, the majority of oxygenated monoterpene consist of Linalool, while in the leaf EO from Central Java and in the common leaf EO, the major oxygenated monoterpene was Citronellal. On the other hand, in the three different fruit EOs, two dominant groups of compounds were monoterpene hydrocarbon and oxygenated monoterpene (Table 3). In the fruit EOs from East Sumba and Central Java, monoterpene hydrocarbon was more dominant (approximately two times higher), while in the common fruit EO, monoterpene hydrocarbon and oxygenated hydrocarbon had relatively the same percentage.

The varied compositions of essential oils in this study can be caused by differences in plant origin, age, morphology, and aroma. Moreover, environmental factors can affect the production of essential oils. Both *C. hystrix* from the BBG collection were planted in the same area and closed to each other. However, *C. hystrix* from Central Java was located in a part that was shaded by other plants, while *C. hystrix* from East Sumba was in an area that was exposed to direct sunlight. According to Yang et al. (2018), plant secondary metabolites accumulation depends on various environmental factors such as light, temperature, and soil water. For most plants, a change in an individual also factor may alter the content of secondary metabolites even if other factors remain constant.

In conclusion, the results showed that leaf and fruit EOs composition analysis of *C. hystrix* from East Sumba and Central Java using GC-MS indicated a variation of detected volatile compounds between *C. hystrix* from the two regions compared to common *C. Hystrix* from reference. The main composition of *C. hystrix* leaf EOs from East Sumba was Linalool, while in *C. hystrix* from Central Java and common *C. hystrix* was Citronellal. Meanwhile, the *C. hystrix* fruit EOs from East Sumbawa and Central Java has almost the same profile with the three main components, namely L-β-Pinene, D-Limonene, and L-α-Terpineol, while in common *C. hystrix* were L-β-Pinene, D-Limonene, and Citronellal.

**Table 3.** Class of compounds in *C. hystrix* fruit essential oils.

| No. | Group of compounds | Total peak (%) | | | | | |
|---|---|---|---|---|---|---|---|
| | | East Sumba | | Central Java | | Common | |
| | | Leaf | Fruit | Leaf | Fruit | Leaf | Fruit |
| 1 | Monoterpene hydrocarbons | 10.4 | 62.5 | 7.2 | 66.9 | 5.0 | 50.1 |
| 2 | Oxygenated monoterpenes | 87,3 | 35.1 | 82.7 | 31.5 | 92.9 | 48.6 |
| 3 | Sesquiterpene hydrocarbons | 1.1 | - | - | 1.7 | 2.0 | 0.65 |
| 4 | Oxygenated sesquiterpenes | 0,4 | - | - | - | - | - |
| 5 | Non-terpene hydrocarbons | - | 0.2 | 2.7 | - | - | - |
| 6 | Oxygenated non-terpenes | - | 2.2 | 7.4 | - | - | - |

## AUTHORS CONTRIBUTION

All authors have an equal contribution to the research and publication. IPA was designed for the study, observed, and collected the research samples from the garden. KDP and FD analyzed the data. IPA, KDP, and FD wrote the manuscript.

## ACKNOWLEDGMENTS

The author would like to thank Professor Anny Sulaswatty, who helped the authors review the manuscript and Mr. Harto from BBG for collecting kaffir lime leaf and fruit samples from the garden.

## CONFLICT OF INTEREST

The authors declare that they have no conflict of interest from this manuscript.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66111
DOI: 10.22146/jtbb.66111

**Short Communications**

# The Potential of *Trichosanthes tricuspidata* Lour. from Bangli, Baturiti, Bali for Free Radicals Scavenging

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**Keywords:**
antioxidant
Cucurbitaceae
DPPH
methanol extract
phytochemical
**Submitted:**
26 May 2021
**Accepted:**
30 October 2021
**Published:**
18 February 2022
**Editor:**
Ardaning Nuriliani

**ABSTRACT**

In addition to the studies on potential medicinal uses of *Trichosanthes*, a screening on phytochemical compounds and antioxidants activity of *Trichosanthes tricuspidata* from Bangli, Baturiti, Bali, Indonesia, was conducted on its leaves, fruits, peels, and seeds. Qualitative phytochemical tests were conducted to find out the chemical constituents of *T. tricuspidata*, while its antioxidant activity was tested by applying DPPH (1,1-diphenyl-2-picrylhydrazyl radical) method. As a result, flavonoid, alkaloid, terpenoid, tannin, and saponin were present in all methanolic extracts of *T. tricuspidata*. Furthermore, the best antioxidant activity was exhibited by peel extract. After all, *T. tricuspidata* contains a prospective compound agent for medicinal use.



*Trichosanthes*, belongs to Cucurbitaceae, is characterized by its climbing habit, mostly branched tendrils, distinct fringed petals, and bright-colored fruits (Duyfjes & Pruesapan 2004). Its natural distribution is widely spread from India eastwards to Japan, Malaysia regions, and downwards to Australia. Among 103 species included in this genus, around 39 species grow in Malaysia regions, and 8 species are reported to be found in Indonesia (Rugayah & Darnaedi 2004). One of the notable species of this genus is *Trichosanthes tricuspidata* Lour. which is potentially believed to treat a broad spectrum of diseases, is spreaded from Asia to tropical Australia (Bhandari et al. 2008; Ahuja et al. 2019).

*Trichosanthes* is used traditionally for food and medical purposes. Kumar et al. (2012) reported that *T. dioica* is used as a vegetable and an ailment of numerous illnesses including asthma, ulcer, and diabetic for traditional uses. It is also used as a traditional medicine in Ayurvedic, Unani, and Thai medical systems to treat fever, inflammation, worm infection, migraine, and is used as laxative agent (Bhandari et al. 2008; Ahuja et al. 2019). Meanwhile, in Indonesia, Windadri et al. (2006) reported the use of *T. tricuspidata* for scabies treatment by Muna tribe people in Southeast Sulawesi.

In addition to the research on potential medicinal uses, phytochemical screening studies have also been conducted. Cycloartane glycosides, cucurbi-

tane, hexanorcucurbitane, octanorcucurbitane glycosides, and cucurbitacins were isolated from *T. tricuspidata* (Kasai et al. 1999; Kanchanapoom et al. 2002; Mai et al. 2002). Phenol and flavonoid were also isolated from *T. anguina* (Marsetya et al. 2009). Meanwhile, *T. kirilowii* extract contains numerous chemical compounds including flavonoid (Xu et al. 2012). Additionally, the antioxidant potential of *Trichosanthes* has been widely reported. Marsetya et al. (2009) documented antioxidant activity of *T. anguina* extracts. Significant antioxidant activity was also shown by water extracts of *T. cucumeriana* aerial parts (Arawwawala et al. 2011).

Although extended research of *Trichosanthes* phytochemical and antioxidant screening studies was presented, a study in this subject for Indonesian *Trichosanthes* is still limited. Known as *anggur memedi* by the locals and considered as weeds, *T. tricuspidata* is also found in Tabanan, Bali. The present study is aimed to examine the phytochemical compounds and antioxidant activity of *T. tricuspidata* methanolic extract. It is expected that this study will enhance our understanding of phytochemistry of *T. tricuspidata* and support its future domestication and conservation efforts.

This research was conducted from November 2020 to January 2021. Specimens of *T. tricuspidata* were collected from a paddy field at *Banjar* Apid Yeh (691 asl), of Bangli Village, Baturiti, Tabanan, Bali, in November 2020 (Figure 1A). Plant extraction was prepared in Bali Botanic Garden Seed Bank and Applied Botany Laboratories. *T. tricuspidata* leaves and fruits were separated (Figure 1B). The fruits were peeled to separate its epicarp (outermost skin/peels), mesocarp (fleshy part/fruits), and seeds. Leaves, fruits, peels, and seeds were washed under running tap water, chopped, and air-dried at room temperature for 3 to 5 days (Figure 1C). The simplicia was then be extracted using methanol as a solvent. Methanolic extract from each simplicia was subjected to be tested for its phytochemical screening and antioxidant activity at Herbal Materia Medica Laboratory, Batu, East Java.

Qualitative chemical tests were conducted to find out the chemical constituents of *T. tricuspidata* following the methanolic extracts, while its antioxidant activity was conducted by applying DPPH (1,1-diphenyl-2-picrylhydrazyl radical) method. The extracts concentrations were used as follow: leaf extract 125, 250, 500, 1000 ppm; peels extract 31.5, 62.5, 125, 250 ppm; fruit extract 500, 600, 700, 800, 900, 1000 ppm; and seed extract 31.5, 62.5, 250, 500, 1000 ppm.

To examine the antioxidant activity, a total amount of 100 mL DPPH solution by 0.1 mM was added to each extract. A spectrophotometer was used to measure the solution absorbance at 515 nm wavelength. The percentage of inhibition obtained was then be arranged in a regression graph to determine the 50% inhibition concentration ($IC_{50}$) of the extract.

The percentage of *T. tricuspidata* methanol extract production ranged from 1.248 to 10.649 percent. The highest extract was yielded from the fruit,


**Figure 1**. *Trichosanthes tricuspidata* A: in its natural habitat at *Banjar* Apid Yeh, Bangli, Baturiti, Tabanan, Bali; B: leaves and fruits were separated; C: seeds were extracted from the fruits and air-dried for 3-5 days.

while the seed produced the lowest extract percentage. The complete yield of *T. tricuspidata* methanolic extract is presented in Table 1.

The phytochemical screening revealed the presence of flavonoid, alkaloid, terpenoid, tannin, and saponin in all *T. tricuspidata* methanolic extracts. Terpenoid was present in the form of triterpenoid while steroid was absent in all extracts. The result of *T. tricuspidata* extracts screening conducted in this study is presented in Table 2.

All of phytochemical compounds detected in *T. tricuspidata* methanol extract are valuable in medicinal sciences. Previously, flavonoid was isolated from *T. anguina* fruit extracts (Marsetya et al. 2009; Tripathy et al. 2014; Aseervatham et al. 2019) to treat cancer and cardiovascular diseases, which act as antioxidants, anti-inflammatory, anti-diabetic, and anti-bacteria (Arifin & Ibrahim 2018). In line with that finding, we found flavonoids in all parts of *T. tricuspidata*. Among natural products, flavonoids are one of the important secondary metabolites in plants. Furthermore, *T. tricuspidata* roots extract showed the presence of biologically active compounds that contribute to its antibacterial activities such as phenol, tannin, alkaloid, triterpenoid, and saponin (Bhardwaj & Rashmi 2015).

**Table 1**. The yield of *Trichosanthes tricuspidata* crude extract.

| Plant material | Weight of simplicia (g) | Amount of extract (mL) | Yield (%) |
|---|---|---|---|
| Leaves | 200 | 16 | 8.000 |
| Fruits | 185 | 19.7 | 10.649 |
| Peels | 245 | 24.7 | 10.082 |
| Seeds | 882 | 11 | 1.248 |

**Table 2.** Phytochemical analysis of *Trichosanthes tricuspidata* extracts.

| Plant materials | Compounds | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Flavonoid | Alkaloids | | | Tannin | Terpenoids | | Saponin |
| | | Mayer test | Dragendorff test | Bouchardat test | | Steroid | Triterpenoid | |
| Leaves | + | + | - | + | + | - | + | + |
| Peels | + | + | + | + | + | - | + | + |
| Fruits | + | + | - | + | + | - | + | + |
| Seeds | + | + | - | + | + | - | + | + |

Note: + = Present, - = Absent

Kavitha (2017) reported the presence of alkaloids in *T. dioica* leaves and fruits methanolic extracts, but in *T. tricuspidata*, this compound found in leaves methanolic extract (Yuvarajan et al. 2015), while Tripathy et al. (2014) found alkaloid in fruit extract. Out of three tests conducted to examine the presence of alkaloids in this study, Mayer and Bouchardat tests showed a positive result in all *T. tricuspidata* extracts, while the Dragendrof test only showed a positive result in the peels extract. The positive results of alkaloid from Mayer's test for all extracts shows that *T. tricuspidata* might contain alkaloids with quaternary nitrogen structure. Alkaloids are best known for their physiological action on the animal organism which placed them as a high valued compound for medicine (Maldoni 1991). All extracts showed a positive result for at least two alkaloid tests except for peels extract that showed positive results in all three alkaloid tests.

Tannin was found in *T. lobata* and *T. dioica* extracts (Kumar et al. 2012; Rajasekaran & Periyasamy 2012). In this study, tannins were detected in all methanolic extracts of *T. tricuspidata*. However, Tripathy et al. (2014) reported that tannin was only found in *T. tricuspidata* fruit aqueous extract, but it was not found in fruit methanol extract. Tannins have been known as a compound that acts against infections and cell abnormalities, as well as possess antibacterial and antifungal potencies (Gupta & Pandey 2020).

Tripathy et al. (2014) reported the presence of terpenoid in *T. tricuspidata* fruit n-butanol extract, but it was absence in the methanol extract. On the contrary, we found the presence of terpenoid in the methanolic fruit extract. Similarly, cucurbitacins, a triterpenoid compound from the Cucurbitaceae, were also extracted from *T. tricuspidata* fruit (Attard & Martinoli 2015). Triterpenoid was also presented in *T. tricuspidata* roots extracts (Bhardwaj & Rashmi 2015). Meanwhile, the steroid was not found in all extracts tested in this study. In contrast, Tripathy et al. (2014) reported the presence of steroids in *T. tricuspidata* fruit methanolic extract. Moreover, triterpenoids and saponin were detected on each extract from *T. tricuspidata,* which potentially obtain antimicrobial activity (Barre et al. 1997; Amaral et al. 1998; Gupta & Pandey 2020). A previous phytochemical study showed that the presence of various phytochemical compounds including terpenoids of *T. tricuspidata* extract exhibited a strong anti-diabetic activity (Kulandaivel et al. 2013).

Leaves and roots extracts of *T. tricuspidata* also contain saponin (Bhardwaj & Rashmi 2015; Yuvarajan et al. 2015). Identically, we found that saponin was present in all extracts of *T. tricuspidata*. However, Tripathy et al. (2014) found that saponin only presented in aqueous fruit extract but was absent from the fruit methanol extract. Saponin is a chemical compound with numerous therapeutic benefits such as anti-inflammatory, antifungal, hypoglycemic, hypocholesterolemic, immunostimulant, cytotoxic, and obesity treatment potential (Marrelli et al. 2016).

To analyze the potential of *T. tricuspidata* as an antioxidant agent, a DPPH scavenging activity test was conducted to each extract. Delocalisation of the spare electron from the DPPH molecule had been measured to have an inhibitory percentage. The inhibitory percentage indicates the sample's ability to bind free radical electrons. DPPH assay is used widely to assess free radical scavenging activity due to its convenience, stability at room temperature, and its production of violet solution in a solvent. The scavenging activity of plant extracts were indicated by the discoloration in the solvent (from violet to yellow) (Singh et al. 2016). The current study results showed different DPPH scavenging activities from different parts of the plant (Table 3).

Leaves, fruits, peels, and seeds extract of *T. tricuspidata* showed the ability of scavenging free radicals. The increase of extract concentration is directly proportional to the percentage of plants that ward off free radicals. This result was supported by Ghasemi et al. (2009) which revealed the radical-scavenging activities of all extracts of peels and tissues of citrus species increased with increasing concentration. In this study, concentrations of leaves, fruits, and seeds extracts of 100 ppm inhibited 67.84, 69.06, and 70.34% free radicals, respectively. Unlike the peels, to inhibit up to 67.26%, free radicals

**Table 3.** Inhibition percentage of *Trichosanthes tricuspidata* extracts toward free radical.

| Extract | Extract concentration (ppm) | Absorbance value | Inhibition | Inhibition percentage (%) |
|---|---|---|---|---|
| Leaves | 125 | 0.461 | 0.453 | 45.302 |
| | 250 | 0.436 | 0.483 | 48.270 |
| | 500 | 0.384 | 0.544 | 54.442 |
| | 1000 | 0.271 | 0.678 | 67.844 |
| Fruits | 500 | 0.331 | 0.573 | 57.267 |
| | 600 | 0.305 | 0.606 | 60.615 |
| | 700 | 0.291 | 0.624 | 62.424 |
| | 800 | 0.273 | 0.647 | 64.725 |
| | 900 | 0.250 | 0.677 | 67.724 |
| | 1000 | 0.240 | 0.691 | 69.055 |
| Peels | 31.5 | 0.466 | 0.447 | 44.697 |
| | 62.5 | 0.416 | 0.507 | 50.668 |
| | 125 | 0.399 | 0.527 | 52.650 |
| | 250 | 0.276 | 0.673 | 67.262 |
| Seeds | 31.5 | 0.471 | 0.441 | 44.139 |
| | 62.5 | 0.449 | 0.467 | 46.655 |
| | 250 | 0.417 | 0.505 | 50.513 |
| | 500 | 0.339 | 0.598 | 59.760 |
| | 1000 | 0.250 | 0.703 | 70.336 |

required a concentration of 250 ppm. Correspondingly, Orak et al. (2012) reported that *Punica granatum* peels extract showed higher scavenging activity than its juice and its seeds extracts. Similarly, Singh et al. (2016) found that the antioxidant activity of four Cucurbitaceae species were significantly higher in peels than pulps.

A higher concentration at 500-1000 ppm on leaves, fruits, and seeds extracts showed a non-significant increase in inhibitory activity. The scavenging activity is considered effective if the requirements of low extract concentration with high inhibition percentage. Therefore, the scavenging activity given below (Table 4) is calculated of the half maximum (50%).

**Table 4.** The $IC_{50}$ value of *T. tricuspidata* extracts on DPPH radical.

| Extract | $IC_{50}$ (ppm) |
|---|---|
| Leaves | 315.970 |
| Fruits | 171.992 |
| Peels | 78.029 |
| Seeds | 208.582 |

The $IC_{50}$ value is obtained through a regression analysis that describes the concentration of a test solution that can reduce 50% free radicals and represents antioxidant activity (Widyasanti et al. 2016). Antioxidant activity or $IC_{50}$ values in leaves, fruits, peels, and seeds extracts were 315.970, 171.992, 78.029, and 208.582 ppm, respectively. Compared to other extracts, *T. tricuspidata* peels extract has the strongest antioxidant activity. According to Molyneux (2004), antioxidant activity is grouped into very strong ($IC_{50}$ < 50 ppm), strong ($IC_{50}$ 50–100 ppm), moderate ($IC_{50}$ 100–150 ppm), and weak ($IC_{50}$ 150–200 ppm).

The peels exhibited the highest ability in the DPPH scavenging activity test with $IC_{50}$ 78.029 ppm, the lowest value compared to the other parts. Our result may suggest that peels have a high concentration of phenolic compounds that contribute to antioxidant activity. This result is similar to the previous study in *T. tricuspidata* that showed antioxidant activity was cumulated in the pericarp region (Mai et al. 2002). Similarly, Singh & Prakash (2013) were also reported that the best $IC_{50}$ of *T. cucumerina* extracts was found in fruits rather than in leaves, stem, and roots. Moreover, our finding in the highest antioxidant activity is specific and narrowed to the epicarp or peels.

Phenols and polyphenolic compounds, such as flavonoids, anthocyanins, and tannins, have proven to exhibit antioxidant properties (van Acker et al. 1996; Hosu et al. 2014). Anthocyanins are flower and fruit dominant pigments that produce their distinctive reddish, bluish, and purple hues (Rice-Evans et al. 1997). As reported by Febrianti et al. (2016), the dark rind contains higher polyphenol. Therefore, the strong antioxidant activity of the peels is thought to be due to their red color. Polyphenols in their chemical activity are reduced as hydrogen or electron donor agents predicting their

potential action as free radical scavengers (antioxidants) (Rice-Evans et al. 1997). Moreover, Wangensteen et al. (2004), Marsetya et al. (2009), Ardekani et al. (2010), Kumar et al. (2014), and Febrianti et al. (2016) stated that total phenols in extracts are positively correlated with the antioxidant activity. Thus, further research is needed to investigate the quantitative phytochemical compounds that act as active antioxidants. However, a strong antioxidant activity of *T. tricuspidata* that resulted in this study could be important scientific evidence supporting the use of this species in traditional medicine for centuries.

To conclude, phytochemical screening on *T. tricuspidata* showed the presence of flavonoid, alkaloid, terpenoid, tannin, and saponin. Leaves, fruits, peels, and seeds showed antioxidant activity. Above all, peel extract showed the highest antioxidant activity. Subsequently, we conclude that this species contains a prospective compound agent for medicinal use.

## AUTHORS CONTRIBUTION

ASL collected the materials, prepared and extracted the plant, composed the manuscript, and evaluated the manuscript; FK collected the materials, composed the manuscript, and evaluated the manuscript; ARUW collected the materials and composed the manuscript; CIMS extracted the plant and composed the manuscript; while PKW collected the materials and extracted the plant. Thus, we declare that ASL, FK, and ARUW are the main contributors, while CIMS and PKW are the member contributors.

## ACKNOWLEDGMENTS

The authors want to express our gratitude to Millenium Seed Bank Partnership, Royal Botanic Garden, Kew, for the grants; the head of Eka Karya Bali Botanic Garden and the head of Research Center for Plant Conservation and Botanic Gardens, National Research and Innovation Agency, for the permit and supports; as well as Resort Pengelolaan Hutan Penebel, Unit Pelaksana Teknis Kesatuan Pengelolaan Hutan Bali Selatan, Dinas Kehutanan dan Lingkungan Hidup Provinsi Bali, for the permit. We also want to thank Gusti Ayu Candiwati, I Gusti Made Sudirga, Hanapi Effendi, Ahmad Fauzi, and I Nyoman Peneng for the technical assistant during this study.

## CONFLICT OF INTEREST

The authors state that there is no conflict of interest regarding the research substance and funding.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb67896
DOI: 10.22146/jtbb.67896

Short Communications

# Antioxidant activity of phenolic compound of *Astraeus hygrometricus*: A Case of Ranchi, India

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**Keywords:**
*Astraeus hygrometricus*
FT-IR
MS
Mushrooms
**Submitted:**
24 July 2021
**Accepted:**
10 October 2021
**Published:**
28 February 2022
**Editor:**
Miftahul Ilmi

**ABSTRACT**
The forest region of Ranchi district of Jharkhand, India, is very rich with different types of wild mushrooms. This study collected two hundred samples. There are nine different species were identifiedand one them was *Astraeus hygrometricus* that chosen for isolation, purification, and characterization of its compounds. This mushroom was chosen because of its nutritive value as for human consumption and also fewer studies done on it. It has got many compounds unrevealed. Various techniques such as solvent extraction including phase separation, TLC, FT-IR, and MS were employed in this study. Also, the total phenol content and antioxidant assay (DPPH) of the purified transparent compound of methanolic extract was carried out. The study showed that unknown transparent phenolic compound, established as astrakurkurol with molecular weight 485 was obtained.



The nutritional and medicinal aspects of different mushrooms of Ranchi were analyzed and communicated for publication somewhere else (article accepted now). Of the several wild mushrooms of Ranchi, *Astraeus hygrometricus* is highly popular and consumable among all the other ones. It contains a very good amount of protein and vitamins, also less amount of carbohydrate and fats. Several medicinal properties such as antimicrobial, antidiabetic antioxidant and anti-AChE activity has also been shown by *A. hygrometricus* (Khan & Chandra 2019).

Considering the importance of above medical aspects and potential of *Astraeus hygrometricus*, the present work was undertaken to investigate the important compound which was present in this wild mushroom. This is the first report on the purification aspect of compounds of wild mushroom, *Astraeus hygrometricus* of Ranchi district.

Mushrooms were collected from various niches of Ranchi district. Macroscopic and microscopic studies were carried out for their identification purposes. Voucher specimens were deposited in the herbarium of the microbiology lab in the Department of Bioengineering, BIT Mesra, Ranchi. On the basis of these studies, the mushroom was identified and it was found to be *Astraeus hygrometricus*.

Extraction was carried out by the method followed by Smedsgaard (1977) with some modifications. 18g of dried mushrooms sample was taken and crushed. It was then mixed in 36 ml of solvent I (consisting of methanol: dichloromethane: ethyl acetate in 1:2:3 proportion). It was then left overnight at 5 ℃. Then it was evaporated and suspended in solvent II (50 ml) [consisting of methanol (43.76 ml), hydrochloric acid (0.04 ml), formic acid (1.2 ml), and water (5 ml)]. This supernatant was stored in refrigerator at 4 ºC for further use.

In this method, the equal volume of n-hexane (10ml) and methanolic extract (10ml) of *Astraeus hygrometricus* were taken in a separatory funnel and blended well. At that point, an equivalent volume of distilled water and sodium chloride were added to a separatory funnel to improve the separation phase. Steadily, the extract got exchanged to the hypo-phase (n-hexane phase). The epi-phase containing methanol and water-dissolvable contaminations were expelled. At long last, n-hexane phase was washed 4 to 5 times with distilled water to expel leftover methanol. The compound gathered was treated with 1N HCl (9:1; v/v) and it was concentrated by evaporation at 40° C.

In this method, the compound was analyzed by thin layer chromatography using silica gel (silica gel G, Himedia). The optimized solvent system for running phase consists of hexane: ethyl acetate (90:10; v/v). The extract was spotted on the silica gel plates and air-dried. After the TLC plates have run, retention factor (Rf) was calculated (Gogoi et al. 2016).

Purified compound was characterized by Fourier transforms infrared spectroscopy (FT-IR) and Mass spectrometry. This technique works in the fact that bonds and groups of bonds vibrate at selected frequencies. The compound was further characterized using Fourier transform infrared (FTIR) spectrophotometer (Model IR-Prestige 21, Shimadzu Corporation, Japan). Dried compound was mixed with KBr powder and pressed into pellets for FTIR spectroscopy with frequency range of 4,000–400 cm−1.

Mass Spectrometry (MS) was adopted for the identification and determination of the molecular weights of the purified bioactive compounds. The methanolic fractions of purified compounds of *Astraeus hygrometricus* were subjected to MS analysis. It was performed on Thermo Fisher LTQ excel.

The quantity of phenol in transparent compound obtained in TLC was determined using a spectrophotometric method (Orhan et al. 2009) with some modifications. Folin-Ciocalteu assay method was applied for the determination of total phenolic content. In the procedure, purified compound (concentration of 0.190 mg/ml) was dissolved in 0.4 ml Folin-Ciocalteu reagent (diluted 1:10 v/v). Sodium carbonate solution (4 ml) was added after 5 minutes. Distilled water was added to make the final volume of the tubes to 10 ml and allowed to stand for 30 minutes at room temperature. Gallic acid was used as the standard. The absorbance of test sample and standard was taken at 550 nm with spectrophotometer.

It was conducted by the method of Orhan et al. (2009) with some modifications. 2.9 ml of DPPH solution (0.15 mM) was mixed with 0.5 ml of purified compound. Then, the mixture was shaken vigorously and was allowed to stand for 30 minutes in the dark. Finally, absorbance was taken at 517 nm.

Standard curve of Gallic acid was used as a reference.

$$\text{Scavenging effect \%} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

The methanolic extract was purified by extracting with n-hexane. In the process of separation phase, the n-hexane hypo-phase extract was taken out and 0.35g of extract was found in 10 ml of n-hexane. Thus, it can be concluded that approximately 9.72g of extract was found in 100g of mushroom sample. Then, thin layer chromatography (TLC) was performed and two compounds were obtained. Out of these two, one was more prominent and intense (compound 2 as shown in Figure 1a) so it was chosen for further study and its Rf value was found to be 0.6. The TLC plate was viewed in the UV chamber at both ranges (254 nm and 366 nm). Then, the desired com-

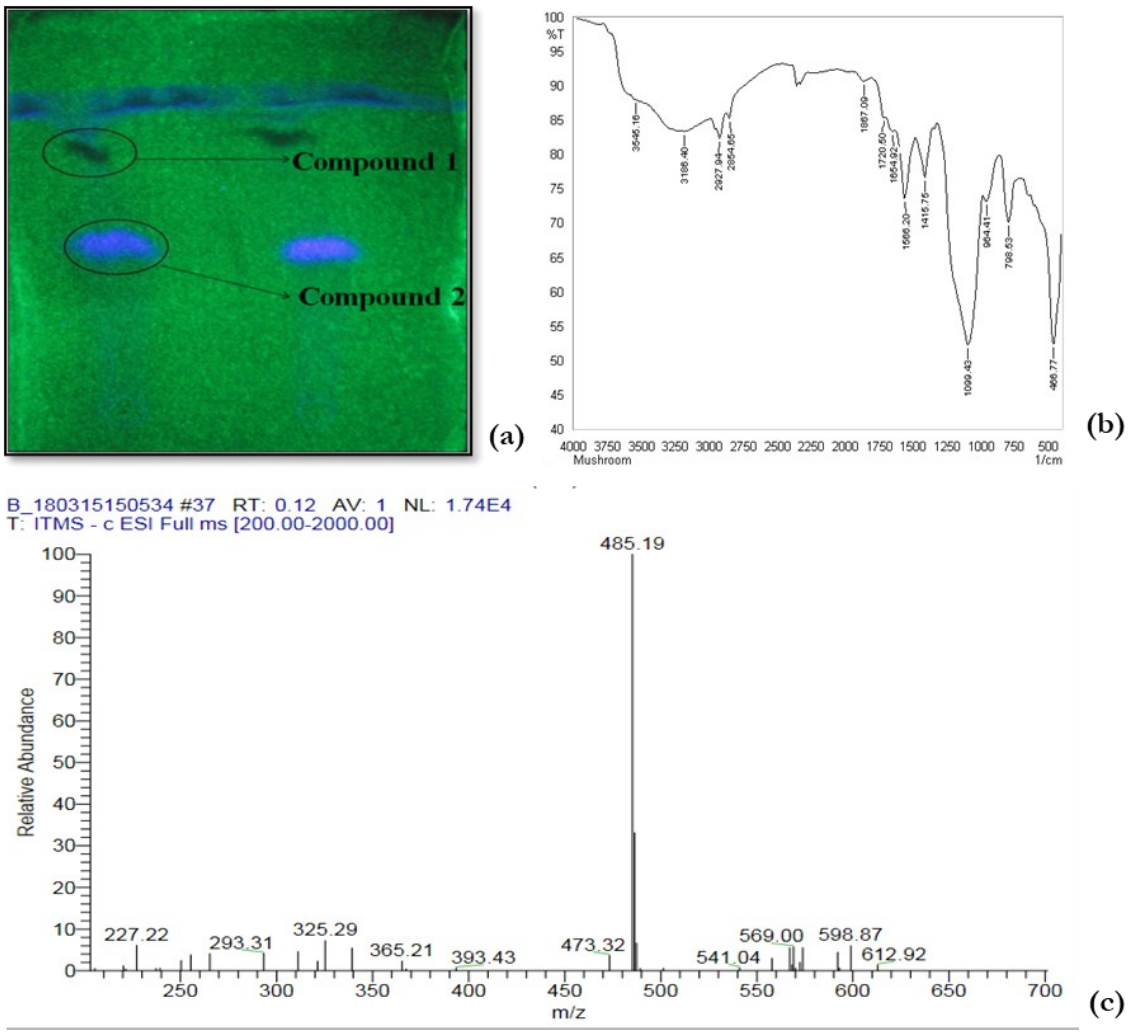


**Figure 1. (a)** Thin layer chromatogram of the compound single band of purified compound at visible range, plate viewed at 256 nm (short wavelength) UV range, **(b)** Fourier transform infrared spectrum (FTIR) of TLC purified transparent compound, **(c)** Mass spectrum of TLC purified transparent compound.

pound was scrapped out and dissolved in methanol; after that, it was then centrifuged to separate silica from the desired compound.

Finally, Fourier transform infrared (FTIR) was performed on the TLC purified sample (Figure 1b). The spectra of the compound showed strong bands at 1099 cm -1 (C-O stretch), 1566 cm–1 (C=O stretch), and 2927 cm–1 (O-H, C-H stretch) which is almost similar to the spectra of astrakurkurol. It was also supported by the studies of Stanikunaite et al. (2008) and Sheldrick (1997). Two sharp bands at 1566 and 1415 $cm^{-1}$ were ascribed to $CH_2$ (bending) and Me(bending) vibrations, respectively.

The purified transparent compound was analyzed and characterized by mass spectrometry (MS). One major peak occurred on the mass chromatogram (Figure 1c). In positive ion mode, most of the m/z data were found in the form of [M+Na]+ and provided molecular mass of 508.9 [M+Na]+ for purified compound. The molecular formulae of transparent compound was established by ESI-MS as $C_{32}H_{54}O_3$ (m/z 508.9 [M+Na]+). Biswas et al. (2017) also purified a compound with the same molecular formulae i.e. $C_{32}H_{54}O_3$. During MS analysis DBE (Double Bond Equivalent) was calculated and on the basis of the number of DBE, it can be concluded that the obtained compound has got five rings and one pi bond in its structure. This analysis concludes a structure for the compound that resembles the structure obtained by Lai et al. (2012), Hill & Connolly (2015), Liziane et al. (2016), and Biswas et al. (2017).

Thus, through the molecular weight, MS and FT-IR analysis of this transparent compound can be named as astrakurkurol as it is found and named by Lai et al. (2012), Hill & Connolly 2015, Liziane et al. (2016), and Biswas et al. (2017).

The Phytochemical analysis of the transparent compound showed the presence of phenols. The phenolic compounds were reported for their direct contribution to anti-oxidative action (Velioglu et al.1998). Content of phenolic compounds correlates with the antioxidant activity as it has already been reported (Blanche et al. 2017). Therefore, for a mushroom to have antioxidant activity presence of phenolic compound is important. Phenolic is one of the major groups of nonessential dietary components that is related to the inhibition of cancer and atherosclerosis (Williams et al. 1997). Total phenol content was found to be 27.85 mg/100g. DPPH activity in this compound was found to be 56.7%. Total phenol content and DPPH activity in crude mushroom extract was 38.91mg/100g dw and 61.1%. Phenolic rich methanolic extract of this mushroom have rich total anti-oxidant activity as observed by Ullah et al. (2015). The phenolic fraction of plants is usually interlinked to their antioxidant and antimicrobial activities. Singh (2010) quantified several phenolic compounds and found this mushroom to be rich in Protocatechuic acid, Ferulic acid, Salicylic acid, Anthralinicacid, and Syringic acid. Total phenolic content was also determined spectrometrically as 1.4% in inner and outer part of the mushroom respectively (Singh 2010) and it all correlates with our study.

As per literature review and studies done it may be concluded that *Astraeus hygrometricus* has several nutritional and medicinal properties. The methanolic extract of this mushroom contains two biochemical compounds as it is clear from our TLC result. Out of both compounds, one was more prominent and intense (compound 2) and this transparent compound was found to be astrakurkurol with a molecular weight of 485.19. This compound also showed good amount of phenol content and DPPH activity.

## AUTHORS CONTRIBUTION

F.K. has done the research under the supervision of R.C.

## ACKNOWLEDGMENTS

The authors are thankful to Centre of Excellence (COE) TEQIP-II (Grant no. - NPIU/ TEQIP II/ FIN/ 31/158) for financial support and Department of Bioengineering, Birla Institute of Technology Mesra, Ranchi for providing R & D facilities.

## CONFLICT OF INTEREST

There is no conflict of interest.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb63820
DOI: 10.22146/jtbb.63820

Research Article

# Herpetofaunal Assemblages in the Lowland Regions of Sumatera Barat

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**Keywords:**
abundance
biodiversity
conservation
species richness
**Submitted:**
02 February 2021
**Accepted:**
30 October 2021
**Published:**
07 January 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT

The habitat destruction and land-use changes caused the decline of animal composition in many tropical regions. Here, we study the diversity of herpetofauna in the lowland areas in Sumatera Barat, a midwestern province in Sumatera island, using a visual encounter survey method. The surveyed habitat included rubber plantations, streams, paddy fields, and peat swamps. We observed 338 individuals representing 44 species from 14 families of herpetofauna with almost 90% individuals were amphibians. Overall, the rubber plantations contained a higher number of species than other types of habitat. For amphibians, Ranidae and Dicroglossidae represented the first and the second highest both in the species and individual number. For reptiles, Agamidae and Colubridae or Gekkonidae accounted for the first and the second highest in the individual number while Colubridae and Scincidae consisted of the highest species number. Our data showed that the diversity index was mostly in moderate level except in paddy field. The species composition in rubber plantations were more similar to those of streams rather than paddy field or peat swamp Sago habitat.



## INTRODUCTION

Most of the decline of fauna diversity occured due to loss of habitat, habitat destruction, and habitat change (Stuart et al. 2004; Todd & Rothermel 2006). Those damages are mainly caused by the conversion of forests to areas for plantations. Indonesia is known to be the second largest rubber exporting country globally (Bruinsma & Food and Agriculture Organization of the United Nations 2003). In exchange, Indonesia lost many more primary forest land than Brazil (Margono et al. 2014). Among the five biggest islands in Indonesia, Sumatera is one of those experiencing the highest loss of primary forests (Gunarso et al. 2013). The conversion of forest land to plantation has raised many animal diversity problems. Changes in the vegetation structure and human activities lead to the loss of many intolerant disturbance species

(Drescher et al. 2016). The conversions also negatively impacted the species richness, abundance, and community structure (Fitzherbert et al. 2008).

Amphibians are the most endangered group of vertebrates by the habitat loss and overutilization (Stuart et al. 2004). On the other hand, reptiles, in the conservation perspective, also receive a little attention on how they response to agricultural activities (Tews et al. 2004). Herpetofauna communities are affected by changes in vegetation for example, from primary forest to plantation (Paoletti et al. 2018). Some anthropogenic activities like oil-palm replanting (Kurz et al. 2016), converting forest into plantation (Konopik et al. 2015) have been known decreasing the number of species, the richness, and abundance of frog communities. Specifically, leaf litter thickness and canopy cover have strongly determined the species richness and abundance (Whitfield & Pierce 2005; Wanger et al. 2009).

Several works partly or fully involving Sumatera Barat as the study site were reported (e.g. Inger & Iskandar 2005; Kurniati 2008; Teynie et al. 2010; Wostl et al. 2017). However, those studies mainly focused on biodiversity discoveries and conservation areas that are administratively regulated by national or local governments. Nonetheless, non-protected areas could not be neglected in terms of amphibians and reptiles conservation because they might contain more diverse and more abundant herpetofauna (Whitfield & Pierce 2005; Luja et al. 2017). Even though the herpetofauna inventory and community assessment outside the protected areas in Sumatera Barat are scarce, such studies seem to have already started to grow (Sumarmin et al. 2019; Nugraha et al. 2021).

This study aimed to analyze the diversity of amphibians and reptiles communities in the lowland regions of Sumatera Barat province. We chose lowland region because many areas are vulnerable to anthropogenic activities (Gunarso et al. 2013), likely impacting the structure of herpetofauna communities.

## MATERIALS AND METHODS

### The study sites

We chose four regions in this study that were located under 400 meters above sea level (masl) in Sumatera Barat Province (Figure 1). City of Padang (CP, 0°51'28.60"S; 100°19'57.07"E, elevation ca.4 masl) is a peat swamp area that is overgrown by Sago plants and is surrounded by the settlement. Sungai Barameh (SB, 1°1'54.71"S; 100°24'43.69"E, elevation ca. 83 masl) and Bukik Kasang (BK, 0°47'7.20"S; 100°21'8.59"E, elevation ca. 351 masl) comprise of streams and rubber plantations habitat. Lubuk Bonta (LB, 0° 31'10.16"S; 100°17'43.16"E, elevation ca. 222 masl) consists of stream, rubber plantation, and paddy field (Figure 2).

Streams in SB and BK were conformable in which they consistof many rocks with medium to large size. The width of the streams is about 8 to 9 meter. While stream in LB is smaller in width (3-5 meter), rocks are obtainable but the size is much smaller and most of them submerged in the

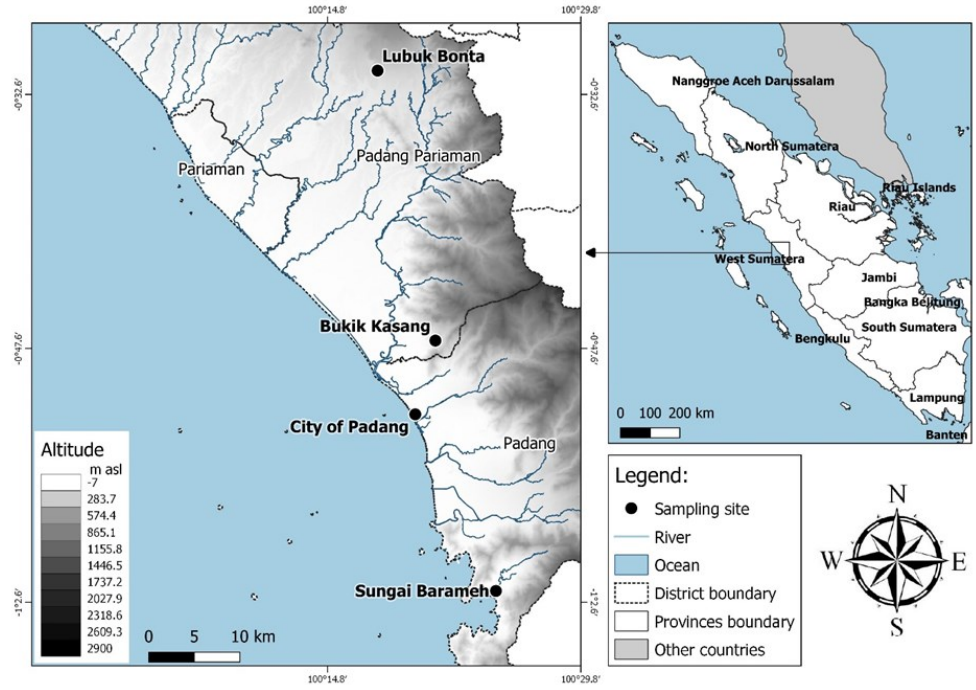


**Figure 1.** Locations of the study indicated by black-filled circle. Lubuk Bonta (LB), Bukik Kasang (BK), City of Padang (CP) and Sungai Barameh (SB).

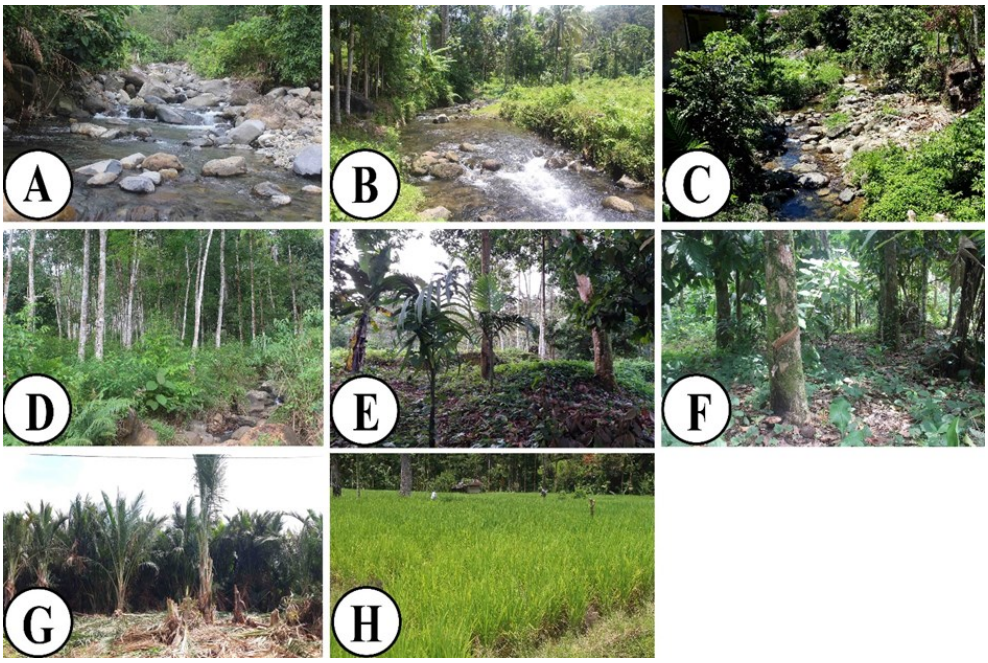


**Figure 2**. Typical habitat in the study site. A - C: streams in BK, LB and SB, respectively. D – F: rubber plantations in BK, LB and SB, respectively. G: peat swamp overgrown by Sago plants in CP. H: paddy field in LB.

streambed. Rubber plantation in BK was not well-treated by the owner thus allowing the shrubs grew among the rubber trees. There was a small-sized stream in the middle of plantation with width of about 0.7 to 1 m and depth of about 0.3 to 0.5 m. In contrast, the rubber plantations in SB and LB were more well-maintained in which vast majority of space among rubber trees were dominated by leaf litter. In addition to rubber plantation in SB, there was a tiny flow of water with width of only about 0.2 m that sometimes no water found within it.

Paddy fields comprised of paddy plants in various stages: early planting, middle age, and post-harvested. Peat swamp contained Sago as the majority of plants surrounded by dense shrubs. Some areas had been destructed due to human activities like harvesting Sago.

## Field survey and data collection

Two times survey was conducted in Sungai Barameh on 13th and 14th April, 2019. Third and fourth sampling were carried out in Bukik Kasang and Lubuk Bonta in April 21st and November 16th, 2019, respectively. Peat swamp habitat in the city of Padang was surveyed twice in November 23th and 30th, 2019.

We used the visual encounter survey technique for a known period of time (Dodd 2009) to explore areas in the study sites, where four to six persons searched systematically in the study area. The search was carried out from 8pm – 11pm by following the stream path for approximately 600-700 m during those three hours. Up to 5 m beside each stream was also surveyed with randomized walk. In each rubber plantation, the movement of surveyors was also randomized covering the area of about 400 to 500 $m^2$ after three hours searching. The fragmented Sago populations were represented approximately 400 to 500 $m^2$, while paddy fields being surveyed were about 200 – 300 $m^2$ in size.

The searches were made in all possible areas including: inside the shrubs, under the rocks, logs, and leaf litter (Dodd 2009), tree stems, tree branches, and among low vegetations. The observed specimens were captured for documentation in the next morning. All the specimens were released back to the site where they were captured. Species identification was performed under the guideline books and articles related to Sumateran herpetofauna (e.g. Das 2015; Frost 2021; Inger & Iskandar 2005; Inger & Stuebing 1997; Iskandar 1998; Kurniati 2008; Teynie et al. 2010).

## Data analysis

The number of species and individuals in each location were subjected for analysis of herpetofauna diversity indices including: Shannon-Wiener's heterogeneity index (H') (Krebs 1998), Margalef's species richness index (Dmg) (Magurran 2003), and Simpson's dominance index (D) (Magurran 1988); we used Jaccards's coefficient to compare species composition similarity among the study sites (Sokal & Sneath 1963) implemented in PAST v3.11 (Hammer et al. 2001).

The number of species and individual was divided into four habitat types: stream, rubber plantation, paddy field, and peat swamp Sago. Shannon -Wiener index is classified into three categories: low (< 1), moderate (1 < H' < 3), and high (> 3) (Odum 1994); dominance Simpson index is classified into three categories: low (0.00 < D < 0.30), moderate (0.30 < D < 0.60), and high (0.60 < D < 1.00) (Krebs 1999).

## RESULTS

### Species composition and sampling effort

Overall, we recorded 338 individuals, of which 306 were amphibians representing 26 species from 6 families, and 32 were reptiles representing 18 species from 8 families (Table 1 and Figures 6-7). Regardless to the locations, the overall species number in each type of habitat showed a variation. Totally, rubber plantation contained the highest number of species (n= 27) that differed slightly from stream (n= 24), while paddy field was the lowest (n= 2). Similarly, if splitted into amphibian and reptile groups, both were more abundant in rubber plantation with 16 and 11, respectively, than in any other types of habitat. The second richest habitat was stream that contained 15 species of amphibians and 9 reptiles. Meanwhile, no reptile was encountered in paddy field and only two species of amphibians were observed (Figure 3).

Looking more detail at species or individual number in each type of habitat in all locations, the richest species number in rubber plantation was in BK (n= 25), differed significantly from rubber plantations in SB and LB at 5 and 7 species, respectively. However, in the stream habitat, BK had the lowest number of species (n= 7) differed markedly from stream in LB with 17 species and stream in SB with 12 species. Although peat swamp Sago habitat comprised of 12 species, the number of individual was the highest among other habitat with 103 individuals. The lowest individual number was found in the stream of BK with only 9 individuals (Figure 3).

Among amphibians, the ranid group was the most abundant with a slight below 50% of the total (n= 156) followed by Dicroglossidae (n= 104), Bufonidae (n= 37), Microhylidae (n= 4), Megophrydae (n= 3), and Rhacophoridae (n= 2). Similar figure to the individual number, Ranidae and Dicroglossidae were represented the highest species number with 8 and 7, respectively (Figure 4).

While among reptiles, Agamidae was the most abundant (n= 11 individuals) ), followed by Colubridae (n= 6), Gekkonidae (n= 6), Scincidae (n= 5), Geomydidae (n= 1), Lacertidae (n= 1), Varanidae (n= 1), and Viperidae (n= 1) (Figure 4). Although Agamidae was the most abundant among individuals, Colubridae and Scincidae accounted for the richest species number (4 species). None of the encountered species was in the Threatened or Data Deficient status under IUCN red list, yet *Limnonectes blythii* and *Cyclemys dentata* were listed as near threatened (NT) species. In addition, two amphibian species were known to be endemic to Sumatera Island (*Wijayarana sumatrana* and *Chalcorana rufipes*).

In the first attempt of the survey, we found 5 species in the rubber plantation in SB. The second survey was in the stream habitat of SB and we observed 9 species. The number of species reached the highest point in rubber plantation of BK at 15 species, then gradually decreased until the final survey. However, overall, the species accumulation curve showed an upward

**Table 1**. List of the species observed in the study including the number of individual. SB: Sungai Barameh; BK: Bukik Kasang; LB: Lubuk Bonta; CP: City of Padang; N: number of specimens. Typed in bold: endemic to Sumatera.

| Family | Species | Rubber Plantation | | | Stream | | | Peat Swamp Sago | Paddy Field |
|---|---|---|---|---|---|---|---|---|---|
| | | SB | BK | LB | SB | BK | LB | CP | LB |
| Bufonidae | *Duttaphrynus melanostictus* | 3 | 5 | 1 | 0 | 0 | 0 | 0 | 0 |
| | *Ingerophrynus divergens* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Leptophryne borbonica* | 2 | 4 | 0 | 3 | 0 | 0 | 0 | 0 |
| | *Phrynoidis aspera* | 0 | 6 | 2 | 2 | 3 | 5 | 0 | 0 |
| Dicroglossidae | *Fejervarya cancrivora* | 0 | 6 | 0 | 5 | 0 | 5 | 3 | 9 |
| | *Fejervarya limnocharis* | 0 | 4 | 0 | 0 | 0 | 0 | 0 | 18 |
| | *Limnonectes blythii* | 0 | 4 | 0 | 4 | 2 | 3 | 12 | 0 |
| | *Limnonectes kuhlii* | 0 | 4 | 0 | 3 | 5 | 0 | 0 | 0 |
| | *Limnonectes macrodon* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| | *Occidozyga lima* | 0 | 0 | 0 | 0 | 0 | 0 | 6 | 0 |
| | *Occidozyga sumatrana* | 0 | 0 | 0 | 0 | 0 | 1 | 8 | 0 |
| Megophrydae | *Megophrys nasuta* | 0 | 2 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Leptobrachium* cf. *hasseltii*. | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Microhylidae | *Kaloula baleata* | 0 | 0 | 1 | 0 | 0 | 0 | 0 | 0 |
| | *Microhyla* sp1. | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| | *Microhyla* sp2. | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Ranidae | *Amnirana nicobariensis* | 0 | 0 | 0 | 0 | 0 | 0 | 17 | 0 |
| | ***Chalcorana parvaccola*** | 2 | 5 | 0 | 4 | 0 | 4 | 37 | 0 |
| | ***Chalcorana rufipes*** | 0 | 7 | 1 | 4 | 0 | 4 | 0 | 0 |
| | ***Wijayarana sumatrana*** | 0 | 0 | 0 | 0 | 4 | 3 | 0 | 0 |
| | *Hylarana erythrea* | 3 | 6 | 2 | 0 | 3 | 4 | 3 | 0 |
| | *Odorrana hossi* | 0 | 0 | 0 | 2 | 6 | 5 | 0 | 0 |
| | *Pulchrana glandulosa* | 0 | 0 | 0 | 0 | 0 | 0 | 11 | 0 |
| | *Pulchrana sundabarat* | 0 | 8 | 1 | 10 | 0 | 0 | 0 | 0 |
| Rhacophoridae | *Polypedates leucomystax* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *Polypedates macrotis* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| Agamidae | *Aphaniotis fusca* | 1 | 1 | 0 | 2 | 0 | 0 | 0 | 0 |
| | *Bronchocela cristatella* | 0 | 3 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *Gonochepalus grandis* | 0 | 0 | 1 | 0 | 0 | 2 | 0 | 0 |
| Colubridae | *Coelognathus radiatus* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Dendrelaphis haasi* | 0 | 0 | 0 | 0 | 0 | 1 | 0 | 0 |
| | *Dendrelaphis pictus* | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 0 |
| | *Xenochrophis trianguligerus* | 0 | 1 | 0 | 1 | 0 | 0 | 0 | 0 |
| Gekkonidae | *Cyrtodactylus* sp. | 0 | 0 | 0 | 3 | 0 | 0 | 0 | 0 |
| | *Hemidactylus frenatus* | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 0 |
| | *Hemiphyllodactylus typus* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Geoemydidae | *Cyclemys dentata* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Lacertidae | *Takydromus sexlineatus* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Scincidae | *Eutropis multifasciata* | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 |
| | *Eutropis rugifera* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| | *Lygosoma bowringii* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 0 |
| | *Spenomorphus* sp. | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Varanidae | *Varanus salvator* | 0 | 1 | 0 | 0 | 0 | 0 | 0 | 0 |
| Viperidae | *Tropidolaemus wagleri* | 0 | 0 | 0 | 0 | 1 | 0 | 0 | 0 |
| **Total number of individuals** | | 11 | 76 | 9 | 43 | 24 | 45 | 103 | 27 |
| **Total number of species** | | 5 | 25 | 7 | 12 | 7 | 17 | 12 | 2 |

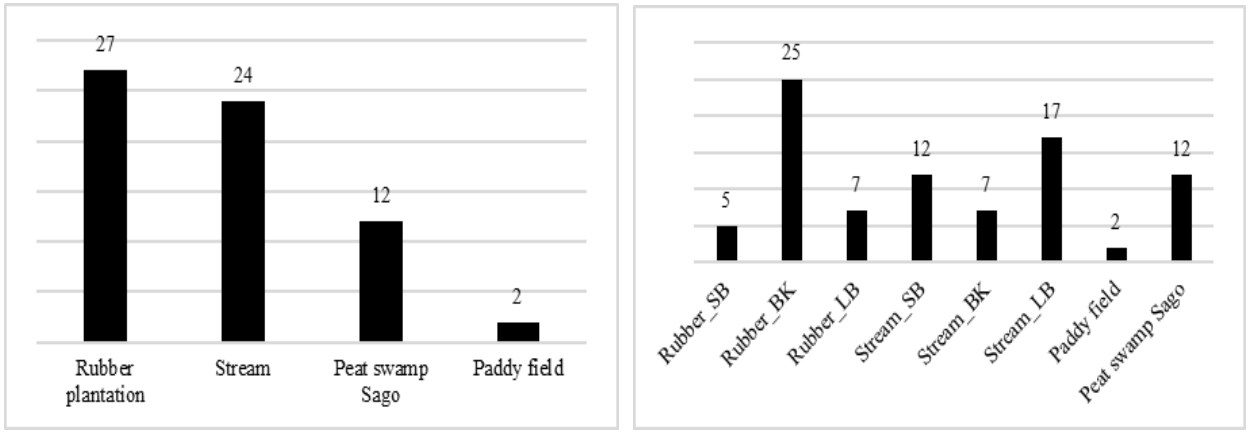


**Figure 3**. Species number of amphibian and reptile collectively (left) and species number of in each habitat (right). Rubber= rubber plantation; SB, BK, LB= referto the study sites mentioned in the previous section.

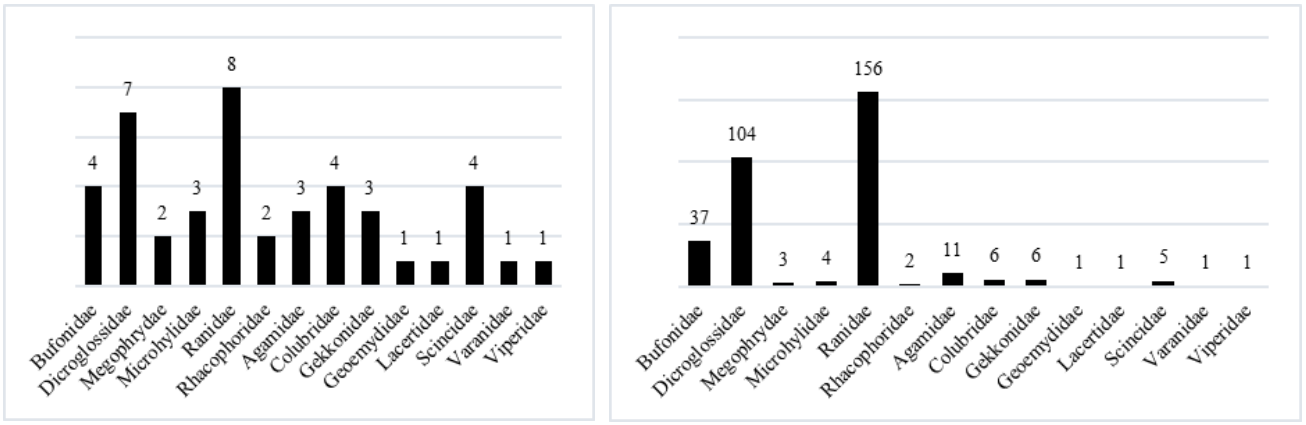


**Figure 4**. The number of species (left chart) and individual (right chart) in each family.

trajectory with a drastic uptick to a slight more than 45 species at the end of study (Figure 5).

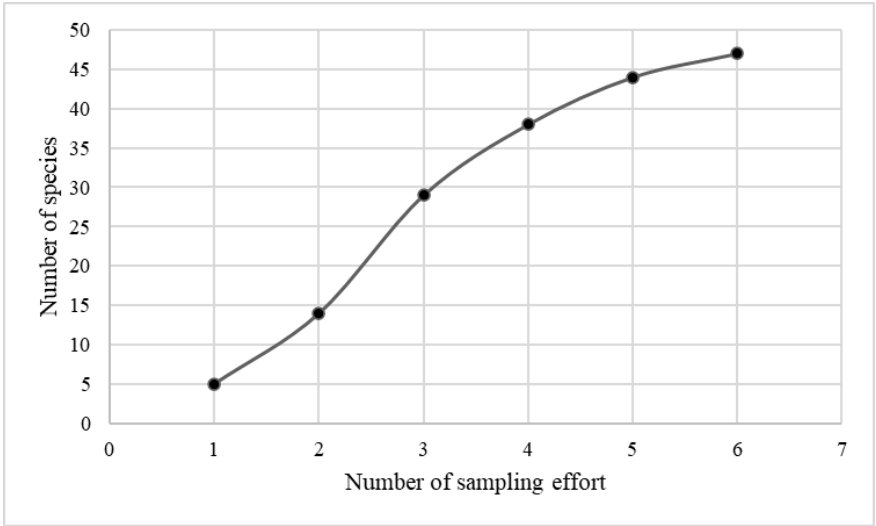


**Figure 5**. Species accumulation curve from each VES-sampling effort. Two visits in SB (sampling effort 1 and 2), one visit in BK and in LB (3 and 4), and two visits in CP (5 and 6).

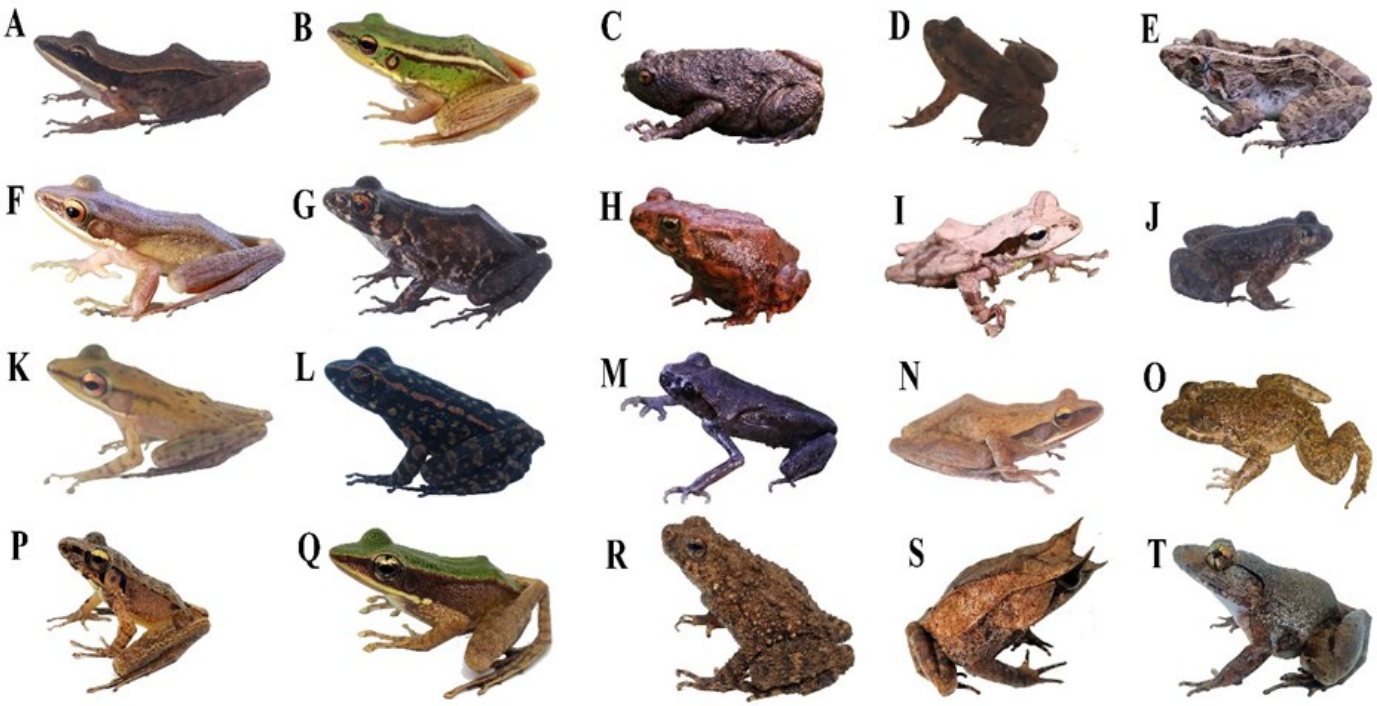


**Figure 6**. List of some anuran species that were observed during the study. Images are not to scaled. A. *A. nicobariensis*, B. *H. erythraea*, C. *K. baleata*, D. *O. Sumatrana*, E. *F. limnocharis*, F. *C. rufipes*, G. *P. glandulosa*, H. *I. divergens* (juvenile), I. *P. macrotis*, J. *O. lima*, K. *C. parvaccola*, L. *P. signata*, M. *Leptobrachium* sp., N. *P. leucomystax*, O. *L. kuhlii*, P. *W. Sumatrana*, Q. *O. hosii*, R. *P. aspera*, S. *M. nasuta*, T. *L. macrodon.*

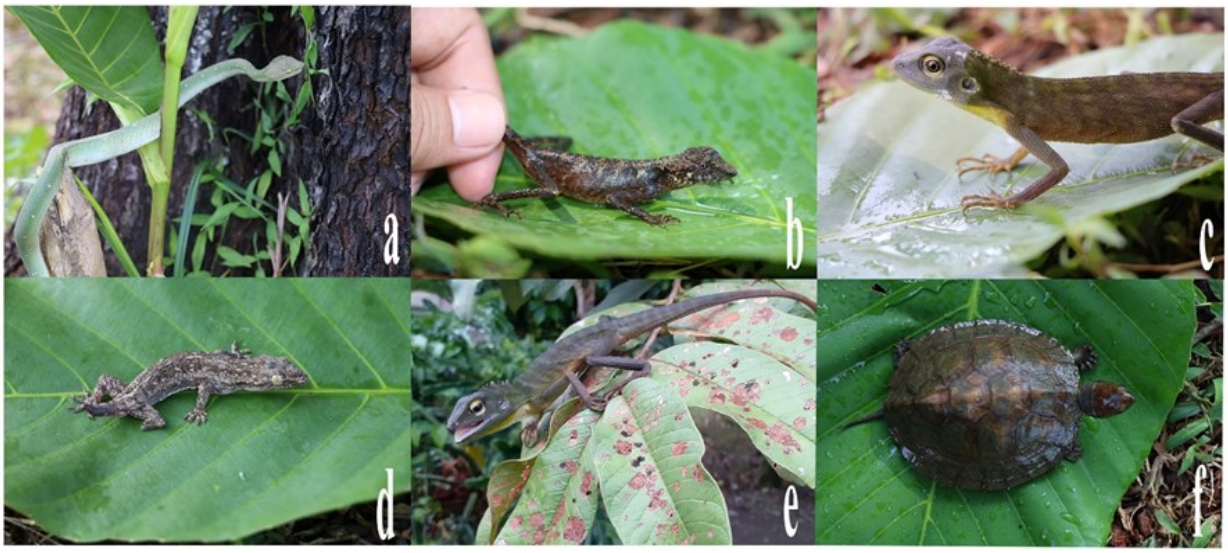


**Figure 7**. List of some reptile species that were observed during the study. Image are not to scaled. a. *T. wagleri*, b. *A. fusca*, c. *B. cristatella*, d. *H. typus*, e. *G. grandis*, f. *C. dentata.*

## Notes on habitat use at the time of observation

Ranidae is generally found along the stream banks or in the middle of streams. However, *W. sumatrana* was sometimes found in a distance away from the stream (up to 5 meters). Some species were found in the amplexus position, such as *W. sumatrana*, *L. blythii*, and *Leptophryne borbonica*. Although many *P. sundabarat* were found on the rocks in the middle or in the edge of the streams, there was one individual found in the middle of the rubber plantation (15 meters away from the nearest stream). *Pulchrana glandulosa* distributed exclusively in the peat swamp Sago, often found perching on the

midribs of the Sago plants or on the its stem that has been cut. The dicroglossid group of the genus *Limnonectes* and *Occidozyga* (in all study sites except CP) was mainly found in the puddle of the stream edge where half portion of the body submerged in the water. Whereas, in the peat swamp Sago, *Limnonectes blythii* perch near the tree of Sago or perch on the cut stem of Sago, while *Occidozyga* spp. never used the same substrate instead of staying in the shallow water. The species of *F. cancrivora* and *F. limnocharis* were commonly found in paddy fields. Megophrydae members were found in more open grassy areas near the streams (5 meters in distance) with some temporary pools resulted from human and animal activities. Whereas, a species of rhacophorid was found in a papaya plant near narrow streams. The species of *Kaloula baleata* (Microhylidae) was found perching on a rubber tree about 3 meters high above the ground.

Among reptiles, Colubrid snakes were mainly found on tree branches adjacent to streams (3.5 meters above the ground and about 3 meters from the stream) except for *C. radiatus* which was found on the roof of a woody house. *Xenochropis trianguligerus* and *Gonocephalus grandis* were observed on tree branches above the stream (0.5 to 4 meters high above it). Another agamid (*B. cristatella*) was observed on a tree branch about 0.5 m high above the ground. Asian leaf turtle was found under water of the small stream (width of 0.7 m; depth of 0.3 m) in the middle of the rubber plantation in BK.

### Diversity indices assessment and similarity

In general, most of habitat type in all locations had the moderate level of heterogeneity ranging from 1.55 to 2.36. The highest level of heterogeneity was in rubber plantation in BK (2.36) followed by stream in LB at 2.13, while the lowest level was in the paddy field in LB with only 0.64. The highest level of richness was in rubber plantation in BK (5.54), while stream in LB placed in the second higest with 4.20. Again, paddy field represented the lowest value of richness at 0.30. Dominance index values indicated that no species dominated in most of all types of habitat in all locations (range values of 0.12 to 0.22), yet the value in the paddy field was relatively higher than others (value of 0.56) (Table 2). Regarding the similarity of herpetofauna communities, all rubber plantations were more conformable for each other, separated from all streams habitat. The pattern of similarity in rubber plantation and stream groups was the same where BK was more similar to SB than LB. Community in paddy field and peat swamp Sago separated from those rubber plantations and streams (Figure 8).

## DISCUSSION

The previous inventory carried out in Sumatera Barat region was completed by previous studies, e.g. Inger & Iskandar (2005), Kurniati (2008), Wostl et al. (2017), and Nugraha et al. (2020). Their inventories mainly focused on conservation forests that administratively managed and protected either by local or national government such as national park, nature reserve, or

**Table 2**. Diversity indices of herpetofauna communities in each type of habitat in Sumatera Barat. SB: Sungai Barameh, BK: Bukik Kasang, LB: Lubuk Bonta, and CP: City of Padang.

| Habitat Type | Location | Heterogeneity | Richness | Dominance |
|---|---|---|---|---|
| Rocky stream | SB | 2,05 | 2,92 | 0,12 |
| Rocky stream | BK | 1,70 | 1,89 | 0,17 |
| Rocky stream | LB | 2,13 | 4,20 | 0,08 |
| Rubber plantation | SB | 1,55 | 1,67 | 0,22 |
| Rubber plantation | BK | 2,36 | 5,54 | 0,06 |
| Rubber plantation | LB | 1,65 | 2,73 | 0,16 |
| Paddy field | LB | 0,64 | 0,30 | 0,56 |
| Peat swamp Sago | CP | 1,80 | 2,37 | 0,19 |

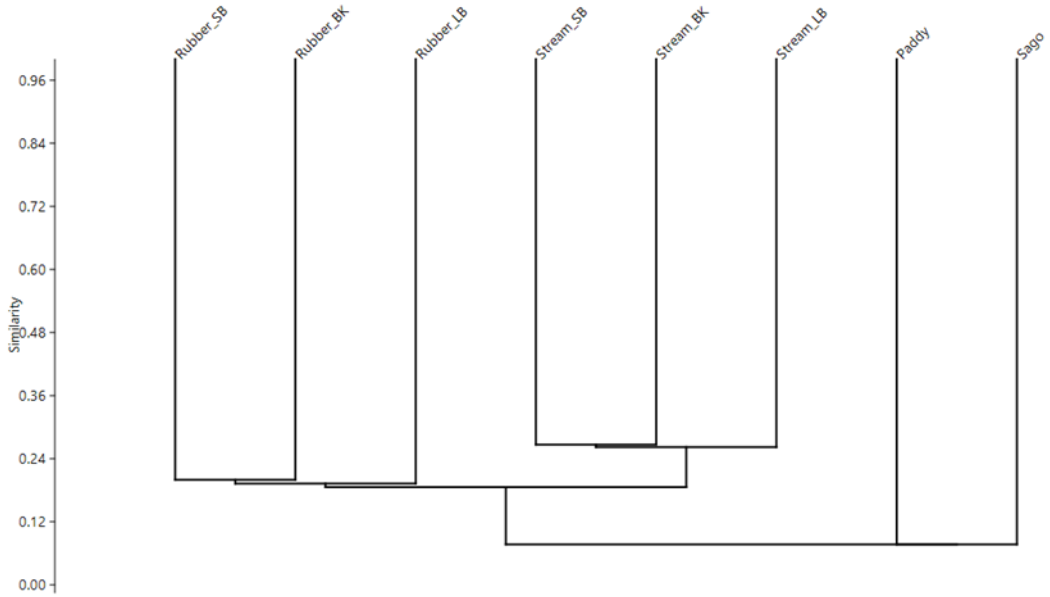


**Figure 8**. Dendrogram of similarity of the herpetofauna species composition between each habitat. Rubber= rubber plantation; SB, BK, LB= refer to the study sites mentioned in the previous section, paddy = paddy field in LB and Sago = peat swamp habitat in CP.

protected forest. However, our knowledge of herpetofaunal communities assessment from non-protected areas in Sumatera Barat started to accrue and has been opened publicly, for example, Nugraha et al. (2021) provided a cheklist of amphibians and reptiles species in a tourism area, Harapan et al. (2020) analyzed the potential distribution of the secretive species of *Ichthyopis*, and Sumarmin et al. (2019) inventoried the anuran species in a paddy field. Our current study provided information on herpetofaunal communities that specifically analyze the diversity index for some regions of lowland habitat in Sumatera Barat. Diversity estimation is important for future protection and management (Snodgrass et al. 2000) and the effect of habitat changes assessment through time (Dodd 2009).

Regarding the sampling effort, the species accumulation curves did not show a plateau trend in the end of survey attempt, thus it can be deemed that more sampling effort would perhaps yield more number of species. Overall, regardless to the locations, rubber plantation contained more species number than other types of habitat. It was similar to what Paoletti et al. (2018) found in Jambi, eastern part of Sumatera that considered the rubber plantations

might offer more niche for amphibian species diversity.

Splitting to each type of habitat, the different number of species in each habitat could be explained through several reasons. For rubber plantation, the number of species in BK differed significantly from SB and LB. It most likely that BK rubber plantation was not well-treated by the owner compared to SB and LB, thus more understory vegetation existed there. Moreover, there is a small-sized stream in the middle of plantation that certainly plays an pivotal role in attracting a number of amphibians and reptiles. Conversely, SB and LB rubber plantations lack of such properties. Although there was a small water flow in rubber plantation of SB, it might be unsufficient for herpetofaunal needs because sometimes it contains no water. For stream habitat, however, the number of species in BK was lower than SB and BK. It might be due to the amount of water it had. Stream in BK in our visit time had much water as we hardly stepped on large rocks in it. The edge of stream is also too high to see during the survey, hence we likely surveyed that area less than in SB or LB. In peat swamp Sago area, the species number is quite comparable to SB and LB streams. Although the area was sometimes disturbed by harvesting activities, the disturbance is might be relatively much fewer because it is not a plantation. Hence, peat swamp Sago area provided better understory vegetation, more permanent puddles and most likely more humid than common type of plantations like rubber or oil palm. The abundance correlated with type of plantation. Oil palm plantations become the most inhabited plantation by large number of amphibians because the harvesting activities by using trucks often made basins filled by water for amphibian eggs. In contrast, rubber plantation contained more species numbers due to vegetation structure that are more stable and diverse (Paoletti et al. 2018).

Based on the heterogeneity index value, most of surveyed habitat was classified as moderate ($1 < H' < 3$). The lowest heterogeneity level was found in paddy field with the value of 0.64 and the highest was in rubber plantation in BK with the value of 2.36. As stated before that the more diverse of habitat in BK may allow herpetofaunal communities to survive and to develop well in the area. On the contrary, paddy field is considered to be the most disturbed habitat because the area is highly modified for plantation purposes. High human activities can reduce the diversity of habitat in an area (Hassan & Hassan 2019) which can directly affect the level of diversity and the abundance of herpetofauna (Carpio et al. 2015). In addition to habitat diversity, the quality of abiotic factors especially water also determines the survival of herpetofauna. It affects the survival of tadpoles, growth, maturation, and physical development (Dodd 2009). For comparison, the diversity level of herpetofauna in plantations and urban areas have been revealed by some authors. Samitra & Rozi (2020) revealed a moderate level of diversity of herpetofauna in rice field and river in Southern Sumatera; Maulidi et al. (2019) also showed that herpetofaunal diversity in Borneo in a rubber plantation was in moderate level.

Likewise for richness level, high level of heterogeneity corresponded to the high level of richness. Dominance index showed that there was no species dominated in all habitat, but paddy field might be getting risk by high modification on the land by the farmers. The richness index in our study site in paddy field was higher than that of in Samitra & Rozi (2020).

The similarity analysis clearly showed that same habitat grouped together, separated from the different type of habitat. It means that the herpetofauna composition in rubber plantations remained similar regardless to the locations. Herpetofauna composition in rubber plantations were more comformable with those in streams, leaving the other types of habitat outside the group. Either in streams or rubber plantations, SB and BK were more similar than those to LB. The elevation of LB was higher than SB and BK that might affect the species composition differentiation among those habitat. For example, we found rhacophorids (*P. leucomystax* and *P. macrotis*) and *Dendrelaphis* spp. only in LB. Another study showed that there was a variety of species composition along geographic elevatinonal gradient. It might be also caused by the elevation-derived abiotic parameters such as temperature and humidity (Sasaki et al. 2005).

## CONCLUSION

The diversity level of herpetofaunal communities in the lowland habitat was revealed. Most of habitat type supported moderate level of heterogeneity index except paddy field that was categorised as low. The highest point in heterogeneity index was in a rubber plantation as well as the richness index. The herpetofaunal communities in paddy field might be disturbed by reguler activities of the farmers as the richness index hit the lowest score and the dominance index peaked the highest. We found that rubber plantations contained the highest number of species followed by streams, peat swamp Sago, and paddy field. Regarding to the number of species per family, the families of Ranidae and Agamidae represented the most abundant group for amphibian and reptile, respectively.

## AUTHORS CONTRIBUTION

F.A.D.N. designed, collected, analyzed the data and supervised all the process, F.K. designed the research and wrote the manuscript, R.S. collected the data, wrote and revised the manuscript, A.M.K analyzed the data and prepared the figures and tables, A.P.A revised the manuscript.

## ACKNOWLEDGMENTS

We are grateful to T. W. Marten, Febrinal, R. Yuliandri, F. A. Chandra, M. Kentino, Y. Amardi, M. Qori for helping us during the field trip. We also thank M. Alvareza for providing vegetation data for BK, Y. Amardi and M Kentino for SB, CP, and LB. We also thank J. Permana for producing a map of study sites. We are also grateful to the anonymous reviewers who commented on this manuscript.

## CONFLICT OF INTEREST

There is no conflict of interest.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb64827
DOI: 10.22146/jtbb.64827

Research Article

# Aboveground Forest Carbon Stock in Protected Area: A Case Study of Bukit Tigapuluh National Park, Indonesia

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**Keywords:**
Forest Carbon Stock
Protected Area
REDD+
**Submitted:**
20 March 2021
**Accepted:**
30 October 2021
**Published:**
12 January 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT

The role of protected areas has been expanded into climate change mitigation, specifically on Reducing Emissions from Deforestation and Forest Degradation (REDD+). A reliable and practical method for measuring, reporting and verifying carbon stock is an essential component for REDD+. This study aims to recognize the characteristic and estimate aboveground forest carbon (AGC) stock in the tropical protected tropical area using a combination of terrestrial forest inventory and spatial data. A 168 cluster plots totaling 33.6 hectares were taken proportionally based on the percentage of forest cover types (dryland primary natural forest/DPF and dryland secondary natural forest/DSF) using a traditional forest inventory method (more than 5 cm dbh). Results showed that Bukit Tigapuluh National Park secured a significant AGC stock which has been estimated to be 269.2 [247.07; 291.43] tC/ha or 35,823,639 [32,872,312; 38,774,966] tC in total, being stored in approximately 133,051 hectares of the tropical rain forest. This result was higher than other studies in non-protected areas but slightly lower than other studies within protected areas. This finding supported the argument that protected areas possess a higher figure of AGC stock than other forest management units. The high amount of forest carbon biomass in the protected areas shall be very important assets for conducting the role of conservation for REDD+.



## INTRODUCTION

The role of protected areas as a valuable tool against the pressures on biodiversity and their related effects on human populations is now well recognized (IUCN 2010). Protected areas vary with respect to governance regimes, and management types, including national parks, nature reserves, wildlife sanctuary, hunting parks and watershed protected forests, among many others (Deguignet et al. 2017; Government of Indonesia 1999). As a world's biodiversity hotspot, Indonesia has established 53 national parks, either terrestrial or aquatic national parks, with a total area of approximately 16 million hectares or about 60% of the total protected areas in Indonesia (Pusat Data dan Informasi KLHK 2017). Within those areas, nearly 80% were forested in

2017, which account for 12% of the overall natural forest in Indonesia (Pusat Data dan Informasi KLHK 2017). However, these parks are at alarming threat of deforestation and degradation, particularly those in Sumatra Island, despite government willingness to protect them (Luskin et al. 2017; Pramudya et al. 2018; Shah & Baylis 2015).

The role of protected areas has been expanded to a climate change mitigation, particularly in the tropical countries, which much of the concept embedded in Reducing Emission from Deforestation and Forest Degradation (REDD+) (Harada et al. 2015; Indonesia Forest Climate Alliance (IFCA) 2007). REDD+ is a commitment under UN Framework Convention on Climate Change (UNFCCC) that introduced a mechanism for acquiring an international fund- or credit-based mechanism for reducing carbon emissions and protecting forest ecosystems (Brofeldt et al. 2014; Harada et al. 2015). REDD+ has received enormous interest from developing countries as a potential source of international funding for the forestry sector. Indonesia has been enthusiastic about the REDD+ initiative following the 13th Conference of Parties (COP13) in Bali and has actively participated in the international REDD+ negotiations. Protected areas, particularly national parks, became a target area for REDD+ in Indonesia (Harada et al. 2015; Indonesia Forest Climate Alliance (IFCA) 2007).

Technically, REDD+ is a carbon payment scheme aiming at mitigating climate change through reducing deforestation, reducing forest degradation, conservation of (existing) forest carbon stocks, sustainable management of forests, and enhancement of forest carbon stocks (e.g. through regeneration and planting in previously forest land) (Gardner et al. 2012; Marshall et al. 2012). Therefore, reliable and practical methods for measuring, reporting and verifying carbon stocks are necessary components of REDD+ (Gardner et al. 2012; Petrokofsky et al. 2012). The IPCC Guideline (IPCC 2006) suggests five carbon pools be included to thoroughly estimate forest carbon stock (i.e. aboveground biomass, belowground biomass, deadwood, litter, and soil carbon). Aboveground biomass (AGB) is the most important carbon pool representing the forest's physical conditions (GOFC-GOLD 2014a; Ministry of Environment and Forestry 2016). Attempts for estimating aboveground tropical forest carbon were mostly related to the type of forest ecosystem (dryland forests, moist forests, peat swamp forests, mangrove forests) and also locations (South East Asia, Africa, South America) (Manuri et al. 2017; Marshall et al. 2012; Yamakura et al. 1986).

Indonesia, through the National Forest Reference Emission Level (FREL) submission to the UNFCCC Secretariat (Ministry of Environment and Forestry 2016), has established a national forest carbon stock divided into seven regions (Sumatra, Kalimantan, Java, Lesser Sunda and Bali, Sulawesi, Maluku, and Papua). This data was claimed to be derived from analyzing the National Forest Inventory data from 1990 to 2013. However, the figures, particularly those in Sumatra (i.e. 135 [125; 145] tC/ha for dry primary forest/ DPF and 85.6 [80.9; 90.3] tC/ha for dry secondary forest/ DSF) and

Kalimantan (i.e. 126.6 [121.4; 131.9] tC/ha for DPF and 95.6 [92.3; 98.8] tC/ha for DSF) were much lower than the other figures in a similar location (Laumonier et al. 2010; Rutishauser et al. 2013; Yamakura et al. 1986). This disparity shall open a wider window to new forest inventory data, particularly those in more stable natural forests, e.g. in protected areas, to support the existing available figures on forest carbon stock. Additionally, estimation of aboveground forest carbon stock in protected areas is fundamental to invest our knowledge to address the role of conservation activity in REDD+, aside from their high biodiversity circumstance.

The present study aims to help fill our gap in knowledge on: (i) the characteristic of forest stands and aboveground forest carbon stocks in a protected area using terrestrial forest inventory; and (ii) estimating the total aboveground forest carbon stock in a protected area using a combination of spatial data and terrestrial forest inventory. We hypothesized that the protected area possessed a relatively higher figure of carbon stocks than the forest under a different type of management, so the role of conservation for carbon stock in a protected area as well as the need for significant activities to maintain this high carbon stock will be demonstrated.

## METHODS

### Study Area

The study area is located within Bukit Tigapuluh National Park (BTNP), Indonesia. Geographically located between E102$^0$13' – E102$^0$46' and S00$^0$42' – S01$^0$18', BTNP is 144,223 hectares of National Park in Eastern Sumatra, consisting of tropical lowland to a hilly undulating forest on mineral soil (Figure 1) at an altitude between 60 to 843 m asl. The climate in Bukit Tigapuluh National Park is a typical of tropical rainforest, i.e. always wet even though it also experiences a dry season with an average rainfall of 2,577 mm per year. The temperature of this area is in the range between 20.8$^0$ – 33$^0$ C. The National Park was established in 1995 after timber concessions had been issued in this forest block. This Park is famous as the last shelter for endangered species such as the Sumatran orangutan, Sumatran tiger, Sumatran elephant, Asian tapir, and many endangered bird species. Unfortunately, this vital ecosystem is threatened by illegal logging, illegal farming, mining, and poaching (Bukit Tigapuluh Wildlife Protection Unit 2017). The Park is also inhabited by indigenous peoples of the Orang Rimba (also called Kubu) and Talang Mamak tribes. The Talang Mamak is a sedentary tribe living only in Bukit Tigapuluh National Park (referred to as the Bukit Tigapuluh landscape). The Orang Rimba people are nomadic because of death, avoiding enemies, and shifting cultivation. The Kubu communities scatter in and around the forest, in huts with walls made of bark and roofs made of leaves. They live in small groups to facilitate mobility and migrate through natural forests depending on forest products and river for their existence (Sitompul & Pratje 2009). The surrounding indigenous peoples (especially the Talang Mamak Tribe) believe that the hills and plants in the national park have magi-

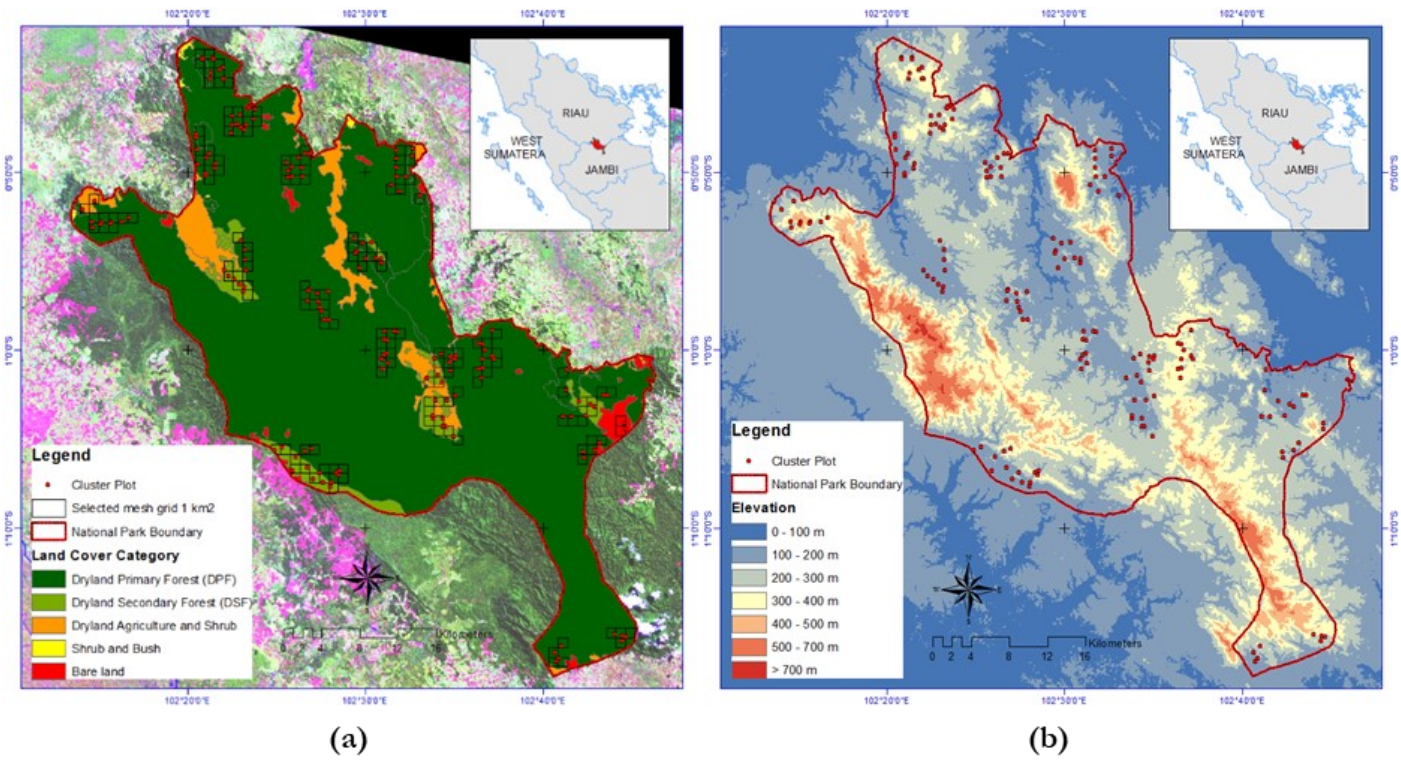


**Figure 1.** (a) The study area of Bukit Tigapuluh National Park (red line) and the location of the 168 cluster plots, which was selected purposively based on the forest types; (b) and overlaid to the DEM.

cal powers in their lives, so that they indirectly participate actively in maintaining and protecting the hills and plants in the national park.

## Data Collection

One hundred sixty-eight cluster plots were taken proportionally based on the area percentage of forest cover types (dryland primary forest/DPF and dryland secondary forest/DSF), following a virtual mesh grid of 1 km² established in the study area (Figure 1). One cluster plot was established within one selected mesh grid regarding forest cover types and access factors. One cluster plot consists of five plots of 400 m² size (in total 2,000 m²) with an arrangement as depicted in Figure 2. This cluster plot is a modification of the conventional single plot of 400 m² (BSN 2011) or 10,000 m² (FAO 2007). The reason for choosing a cluster plot is that the larger the area of the sample plot, the greater the proportion of total variation that falls within the plot, and as a result the smaller the standard errors (Baraloto et al. 2013; Henttonen & Kangas 2015; Picard et al. 2018). Thus, the cluster plot was designed for compromising the larger sample plot's need and complying with the national standard.

We limit our analysis for aboveground biomass and necromass (deadwood) since these carbon pools account for more than 75% of the total forest biomass in mineral soil (GOFC-GOLD 2014b; Manuri et al. 2016; Ministry of Environment and Forestry 2016). In this study, aboveground biomass and deadwood in carbon estimates were combined and called aboveground forest carbon (AGC, in tC/ha). We omit other carbon pools (i.e. be-

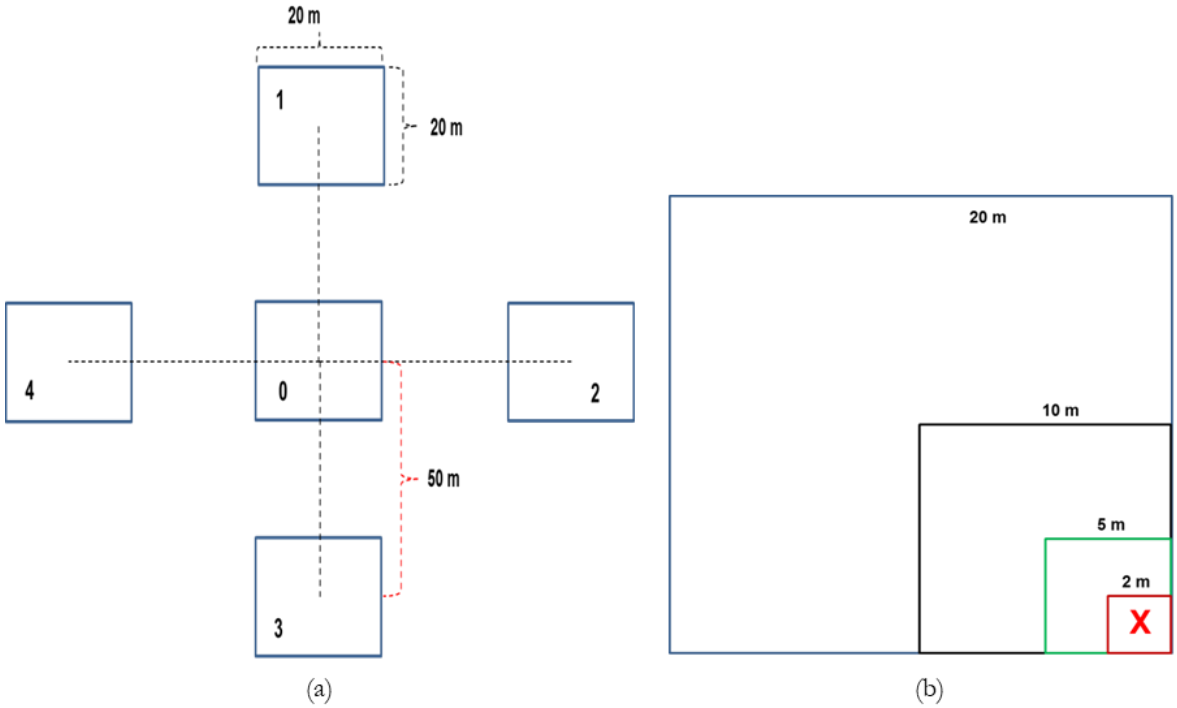


**Figure 2.** (a) The arrangement of plots in a cluster plot, the distance of center plot and the side plots was 50 m; (b) Each plot was comprised of subplots of 400 m², 100 m² and 25 m².

lowground biomass, litter, and soil carbon) because the study area located on the mineral soil where the fraction of soil carbon is mostly less than 20% and belowground biomass is mostly estimated through a relationship to aboveground biomass as indicated by (GOFC-GOLD 2014b), which does not have critical influence to the variation of data.

The 400 m² sub-plot consists of 400 m², 100 m² and 25 m² of plots to record the diameter at breast height (dbh) of the tree (greater than or equal to 20 cm dbh), pole (greater than or equal to 10 cm dbh and less than 20 cm dbh) and sapling (greater than or equal to 5 cm dbh and less than 10 cm dbh) plant categories, respectively. When the tree was buttressed, the tree diameter was measured approximately 20 cm above the buttress. The dbh of deadwood was also recorded within 400 m² plot using standing deadwood categories (BSN 2011), i.e. slight (dead tree without leaves, 0.9 carbon offset factor), moderate (dead tree without leaves and twigs, 0.8 carbon offset factor), and intense (dead tree without leaves, twigs and branches, 0.7 carbon offset factor) as depicted in Figure 3. Downed deadwood was planned to be measured, but we did not find it during the field measurement. In total, a 33.6 hectare of plots was measured from November 2016 to July 2017. A supporting smartphone application was used to assist the surveyor in capturing locations' coordinates and taking on-site photos heading north, east, south, west and looking upward for each cluster plot.

The 2014's land cover data of BTNP on 1:250,000 scales was collected from the Ministry of Environment and Forestry. This data was modified by referring to the 2016 Landsat 8 Image to get the newest condition of land cover so that it relatively parallel to the time of terrestrial forest inventory was carried out (Figure 1).

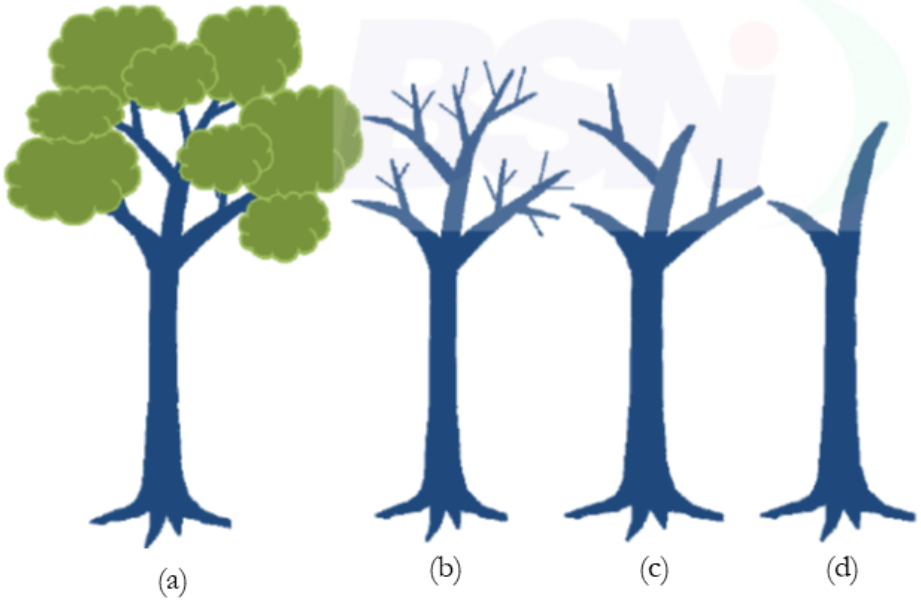


**Figure 3.** Standing deadwood category of individual tree according to BSN (2011), (a) Living tree; (b) slight deadwood; (c) moderate deadwood; and (d) intense deadwood. This category is used for i.e. the carbon offset factor of individual trees, 1, 0.9, 0.8 and 0.7, respectively.

## Data Analysis

Data analysis was carried out in three phases. First, we consolidated the forest inventory data into a spreadsheet. We adopted an allometric equation from Chave et al. (2005) for the moist tropical forest ecosystem to calculate aboveground biomass of each tree since most of the forest stands on mineral soil. This allometric equation was selected to follow similar equation used by the Indonesia's FREL (Ministry of Environment and Forestry 2016) so that a direct comparison between results can be done. The allometric equation is expressed as follows:

$$AGB = \exp(-1.499 + 2.148\ln(D) + 0.207(\ln(D))^2 - 0.0281(\ln(D))^3) \times WD$$

where AGB is aboveground biomass (in kg), D is dbh (in cm), and WD is wood density (in g/cm$^3$). Wood density for each species was derived from International Centre for Research in Agroforestry (ICRAF) wood density database (http://db.worldagroforestry.org/wd). When no botanical identification was available, we used 0.66 as a default wood density referred to Biomass Conversion and Expansion Factor (BCEF) for tropical forest (IPCC 2006). The carbon offset factor (0.9; 0.8 or 0.7) was multiplied to the AGB of a single dead tree (using a similar allometric equation). Aboveground biomass estimates were converted into carbon mass (C) by multiplying AGB with 0.47 (IPCC 2006).

Second, statistical analyses were performed to examine forest stand and forest carbon stock characteristics in the study area. This includes mean, standard deviation, and sampling error estimates as described in Table 1. ANOVA was used to see the significant difference between AGC and geo-

**Table 1**. Statistical analysis of the sample plot data. Uncertainty of estimates is characterized by Sampling Error (SE).

| Forest Cover type | Statistical Analysis | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean ( $^{Mj}$ ) | Standard deviation (SD) | Sample Count (n) | *t-statistic at* 95% (t) | Confidence Interval | Lower Bound | Upper Bound | *Sampling Error* (%) |
| Forest type-j | $\frac{1}{n}\sum_{i=1}^{n} Mi$ | $\sqrt{\frac{1}{n-1}\sum_{i=1}^{n}(Mi+Mj)^2}$ | 3<br>5<br>8<br>10<br>50<br>100<br>∞ | 4,30<br>2,78<br>2,37<br>2,26<br>2,01<br>1,98<br>1,96 | $\frac{SD \times t}{\sqrt{n}}$ | $Mj - CI$ | $Mj + CI$ | $\frac{CI}{Mj} \times 100\%$ |

*Mi* is the amount of aboveground carbon stock (in tC/ha) of cluster plot-*i* in forest type-*j*, n is the number of plots in forest type-*j*.

graphic variables (i.e. elevation and forest cover types). The analysis was divided into two approaches. The first approach was that the forest in the study area is categorized into one forest category (i.e. natural forest). The second approach was that the forest in the study area is categorized into dryland natural primary forest (DPF) and dryland natural secondary forest (DSF), following the land cover category of the Ministry of Environment and Forestry (MoEF).

Third, we estimated the total AGC stock in the forest of BTNP by multiplying the total forest cover area (in ha) and the AGC (in tC/ha) under the two approaches earlier.

## RESULTS AND DISCUSSION

### Results

We recorded 14,127 individual trees with dbh (diameter at breast height) ranging from 5 cm to 295 cm. There were 600 individuals classified as unidentified, and other individuals could be identified at least up to the family name. Dipterocarpaceae was the dominant family with 32 total species and 2,572 individuals.

#### Forest stand characteristic

The distribution of basal area and AGC of sample plots by diameter class is described in Figure 4. The fifth biggest contribution for AGC stock was made by 30 to 70 of diameter classes. These diameter classes accounted for more than 50% of the AGC of the sample plots. Big trees (diameter class more than 150 cm) contributed less than 10% of the overall AGC stock of the sample plots. Overall, the average percentage of AGB and deadwood that constitutes AGC was 96.5% and 3.5%, respectively.

Figure 5 describes the profile of stand basal area against AGC of the sampling plots. The relationship between basal area and AGC stock was relatively linear. Some plots possessed a higher basal area but resulted in low carbon stock because the plots were dominated by low to moderate wood density tree species.

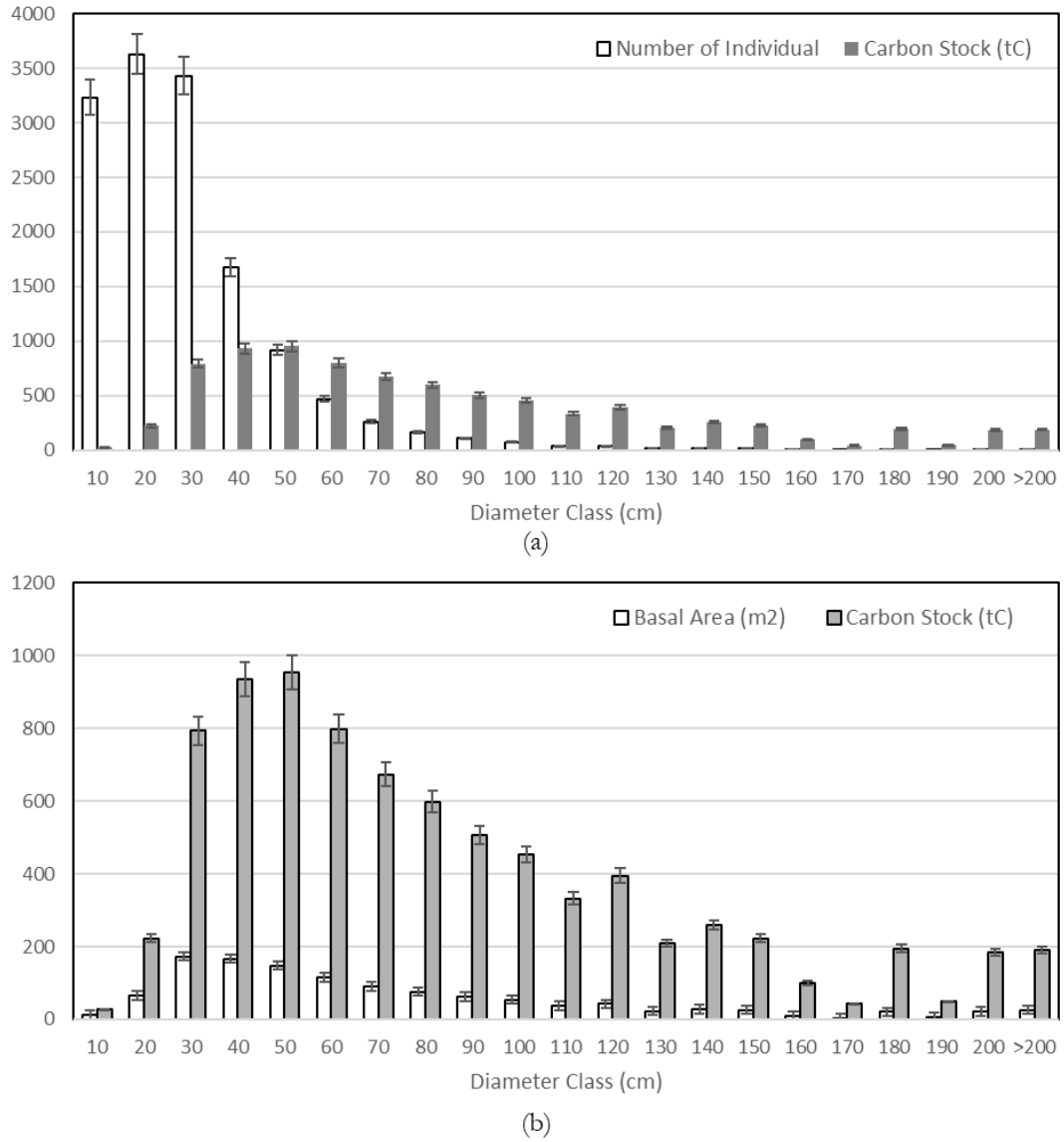


**Figure 4.** The data distribution of number of individual and AGC (a); and basal area and AGC (b) in the sample plots by diameter class. Each unit of measurements is presented in parentheses.

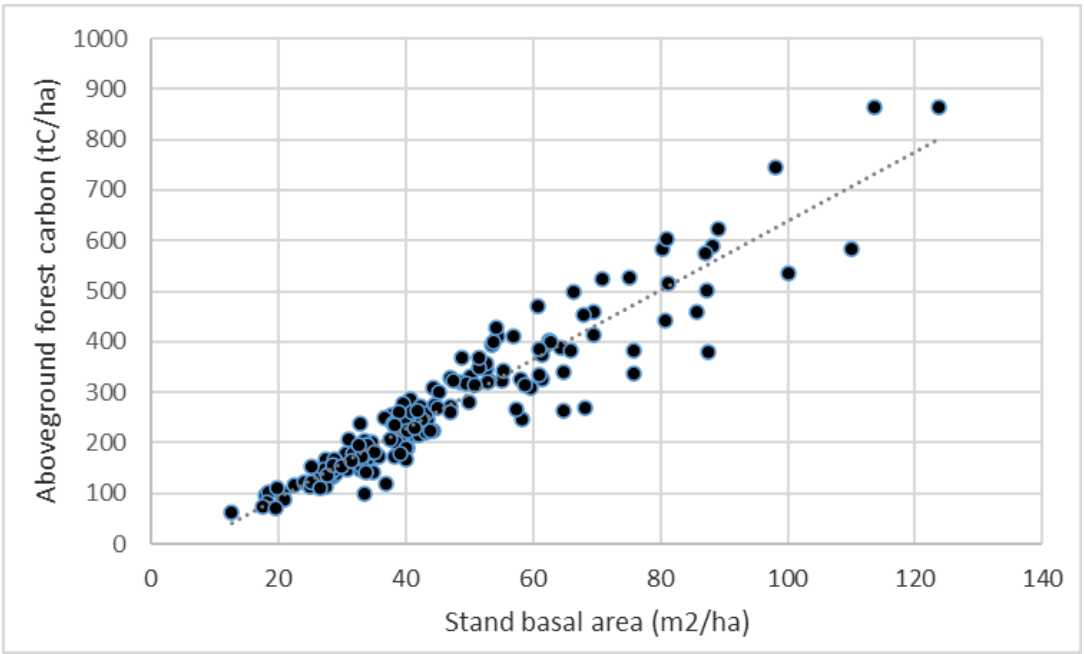


**Figure 5.** The profile of stand basal area against aboveground carbon stock of the sampling plots. The line represents a linear regression ($R^2 = 0.8919$, $F_{1,166} = 1370$, $P < 0.0001$).

The characteristic of sample plots with regard to elevation was described in Table 2. Most of the plots were established at 100 – 200 m asl elevation, while only one plot was set up below 100 m asl elevation (Table 3). Both basal area and carbon stock had a relatively similar increasing trend until 200 – 300 m asl, but then decreasing pattern until the highest elevation (above 400 m asl). However, ANOVA did not show a significant relation among elevation and aboveground forest carbon, where Pr(>F) was 0.1825, and the F value was 1.5783.

## Aboveground carbon stock

The First approach of AGC estimates resulted in 269.25 [247.07; 291.43] tC/ha with 8.24 % of sampling error (SE) (Table 3). By the Second approach, AGC estimates in DPF resulted 287.03 [258.80; 315.26] tC/ha, while DSF resulted 230.67 [197.82; 263.52] tC/ha. ANOVA showed significant relation among forest types (DPF and DSF) and aboveground forest carbon, where Pr(>F) was 0.0202, and the F value was 5.4987. However, SE estimates were rising into 9.84% and 14.24% of DPF and DSF, respectively.

Total AGC in the study area using the first and second approaches are presented in Table 4. The total forested area in BTNP based on the land cover map of 2016 was 133,051 ha which includes 126,992 ha of DPF and 6,059 ha of DSF. Using the first approach, the estimate of total AGC was lower than the second approach, i.e. 35,823,639 tC and 37,847,600 tC for first and second approaches.

**Table 2**. The characteristic of sample plots about elevation. Lower and upper are the 95% confidence interval (CI).

| Height (m asl) | Number of Plot | Basal Area ($m^2$/ha) | | | Carbon Stock (tC/ha) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Lower | Upper | Mean | Lower | Upper |
| below 100 | 1 | 68.1 | NA | NA | 268.8 | NA | NA |
| 100 – 200 | 77 | 45.5 | 43.4 | 47.6 | 259.5 | 230.0 | 289.1 |
| 200 – 300 | 61 | 49.3 | 44.5 | 54.1 | 299.9 | 256.8 | 343.0 |
| 300 – 400 | 24 | 40.4 | 30.9 | 49.8 | 240.5 | 184.9 | 296.1 |
| 400 – 500 | 5 | 33.6 | 13.5 | 53.8 | 183.1 | 111.2 | 255.0 |

**Table 3.** Statistical analysis of aboveground forest carbon (AGC) stock.

| Forest Cover type | Statistical Analysis | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Mean ( $Mj$ ) | Standard Deviation (SD) | Sample Count (n) | *t-statistic at* 95% (t) | Confidence Interval (CI) | Lower Bound | Upper Bound | *Sampling Error* (%) |
| *First approach* | | | | | | | | |
| Forested area | 269.25 | 146.69 | 168 | *1.96* | 22.18 | 247.07 | 291.43 | *8.24* |
| *Second approach* | | | | | | | | |
| DPF | 287.03 | 154.46 | 115 | *1.96* | 28.23 | 258.80 | 315.26 | *9.84* |
| DSF | 230.67 | 120.77 | 53 | *1.98* | 32.85 | 197.82 | 263.52 | *14.24* |

**Table 4.** Aboveground forest carbon (AGC) stored in Bukit Tigapuluh National Park

| Land cover category | Area (ha) | Carbon density/stock (tC/ha) | | | Total carbon stock (tC) | | |
|---|---|---|---|---|---|---|---|
| | | Mean | Lower | Upper | Mean | Lower | Upper |
| *First approach* | | | | | | | |
| Forested area | 133,051 | 269.25 | 247.07 | 291.43 | 35,823,639 | 32,872,312 | 38,774,966 |
| *Second approach* | | | | | | | |
| DPF | 126,992 | 287.03 | 258.80 | 315.26 | 36,449,909 | 32,864,849 | 40,034,969 |
| DSF | 6,059 | 230.67 | 197.82 | 263.52 | 1,397,691 | 1,198,664 | 1,596,717 |
| Total | 133,051 | | | | 37,847,600 | 34,063,514 | 41,631,686 |

## Discussion

### Forest stand and carbon stock characteristics

We evidenced a high diversity of vegetation in the study area by 59 families of trees (5 cm up, i.e. including saplings and poles) covering at least 331 species. The forest ecosystem in BTNP was dominated by the dipterocarp family as the flagship of tropical lowland rainforest in South East Asia (Kuswanda & Barus 2019; Laumonier et al. 2010; Manuri et al. 2016; Yamakura et al. 1986). This forest showed a decent condition of vegetation structure which is characterized by an inverted J graph (negative exponential) of the distribution of the number of individuals by diameter class. This structure is the characteristic of a stable natural forest, where small trees that make up the ecosystem tend to be more dense than large trees (Gunawan et al. 2011). This trend is unlike the distribution of basal area and AGC, where the tendency is more like a normal curve (inverted bell) with the highest value in the diameter class 30 – 70 cm. Stand characteristics like this indicate a natural regeneration process that runs properly where the number of saplings and poles are abundant, and the highest productivity is in the middle classes of diameter which then decreases in the larger diameter classes.

Our analysis of carbon stock estimation showed that using a single class of forest (i.e. natural forest) is more consistent, as demonstrated by low sample error compare to that separating the natural forest class into DPF and DSF (higher SE). This result revealed that detailing forest cover into more specific forest classes in the study area did not improve estimates' uncertainty. There are two reasons for this. The first reason is that the decreasing number of plots in DPF and DSF, increases the data disparity as indicated by the increase of SE. The second reason is the differentiation between DSF and DPF are based only on the visual characteristic of remote sensing data, so it was not related to the type of carbon stock in each forest class.

Ministry of Environment and Forestry (2016) stated that the difference between DPF and DSF is merely related to an exhibit sign of logging activities indicated by patterns and spotting of logging (appearance of roads and logged-over patches), hence difficult to distinguish through Landsat 8 image although some areas of BTNP were a logging concession in the past (Kuswanda & Barus 2019). So, it is possible that DPF and DSF does not necessarily relate to the actual amount of carbon stocks. On the other hand,

Romijn et al. (2013) pointed out that countries shall select the major GHG emissions from land-use changes (e.g. forest cover change) through robust methodology and definitions. This allows them to make a land cover classification that differentiates between different forest types and other important land cover classes. Therefore, to decide forest or land cover classification, attention on how carbon stock has been included into consideration needs to be addressed.

We selected the first approach based on the above considerations. Using this selection, we estimated AGC stock in BTNP is 269.2 ± 22.2 tC/ha. In total, forested area in BTNP stored 35,823,639 ± 2,951,071 tC of AGC. This result was higher than other studies conducted in non-protected area (e.g. Laumonier et al. 2010; Ministry of Environment and Forestry 2016; Rutishauser et al. 2013; Slik et al. 2010; Yamakura et al. 1986), but lower estimates than other studies located in the protected area, i.e. Gunung Palung National Park, West Kalimantan (Paoli et al. 2008) (Table 5). Our results were higher than Avitabile et al. (2016), which produced a pan-tropical biomass map covering Bukit Tigapuluh National Park.

**Table 5.** Forest stand and carbon stock characteristics in various tropical lowland evergreen forests. Each unit of measurements is presented in parentheses.

| No. | Locality | Methodology | Stand Basal Area (m2/ha) | Forest Carbon (tC/ha) | Range of dbh (cm) | Sample area | Authors |
|---|---|---|---|---|---|---|---|
| 1. | Borneo (Sebulu, East Kalimantan) | Terrestrial sampling for AGB, allometric equation (Ogawa & Kira 1977) | 36.8 | 239.23 | ≤152 | 1 ha | Yamakura et al. (1986) |
| 2. | Sumatera Landscape (Jambi, Bengkulu, South Sumatra, Lampung) | Terrestrial sampling for AGB, allometric equation (Brown 1997; Yamakura et al. 1986) | 31.7 [31.2; 32.2] | 180 [135; 240) | 10 – 210 | 70.2 ha | Laumonier et al. (2010) |
| 3. | East Kalimantan, Pasir Mayang Sumatra | Terrestrial sampling for AGB, allometric equation (Chave et al. 2005) | 30.1 | 160 148; 164) | 10 – 140 | 12 ha | Rutishauser et al. (2013) |
| 4. | NFI Sumatra (DPF) | Terrestrial sampling for AGB, allometric equation (Chave et al. 2005) | NA | 135 [125; 145] | NA | 92 ha | Ministry of Environment and Forestry (2016) |
| 5. | NFI Sumatera (DSF) | Terrestrial sampling for AGB, allometric equation (Chave et al. 2005) | NA | 85.6 [80.9; 90.3] | NA | 265 ha | Ministry of Environment and Forestry (2016) |
| 6. | Borneo | Terrestrial sampling for AGB, allometric equation (Chave et al. 2005) | 26 – 49 | 214.8 | ≥10 | 83 plot | Slik et al. (2010) |
| 7. | Gunung Palung NP, West Kalimantan | Terrestrial sampling for AGB, allometric equation (Brown 1997; Chave et al. 2005) | 39.6 ± 1.4 | 292.3 [276.8; 307.8] | ≥10 | 4.8 ha | Paoli et al. (2008) |
| 8. | Bukit Tigapuluh NP | Data fusion approach of two pantropical biomass maps | NA | 160 [114; 206] | NA | NA | Avitabile et al. (2016) |
| 9. | Bukit Tigapuluh NP, Riau – Jambi | Terestrial sampling plot and spatial data | 45.93 | 269.2 [247.1; 291.4] | 5 – 295 | 33.6 ha | This study |

The three highest estimates on forest carbon stock were Paoli et al. (2008), this study, and Yamakura et al. (1986), while the lowest estimates were from the (Ministry of Environment and Forestry 2016). A conservative estimate from (Ministry of Environment and Forestry 2016) probably occurred because of their data selection mechanism. (Ministry of Environment and Forestry 2016) stated that the data validation included, among others, checking measurement data through abnormality filtering of DBH and species name of individual trees in the plots. This filtering mechanism can reduce data variation, thus reducing the number of oversized trees. However, as estimates from (Ministry of Environment and Forestry 2016) was the lowest (both DPF and DSF), a re-enumeration of this national carbon stock with newly available data is advisable, among others, with the inclusion of public participation such as university, research center and other non-state actors (e.g. Boissière et al. 2017).

### Implication to the management of protected area

This study and Paoli et al. (2008) supported the argument that protected areas possess a higher figure of carbon stock compared to other forest management unit. The national government administers national parks in Indonesia strictly prohibits the access of people to the parks to ensure the integrity of forest ecosystems (Harada et al. 2015), so a purely intact forest or an old secondary forest are typically found. Collins and Mitchard (2017) have estimated carbon emissions in the large forest protected areas in tropical countries (N=2018) and found that 36 ± 16 Pg C is stored in protected area's trees, representing 14.5% of all tropical forest biomass carbon. These results suggest that protected areas have been a successful instrument in protecting carbon biomass, thus a subset causing a disproportionately high share of emissions should be an urgent priority for management interventions.

Protected areas aim at protecting multiple ecosystem services (Collins & Mitchard 2017). Apart from its role in biodiversity conservation, the benefits they deliver to society include water, food and medicine, and they also provide important recreational, educational, spiritual and cultural places (Deguignet et al. 2017). We have demonstrated that protected areas in the tropics secure exceptionally high amount of AGC, which is very important to be conserved in the perspective of climate change mitigation. The high amount of AGC stock in the protected areas shall be very important assets for conducting the role of conservation for REDD+. Therefore, the management of BTNP shall enlarge their perspectives on climate change mitigation action apart from merely biodiversity conservation and life-support system. REDD+ readiness for protected areas needs to be completed as soon as possible since REDD+ has been a commitment of Indonesia's Government for conducting Nationally Determined Contribution (Republic of Indonesia 2016).

Many national parks in Indonesia have frequently been suffering from conflicts between government and local people (Harada et al. 2015).

REDD+ initiatives may become a way to tackle social and political problems and guarantee people's right to use and manage forests. REDD+ initiatives are expected to resolve such forest tenure issues, which may become a key precondition to implementing REDD+ projects effectively. Harada et al. (2015) confirmed that the REDD+ demonstration activities (DA) project in Meru Betiri NP could secure land use inside the national park and the participation of local people in the REDD+ DA project in the park, which national regulations in Indonesia had strictly prohibited. Consequently, the project in the national park could successfully introduce alternative livelihoods to improve income, particularly for economically disadvantaged people, by implementing a rehabilitation program with agroforestry while conserving forests. Harada et al. (2015) also demonstrated the necessity of further discussion of effective benefit-sharing of REDD+ incentive while realizing local participation in REDD+ projects and improving local livelihoods. These project outputs can become a model for collaborative forest management with multiple stakeholders in different national parks, such as Bukit Tigapuluh National Park.

## CONCLUSION

Stand characteristics in Bukit Tigapuluh National Park indicate that the natural regeneration process is going well. The highest AGC was found in the middle diameter class which then decreases in the larger diameter classes. This stable forest ecosystem secured a significant forest carbon stock estimated as 269.25 [247.07; 291.43] tC/ha or in total 35,823,639 [32,872,312, 38,774,966] tC being stored in approximately 133,051 hectares of the tropical rain forest. This result was higher than other studies in non-protected areas, but was lower than other studies in protected areas, such as Gunung Palung National Park, West Kalimantan. This study and Paoli et al. (2008) supported the argument that protected areas possess higher carbon stock figures compared to other non-protected forest management units. The high amount of forest carbon biomass in the protected areas shall be very important assets for conducting the role of conservation for REDD+. Therefore, the management of BTNP shall enlarge their perspectives on climate change mitigation aside from merely biodiversity conservation and life-support system.

## AUTHORS CONTRIBUTION

A.D. is lead researcher, conducting overall strategy to conduct this research from planning, data collection, analysis and writing report and paper. Z.W. is giving general suggestion and managing the field data collection. E.M. is analyzing spatial data. A.V. is giving suggestion and English proofread. M.I.F. is giving suggestion on database and data analysis. G.D.W. is giving suggestion on data analysis and discussion. B.W. is giving suggestion the data analysis and discussion especially on National Park Management. T.R. is giving suggestion on the data analysis and discussion on carbon inventory. S.T. is giving suggestion on the general part of the manuscript and English proofread.

## ACKNOWLEDGMENTS

We are grateful to the Balai Taman Nasional Bukit Tigapuluh for supporting the ground survey of carbon measurement and data entry to facilitate this study in collaboration with WWF Indonesia. This study was funded by WWF France.

## CONFLICT OF INTEREST

No competing interest among author and co-authors.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66823
DOI: 10.22146/jtbb.66823

Research Article

# Morphological and Anatomical Variations among *Alocasia alba* Schott Accessions in Bali Botanic Garden

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**Keywords:**
Aroid
environment
leaves
phenotypic
stomata
**Submitted:**
21 June 2021
**Accepted:**
12 September 2021
**Published:**
17 January 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT
*Alocasia alba* Schott is a member of Macrorrhizos group from Aroid family that has conserved in Bali Botanic Garden. On its development, the collections showed varied morphological diversity on leaves and flowers. The aim of this study is to fill the knowledge gap in morphology and anatomy of the species *A. alba* and to know the phenotypic variation in this species. A total of eight *A. alba* accessions from Java, Bali and West Nusa Tenggara were observed in morphological and anatomical characters. The result showed that the eight accessions of *A. alba* have some variations in morphological and anatomical characters. These variations might be caused by genetic factors that resulted from plant adaptation to the different environments.



## INTRODUCTION
*Alocasia* is one of plant group which is very popular among ornamental plant hobbyists and plant breeders. The genus has variety in leave shapes and colors, potentially for exotic plants breeding. *Alocasia* have estimated 121 species that spread around the world, but only 78 species have been described (Boyce & Croat 2011). The *Alocasia* distributions and diversities in Indonesia remain unknown. However based on herbarium tracking and field observation, it is estimated about 36 *Alocasia* species origin from Indonesia (unpublished data). This number may change if the study about *Alocasia* diversity in its nature habitat is increased. Hay (1998) grouped *Alocasia* according to similarity on its special character *Alocasia i.e* Puber, Scabriuscula, Princeps, Macrorrhizos, Longiloba and Cuprea group.

*Alocasia alba* Schott is a member of Macrorrhizos group with large figures and leaves (Figure 1) which first described by Schott based on cultivated plant from Malesiana region. Botanist identified that *A. alba* is originated from Java (Hay 1998). Exploration biodiversity in some regions found the new distribution of *A. alba* in Bali, Lombok (Kurniawan et al. 2013) and Lampung (Mustaqim & Setiawan 2019). In Bali, *A. alba* has a wide distribution from altitude 196 - 1300 m asl on humus to sandy land.


**Figure 1.** *Alocasia alba* Schott (A) Plant (B) Flower (Photograph by I Gede Wawan Setiadi)

Bali Botanic Garden as *ex situ* conservation institution of Indonesian Institute of Sciences, has conserved *A. alba* collected from Java, Bali and West Nusa Tenggara. On their development, the collections showed varied morphological diversity on leaves and flowers. Kurniawan et al. (2013) reported about the variation on flower structures and leave shapes in *A. alba*. Similar research on collected *Begonia areolate* in Cibodas Botanic Garden from various regions, showed the diversity on their leaves while maintained in the same environmental conditions (Efendi et al. 2020). This showed the genetic influence in plant collection is still remaining although being cultivated away from its natural habitat.

There were less study on phenotypic variation in morphology and anatomy in *Alocasia* genus. Only two reported articles on *Alocasia macrorrhizos* (L.) G. Don. such as morphology and reproductive characteristic in Vanuatu (Garcia et al. 2008) also on morphology, anatomy and isozyme variation in Central Java (Suratman et al. 2016). Meanwhile, the morphological and anatomical character research has very important aspect as the parameter to determine diversity level in *A. alba*. Morphological character often used to represent and identified intra-species together with phenotypic variation because they are fast, simple and inexpensive (Jingura & Kamusoko 2015; Suratman et al. 2016). Anatomical character also useful for systematic study, species identification and solve the taxonomic problem (Chikmawati 2013). The aim of this study is to fill the knowledge gap in the morphology and anatomy of species *A. alba* and to understand the phenotypic variation in this species.

## MATERIALS AND METHODS

### Materials

A total of eight *Alocasia alba* Schott accessions from, Java, Bali and West Nusa Tenggara were observed (Table 1, Figure 2). They were planted in Bali Botanic Garden (BBG) after one year acclimatization in nursery and the plant growth was good. BBG is situated in mountain area at 1,250-1,400 m asl so that the temperature was relatively low.

**Table 1.** Collection and access number of eight accessions *A. alba* in Bali Botanic Garden.

| No | Collection/Access Number | Location | Altitude (m asl) |
|---|---|---|---|
| 1 | PSA.222/E2014120016 | Sendang Gile Waterfall, North Lombok, WNT | 471 |
| 2 | JQ.1143/E2017080061 | Benang Kelambu Waterfall, Central Lombok, WNT | 577 |
| 3 | MBA.121/E2016050041 | Rinjani Mountain National Park, East Lombok, WNT | 911 |
| 4 | PSA.226/E2014120020 | Benang Kelambu Waterfall, Central Lombok, WNT | 537 |
| 5 | BA.753A/E20110952 | Munduk Pengubengan, Karangasem, Bali | 1060 |
| 6 | RS.136/E2014020001 | Merapi Mountain National Park, Sleman, Yogyakarta | 1004 |
| 7 | PSA.215/E2014120009 | Seraya Mountain, Karangasem, Bali | 788 |
| 8 | DL.99/E2015110012 | Grojogan River, Jembrana, Bali | 196 |


**Figure 2.** Distribution of *A. alba* in Java, Bali and West Nusa Tenggara. (Google earth and modified by Ni Putu Sri Asih (unpublished data)).

## Methods

Observation of morphology characters was carried out by direct observation of both vegetative and generative characters and character state. The observation of characters included the shape and color of petiole, leaf, peduncle and flower. The observation of character states included the plant height, petiole, peduncle, leaf length and the length of each zone of flower. The plant leaves that used as the main research were the second or third leaves from the top. The color of each part of plants was identified by RHS (Royal Horticultura Society) Color Chart.

Anatomy of leaves anatomy was obtained with modified paraffin-tert-butanol method (Sass 1951). The sections were stained with safranin and fast green. There are two methods for epidermal character observation. We used $HNO_3$ solution (Cutler et al. 2007) to obtain leaf surface and to measure length, width of stomata and simple nail varnish to examine stomatal density (number of stomata/mm$^2$ leaf area) on both abaxial and adaxial surfaces.

## RESULTS AND DISCUSSION

### Morphological analysis

Morphological vegetative characters, both quantitative and qualitative characters, showed several variations (Table 2). All quantitative characters showed the differences in plants height, petiole and sheath length, leaf length and width, posterior costae diverging, primary lateral vein, peduncle and spathe length, spadix and stipitate length, female zone, male zone, also sterile interstice zone and appendix length.

These color variations also occured in some of plant parts *i.e.* in petioles, pattern or line in petioles, leaf colors, axillary glands, peduncles, spathes and each zone in spadix. Some plant characters might have or not patterns or line of petiole. This absence of pattern only found in *A. alba* accession from West Nusa Tenggara. Mostly, leaf characters of accessions from those three locations were similar, but all accessions showed different leaf forms. (Figure 3). Some *A. alba* leaves from Bali accession have suborbicular-sagittate shape, unite posterior lobe, and lanceolate inner-side posterior. *A. alba* from Java accession, has leaf edge which has sinuate character, otherwise West Nusa Tenggara has slightly undulate character that different from others.

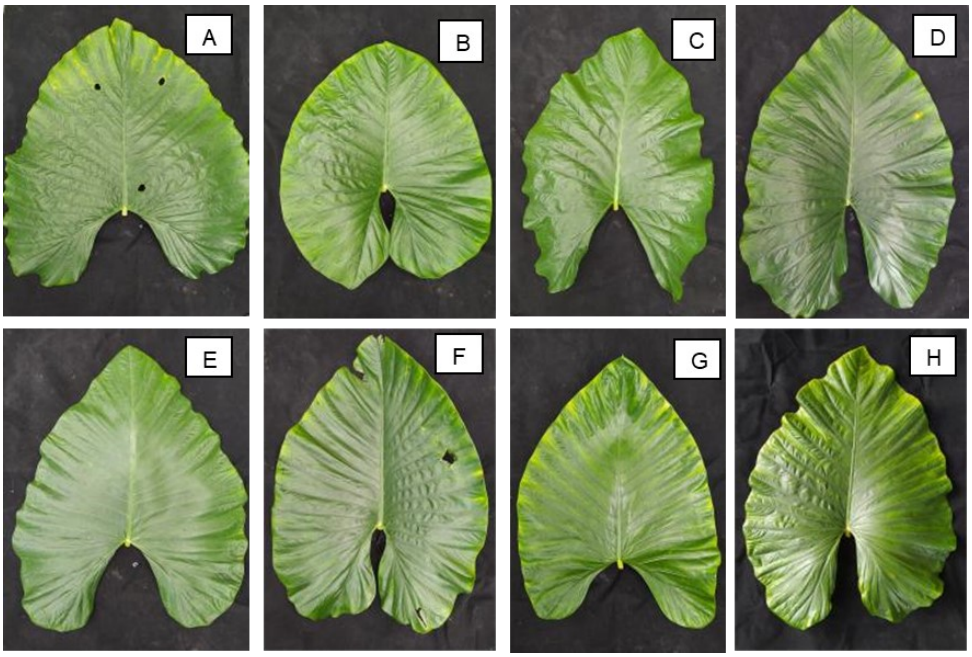


**Figure 3.** Variation of leaf. A. RS.136 (Java). B. BA.753A (Bali). C. DL.99 (Bali). D. PSA.215 (Bali). E. PSA.222 (Lombok). F. PSA.226 (Lombok). G. MBA.121 (Lombok). H. JQ.1143 (Lombok) (Photograph by Ni Putu Sri Asih).

The generative characters, both quantitative and qualitative, showed different sizes and colors in all part of flower and peduncle (Table 3). The only similarity of those accessions is the number of inflorescence, whether it presents in several or a pair of inflorescence. Peduncle color of Bali accession shows more varied than Java and West Nusa Tenggara accession. While the limb, lower spathe, ovary and stigma color of West Nusa Tenggara accession show the most varied. The number of stigma lobe shows the same number

**Table 2.** Vegetative characters of *A. alba* in Bali Botanic Garden based on collections origined Java, Bali and West Nusa Tenggara.

| No | Characters | Java | Bali | West Nusa Tenggara |
|---|---|---|---|---|
| 1 | Plant height (cm) | 129.8-132 | 114-300 | 82.5-242 |
| 2 | Petiole length (cm) | 83.4-96.8 | 66.9-99.2 | 77.2-102.5 |
| 3 | Petiole color | Moderate yellow-ish green 138A | Strong yellow green C N144C<br>Greyish olive green A NN137A<br>Greyish olive green B NN 137B | Strong yellow green 145A<br>Moderate yellow green C 138 C<br>Moderate yellow green B 146A<br>Greyish olive green A NN137A |
| 4 | Pattern or line on petiole | Present | Present | Absent-present |
| 5 | Pattern/line color | Dark purplish grey A N187A | Greyish reddish brown B 200<br>Dark greyish reddish brown A 200 | Greyish reddish brown B 200<br>Dark purplish grey A N 186A<br>Dark greyish reddish brown A 200 |
| 6 | Sheath length (cm) | 34.9-40.3 | 26.8-65 | 31-49 |
| 7 | Leaf shape | Ovate-sagittate | Suborbicular-sagittate<br>Ovate-sagittate<br>Cordate-sagittate | Ovate-sagittate<br>Cordate-sagittate |
| 8 | Leaf color | Greyish olive green NN137A | Greyish olive green B NN137B<br>Greyish olive green A NN137A<br>Greyish olive green B NN 137A | Greyish olive green A MM37<br>Greyish olive green B NN137 B<br>Greyish olive green A NN137A |
| 9 | Leaf edge | Sinuate | Undulate | Undulate<br>Slightly undulate |
| 10 | Spread of posterior leaf | Separated | United- separated | Separated |
| 11 | Leaf length (cm) | 60-68 | 51.8-94 | 48.5-94 |
| 12 | Leaf width (cm) | 51.8-62.5 | 51.8-94 | 36.6-67 |
| 13 | Apex | Shortly acumin-ate | Shortly acuminate<br>Acuminate | Shortly acuminate<br>Acuminate |
| 14 | Inner side of posterior lobe | Obovate | Obovate<br>Narrowly obovate<br>Lanceolate | Obovate<br>Narrowly obovate |
| 15 | Posterior costae diverging (°) | 135-150 | 65-110 | 75-135 |
| 16 | Primary lateral vein | 7-10 | 8-12 | 8-14 |
| 17 | Axillary glands color | White NN155D | White NN155D<br>Brilliant yellow green 149 C | White NN155D<br>Strong yellow green 145 A |

with Bali and West Nusa Tenggara accession (2-4 lobes), while the Java accession has different number (2-3 lobes).

## Anatomical analysis

Epidermal examination on leaf anatomy of eight accessions of *A. alba* showed that cell wall on adaxial epidermal has anticlinal straight, angular or rounded and undulate anticlinal cell wall, whereas on abaxial, it is undulate, sinuous, straight and rounded anticlinal cell wall (Figure 4). Both abaxial and adaxial on periclinal wall are smooth. In this study, all accessions of *A. alba*

**Table 3.** Generative characters of *A. alba* in Bali Botanic Garden based on collections origined Java, Bali, and West Nusa Tenggara.

| No | Characters | Java | Bali | West Nusa Tenggara |
|---|---|---|---|---|
| 1 | Inflorescences | several at the centre of leaf crown, occasionally a pair | several at the centre of leaf crown, occasionally a pair | several at the centre of leaf crown, occasionally a pair |
| 2 | Peduncle length (cm) | 21-28 | 25-37 | 23.5-40 |
| 3 | Peduncle color | Light yellow green D144 | Moderate yellow green D 139<br>Moderate yellow green C 139<br>Moderate yellow green D 137<br>Strong yellow green C 143<br>Strong yellow green C 144<br>Strong yellow green C 145<br>Brilliant yellow green C 142 | Strong yellow green A 144<br>Strong yellow green A 143<br>Light yellow green B 145<br>Strong yellow green C 143<br>Strong yellow green C 144 |
| 4 | Spathe length (cm) | 12.7-14.6 | 13.6-18.6 | 10.7-17.2 |
| 5 | Limb color | Brilliant yellow green C 150 | Light yellow green B 146<br>Strong yellow green D N144<br>Strong yellow green D N145 | Strong yellow green C 144<br>Strong yellow green A 145<br>Moderate yellow green A 138<br>Strong yellow green A 144<br>Light yellow green B 145<br>Light yellow green D 154 |
| 6 | Lower spathe color | Moderate yellow green B 146 | Brilliant yellow green C 154<br>Brilliant yellow green C 150 | Strong yellow green A 144<br>Strong yellow green B 144<br>Strong yellow green C 144<br>Strong yellow green A 145<br>Strong yellow green B 145<br>Brilliant yellow green C 150<br>Brilliant yellow green C 154 |
| 7 | Spadix length (cm) | 9.3-11.1 | 10.6-13 | 7.8-11.6 |
| 8 | Stipitate (mm) | 2-6 | 1-4 | 1-7 |
| 9 | Female zone length (cm) | 2-2.4 | 1.9-2.9 | 1.5-2.3 |
| 10 | Sterile interstice length (cm) | 0.7-0.8 | 0.6-1.4 | 0.5-1.6 |
| 11 | Male zone length (cm) | 2.4-3 | 2.3-3.4 | 1.8-3.5 |
| 12 | Appendix length (cm) | 3.6-5.4 | 5.3-6.9 | 3.2-5.4 |
| 13 | Ovary color | Strong yellow green C N144<br>Strong yellow green C N144 | Light yellow green C 145<br>Strong yellow green D N144<br>Light green yellow C1 | Light yellow green D150<br>Light yellow green B 145<br>Brilliant yellow green C 149<br>Strong yellow green D N144<br>Strong yellow C 144<br>Strong yellow Green A 145 |
| 14 | Lobe number of stigma | 2-3 | 2-4 | 2-4 |

**Table 3.** Contd.

| No | Characters | Java | Bali | West Nusa Tenggara |
|---|---|---|---|---|
| 15 | Stigma color | Pale green yellow D 2<br>Pale green yellow D 3 | Light yellow green D 150<br>Pale greenish yellow D1 | Light greenish yellow D7<br>Light greenish yellow D8<br>Light yellow green D 150<br>Strong green yellow B 151<br>Strong green yellow B 152<br>Brilliant greenish D 151<br>Pale greenish yellow D1 |
| 16 | Sterile interstice color | Pale yellow pink D159 | Yellowish white D 158 | Pale yellow B 158<br>Yellowish white B 155<br>Yellowish white D 158 |
| 17 | Male zone color | Pale yellow pink D159<br>Yellowish white C158 | Yellowish white D 158<br>Yellowish white D 155<br>Yellowish white C 155 | Pale yellow B 158<br>Yellowish white D 155<br>Yellowish white B 155<br>Yellowish white C 158 |
| 18 | Appendix color | Light yellow pink A 159<br>Pale yellow A 158 | Yellowish white C 158<br>Yellowish white D 158<br>Pale yellow A 158 | Pale yellow B 158<br>Pale yellow A 158<br>Yellowish white D 155<br>Yellowish white D 158 |

have similar of anatomical characters. *A. alba* leaf type is ampishomatic which means that stomata occur in both surfaces, but the stomatal density on adaxial is less than abaxial surface. Types of stomata on *A. alba* are anomocytic, anisocytic, paracytic and brachyparatetracytic (Figure 4). The latter stomata type was based on Dilcher (1974). Transversal section of leaf showed 1-2 layer of palisade on adaxial side of the leaf and sponge tissue arranged below the palisade (Figure 5 A). All accessions have druse shape of $CaCO_3$ crystal (Figure 5 B).

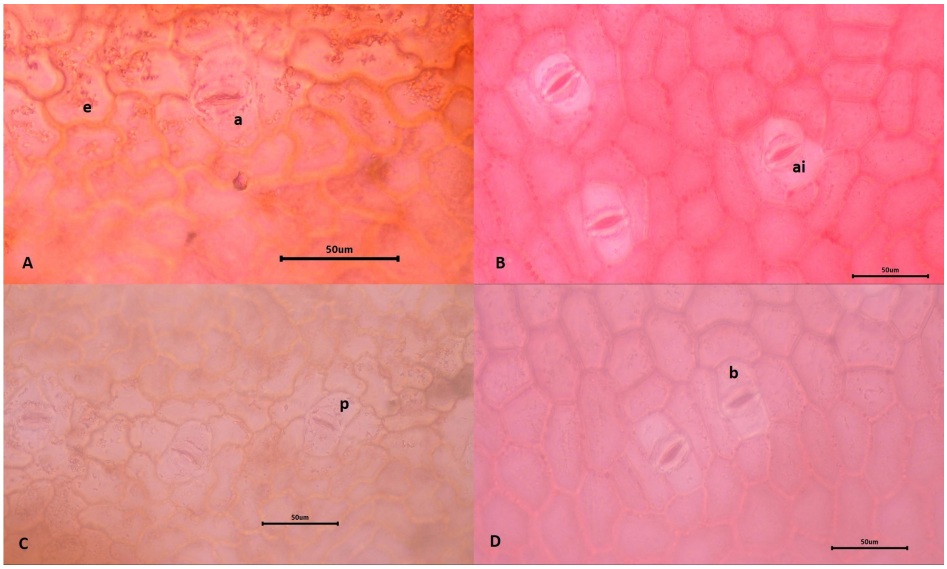


**Figure 4**. Leaves epidermal on *A. alba*. Anticlinal epidermal wall with undulate cell (A). angular and rounded cell (B). sinuous (C). Stomata type of brachyparatetracytic (b) also presents in *A. alba* (D). There are stomata types *i.e.* anomocytic (a), anisocytic (ai) and paracytic (p). Epidermal cell (e). Scale bar 50 µm.

The transversal section of *A. alba* showed that the leaf consists of cuticle, epidermal, palisade and sponge cells (Figure 5). The cuticle is situated in

**Table 4**. Anatomical characters of *A. alba* in Bali Botanic Garden based on collections origined Java, Bali, and West Nusa Tenggara.

| Parameter | | Collection Origin | | |
|---|---|---|---|---|
| | | Java | Bali | West Nusa Tenggara |
| Density of stomata (number of stomata/mm²) | Adaxial | 54.78 ± 2.88 | 41.7 ± 6.33 | 25.2 ± 7.26 |
| | Abaxial | 106.11 ± 8.86 | 80.95 ± 16.93 | 87.27 ± 10.43 |
| Length of stomata (µm) | Adaxial | 32.31 ± 2.20 | 34.81 ± 3.27 | 32.8 ± 2.00 |
| | Abaxial | 29.52 ± 2.13 | 33.05 ± 2.79 | 31.53 ± 1.76 |
| Width of stomata (µm) | Adaxial | 28.37 ± 2.67 | 25.71 ± 2.97 | 24.93 ± 1.86 |
| | Abaxial | 23.62 ± 2.28 | 25.99 ± 2.46 | 23.91 ± 2.55 |
| Epidermal Thickness (µm) | Adaxial | 22.63 ± 2.99 | 23.53 ± 4.14 | 20.05 ± 2.98 |
| | Abaxial | 19.35 ± 3.97 | 17.51 ± 3.28 | 16.73 ± 3.78 |
| Palisade Thickness (µm) | | 49.53 ± 10.31 | 52.52 ± 12.77 | 49.61 ± 10.27 |
| Sponge Thickness (µm) | | 161.70 ± 25.59 | 193.60 ± 26.35 | 156.17 ± 20.29 |
| Leaves Thickness (µm) | | 253.22 ± 23.40 | 287.62 ± 30.64 | 242.56 ± 25.66 |

adaxial surface, while the one layer of epidermal cell is situated in both surfaces. The sponge cell has the thickest part in leaf tissue.

Table 4 showed stomata and epidermal measurement. Stomatal density from Java is higher than from Bali and West Nusa Tenggara. The stomata are longer and wider as well as the epidermal cells are thicker in the adaxial than abaxial side. This study also showed that Bali's accessions have longer stomata; thicker epidermis, palisade and sponge compared to accessions of other locations. But, the adaxial stomata of Java accession are the widest.

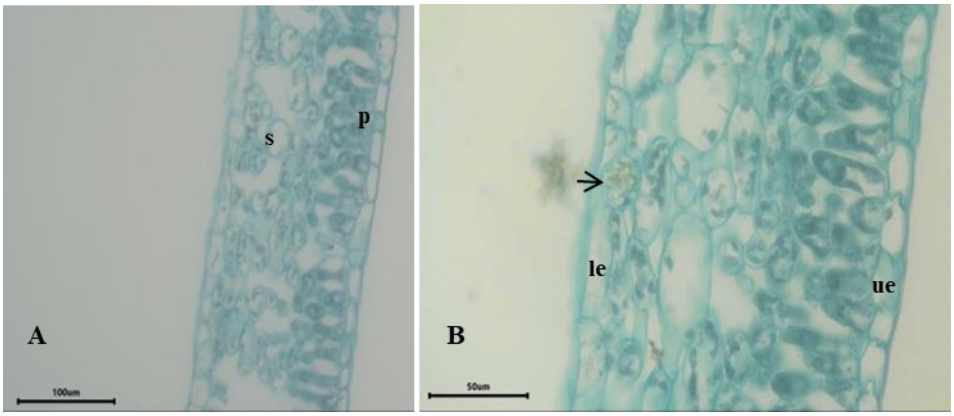


**Figure 5**. The transversal section of *A. alba* leaf. A.) Bar scale 100 µm. B.) s: sponge; p: palisade; le: lower palisade; ue: upper palisade. Scale bar 50 µm.

## Discussion

Eight accessions of *A. alba* from Java, Bali and West Nusa Tenggara were observed based on morphological and anatomical characters. Predominantly, the variations in morphological are the color of petiole, pattern of petiole, leaf, peduncle, spathe and spadix of *A. alba* (Table 2 and Table 3). Related to the present of patterns in petiole, it is divided into two variations *i.e.* petiole with pattern and petiole without-pattern. Petiole without-pattern only found in accession from West Nusa Tenggara. These kinds of variations have never been studied but have founded in several variations in *Alocasia longiloba* Miq.

*A. longiloba* have seven peak variations and mostly have mottled petiole, but the petiole of watsoniana variation is not or faintly mottled. This immottled petiole sometime also founded in lowii variation. The cause of variations are still not understood (Hay 1998).

According to the color of petiole pattern, there are four variations of color. These variations of colors have never been reported in *Alocasia* genus, but has reported in *Colocasia esculenta* (Maretta et al. 2020) and other family, Begoniaceae (Efendi et al. 2020). The differences of colors might be as a response to different light intensities that are obtained by the plant (Zhang et al. 2018).

This study also found some variations in leaf shapes, sizes of petioles, leaves, peduncles, spathes and each zone of spadix. This phenotypic variation within species is the result of the interaction of environmental and genetic factors that was gradually inherited to the offspring (Ramsey et al. 1994; Gonzalez et al. 2012; Albarrán-Lara et al. 2018; Li et al. 2018; Alcántara-ayala et al. 2020; Ren et al. 2020). The leaf size and shape indicated the diversity of leaf morphological phenotypes (Ren et al. 2020).

Meanwhile, the epidermal character of the eight accessions of *A. alba* showed similarities, especially in qualitative parameters. The similarity of cell form in the adaxial and abaxial surface is commonly found in plants, even though it is also found the different forms between those two surfaces (Cutler et al. 2007). The leaf anatomy of *A. alba* had been observed by Erlinawati & Tihurua (2013). The observed characters were epidermal cell shape, anticlinal wall, distribution of stomata, and the present of trichome. Erlinawati & Tihurua (2013) mentioned that the anticlinal wall of *A. alba* was straight but, the eight accessions of *A. alba* on this study, showed that it is also found the undulate and sinuous anticlinal cell wall. These differences can give new information about the range or variation of *A. alba* epidermal characters.

The stomata of *A. alba* are found in adaxial and abaxial, and it has four types of stomata *i.e.* anomocytic, anisocytic, paracytic, and brachyparatetracytic (Figure 4). Some studies about the stomata type of Araceae have been conducted in *A. cucullata, A. macrorrhiza* and *A. plumbea* (Suratman et al. 2016; Arogundade & Adedeji 2019), some Araceae species in Bombay and Maharashtra (Vaidya 2016b), and some species of *Alocasia*, *Colocasia* and *Remusatia* in Indonesia (Erlinawati & Tihurua 2013). Those three studies found one type of stomata in each species, but other research discovered two types of stomata (Sookchaloem et al. 2016; Vaidya 2016a). Those agreed to Cutler et al. (2007) which stated that although most species only have one type, but some species can have several types of stomata.

The other stomata character, density of stomata, showed that the highest density belongs to Java accession. The fact that the stomata on the abaxial side are denser than adaxial side has also confirmed by several research in *Alocasia* (Arogundade & Adedeji 2019; Suratman et al. 2016). Kondo et al. (2010) mentioned that environment condition is one of factor that affects the

density of stomata in plants. The dependency of this character to the environment condition can be used as indicator of transpiration and photosynthesis rate; also on absorption of water and mineral by the plant (Suratman et al. 2016; Rindyastuti & Hapsari, 2017). The quantitative data such as stomata length and width, epidermal thickness, palisade thickness, and sponge thickness and leaf thickness showed the variation amongst examined accession from three locations. Commonly, the Bali accession has the highest of all characters measurement and it might be caused by the adaptation of plant to the environment factors (Suratman et al. 2016).

The fact that the character variations of *A. alba* accessions from different locations, Java, Bali and West Nusa Tenggara, which planted in Bali Botanic Garden Conservatory that relatively has same environment condition might be caused by the genetic factor that the plant inherited from the parental and adaptation to the different physical condition for long time. Research about plant variation in different environments is important to understand the genetic diversity, genetic breeding and basis of conservation biology (Li et al. 2018) completed with the evolutionary processes that might promote speciation and maintain diversity (Alcántara-ayala et al. 2020). Furthermore, for biology conservation, plant variation research can give more specific information about the species that has to be conserved, especially the wild species to prevent genetic diversity loss (Santos et al. 2012).

## CONCLUSION

This study showed that there are some variations within species of *A. alba* from different locations based on their morphological and anatomical characteristics. These variations can be caused by genetic factors as a result from plant adaptation to different environments. Therefore, to prove the genetic factors on these variations, more data of morphology, anatomy and molecular are needed to enrich the information of *A. alba*.

## AUTHORS CONTRIBUTION

N.P.S.A did the morphological observation, analysis and write manuscript. E.H. did the morphological observation, stomatal density measurement and write manuscript. E.F.T. did the anatomical preparation, observation, analysis and write manuscript.

## ACKNOWLEDGMENTS

The authors acknowledge gratefully to Bali Botanic Garden and Herbarium Bogoriense for the permission and support of this research. We also thank to Exploration Team for providing the *A. alba* collection and the staff of Conservatory Garden for maintaining the life and growth of *A. alba*.

## CONFLICT OF INTEREST

The authors state that there is no any conflict of interest regarding the research or the research funding.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66510
DOI: 10.22146/jtbb.66510

Research Article

# *COI*-Based DNA Barcoding of Selais Fish from Arut River, Central Kalimantan, Indonesia

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**Keywords:**
Selais Fish
Siluridae
DNA Barcoding
*COI* gene
Phylogenetics
**Submitted:**
10 June 2021
**Accepted:**
02 September 2021
**Published:**
26 January 2021
**Editor:**
Miftahul Ilmi

## ABSTRACT
Selais fish belongs to the family Siluridae consisting of 12 genera with 104 properly validated species. The human need for these fish has sharply increased due to the benefits provided, especially for consumption. However, morphologically the selais fish are slightly challenging to differentiate among other silurid fish for non-specialist experts. Thus, a DNA barcoding approach using the mitochondrial *COI* gene as a molecular marker in this study was applied to clarify a taxonomic position and classification species of selais fish from Arut River (Central Kalimantan, Indonesia) and was also to assembly fish *COI* database storage from Indonesia. In this research, the method used was a PCR (Polymerase Chain Reaction) method with a pair of universal barcoding primers, FishF2 and FishR2. Based on partial *COI* fragment-based DNA barcoding, the whole samples showed no sequence differences (only 1 haplotype) within the population and this confirmed that these fish only consisted of one identical species. Furthermore, phylogenetic analysis (NJ / ML / BI) revealed that selais fish in this study had a close genetic relationship with *Ompok hypophthalmus* compared to other *Ompok* groups. This relationship was supported by the genetic distance value not exceeding 3.6% and this evaluated the undetermined naming of the selais fish from Arut River which was previously still unclassifiable.



## INTRODUCTION
Siluridae or commonly known as sheatfish is a part of the order Siluriformes consisting of 12 genera (*Belodontichthys, Ceratoglanis, Hemisilurus, Kryptopterus, Micronema, Ompok, Phalacronotus, Pinniwallago, Pterocryptis, Silurichthys, Silurus,* and *Wallago*) and 104 properly validated species (Fricke et al. 2021). In Indonesia, species members of the family Siluridae are commonly referred to as selais fish, such as *Ompok hypophthalmus, Ompok myostoma, Kryptopterus apogon, Kryptopterus micronema, Kryptopterus limpok,* and *Kryptopterus bicirrhis* (Jusmaldi et al. 2018). This is due to among selais fish almost possess similar morphological characters. In general, selais fish are characterized by a total length of about 30 cm to 45 cm, a brightly colored body, and have maxillary and mandibular barbel (Kottelat 2013). Mostly, these fish inhabit rivers in the Sunda Shelf area such as Sumatra, Java, and Borneo (Ng 2003). Selais fish, for local communities, have mostly been being caught for consumption

because they not only have a delicious taste but are also highly nutritious, so they can be used to meet animal protein needs.

Many research types on selais fish were carried out, but only a few studies at the molecular level. Arut River, one of the rivers in Central Kalimantan Province inhabited by selais fish, has become a concern area in this study. Overexploitation and overfishing have the potential to reduce the diversity and population number of selais fish. This is also supported by data at the IUCN which revealed there has been a decrease in the population for several species of selais fish. For conservation management efforts of Selais fish, especially in the Arut River, it is necessary to carry out initial data collection such as molecular identification of species names. Arisuryanti et al. (2020a) reported that the boundaries status and classification by identifying the selais fish using the *16S* mitochondrial gene were still undetermined due to low similarity and genetic distance values compared to the GenBank database. Although the *COI* mitochondrial gene is a high conserve region and highly acceptable for identification of almost all animals (Mitani et al. 2009), limited data in the GenBank database (unregistered) causes a low percentage of similarity and query cover for comparison, and this is a constraint in species determination.

Recently, a DNA barcoding approach using the mitochondrial *COI* gene as a molecular marker has been applied as a rapid alternative bio-identification method in clarifying taxa from the animal kingdom as a whole (Hebert et al. 2003), including ichthyofauna (Panprommin et al. 2019; Pandey et al. 2020). For example, Malakar et al. (2012) explained that three *Ompok* species from India were successfully identified as *Ompok pabda, Ompok pabo,* and *Ompok bimaculatus*. Arisuryanti et al. (2018) added that two cryptic fish species from Indonesia *Periophthalmus argentilineatus* and *Periophthalmus kalolo* were successfully confirmed. In addition, Chen et al. (2021), Chang et al. (2016), and Cline (2012) verified cases of mislabeling fish names from food products being sold.

Therefore, we tried to re-evaluate the uncertain taxonomic degree and systematic of selais fish from the Arut River using *COI* gene as taxonomic DNA by examining genetic relationships of three different phylogenetic tree approaches and this finding was also to compile the *COI* database library of the fish in Indonesia.

## MATERIALS AND METHODS

### Sample Collection

Wild selais fish with a total of 10 individuals from Arut River, Central Kalimantan (2°40'10.6"S and 111°38'08.3"E) was collected (Figure 1) by asking local fisherman for help using large fishing nets and documented for this study (Figure 2). Approximately around 50mg muscle tissue of these freshly caught fish was sampled and placed into 1.5 ml labelled tubes containing 99% absolute ethanol. The samples were transferred and then frozen at a

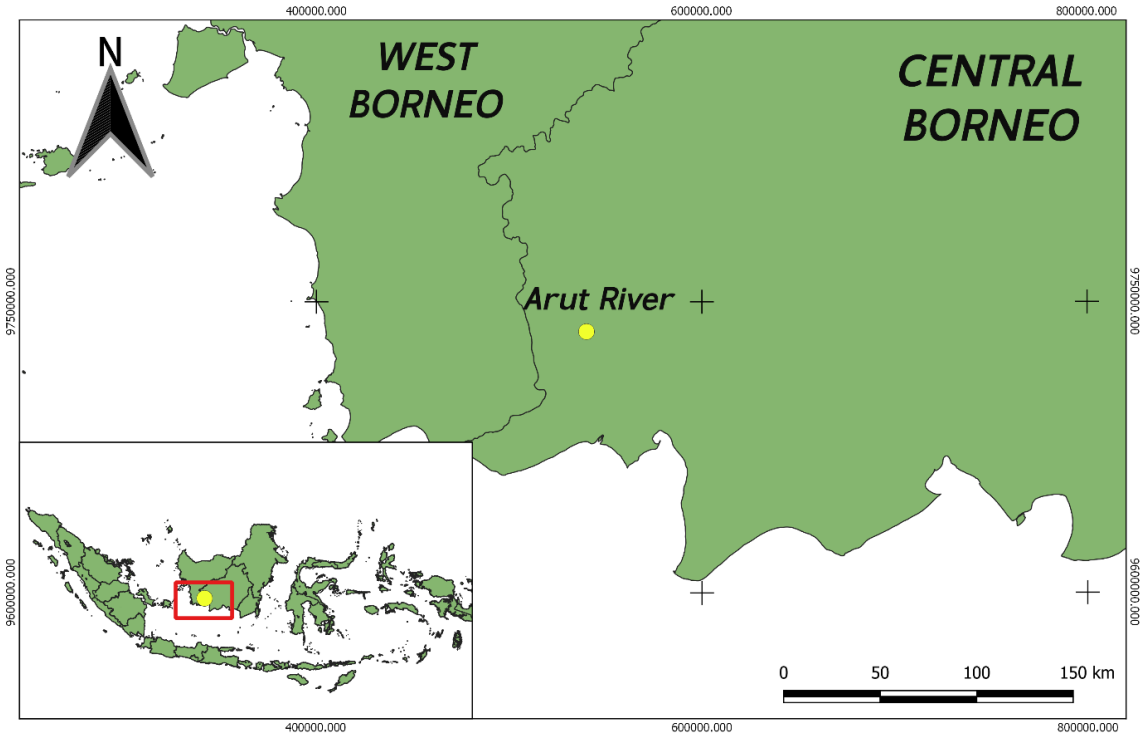


**Figure 1**. Yellow spot showed sampling area in Arut River, Central Kalimantan. Inset: Indonesian map.

**Figure 2**. Selais fish documented from Arut River, Central Kalimantan (bar = 1 cm).

temperature of -20$^{O}$ C in the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada, for further molecular analysis.

## DNA Isolation, Amplification, and Sequencing

The complete genomic DNA (gDNA) of preserved samples was isolated from the muscle tissue (cells) near the ventral fin using the DNeasy Blood and Tissue Kit (QIAGEN, Valencia, USA) following the factory's protocol. The single *COI* fragment was amplified using a PCR machine with barcode primer FishF2 and FishR2 (5'-TCGACTAATCATAAA GATATCGGCAC-3' and 5'-ACTTCAGGGTGACCGAAGAATCAGAA-3') respectively (Ward et al. 2005). Of 50 µl total volume of PCR reaction, 10-100 ng was gDNA, 25 µl was My Taq HS Red Mix PCR (bioline), 2 mM was $MgCl_2$, 3 µl was the two sets of *COI* primer, and 11 µl was $ddH_2O$. The PCR machine was conditioned at 95$^{O}$C in 1 min for pre-denaturation followed by 35 repeated cycles for denaturation at 95$^{O}$C in 15 sec, primer annealing at 50$^{O}$C in

30 sec, elongation at 72$^{O}$C in 30 sec, and at 72$^{O}$C for 1 min for final elongation and ended by the hold at 4$^{O}$C.

The PCR product was visualized using 2 µl staining FloroSafe in 1% agarose gel. The amplicon was then purified and sequenced at 1st BASE company using a pair of the same universal primers during amplification process. Bi-directional *COI* gene sequencing with sanger dideoxynucleotide sequencing method was performed using the ABI 3730XL Genetic Analyzer machine (Applied Biosystems).

## Data Analysis

Sequence data set were processed and set manually using the SeqMan and EditSeq programs (Lasergene, DNASTAR). The appropriate consensus sequence was analyzed using the Identification Engine program in the BOLD website and the BLAST program in the NCBI website to determine percentage identity. For either intra-population (population of selais fish from Arut River in this study) or intra-species analysis (sample combination between this study and GenBank database), sequence data were further aligned in MESQUITE ver. 3.51 (Maddison & Maddison 2018). The nucleotide composition and genetic distance with the Kimura 2-Parameter (K2P) substitution model were then analyzed in MEGA X (Kumar et al. 2018). Next, data of genetic variations (number of haplotypes, polymorphic sites, parsimony sites, transition and transversion, haplotype diversity, and nucleotide diversity) was processed in DnaSP ver. 6 (Rozas et al. 2017). The haplotype network based on the Median Joining Network method was visualized in NETWORK ver. 10.1 (https://www.fluxus-engineering.com). Furthermore, Principal Coordinate Analysis (PCA) was analyzed in GenAlEx ver. 6.5 (Peakall & Smouse 2012) to obtain a simple separation model among haplotypes.

The phylogenetic tree character was defined using three different approaches. The NJ (Neighbor-Joining) and ML (Maximum Likelihood) trees were characterized using the Kimura 2-Parameter (K2P) model with 1,000 bootstrap replications in MEGA X. Bayesian Inference (BI) tree topology was analyzed using the Bayesian Information Criterion (BIC) approach in the jModelTest ver 2.1.10 program (Darriba et al. 2012) to select the most suitable nucleotide substitution model. Furthermore, the BI tree was analyzed in BEAST ver. 1.10 program under the best appropriate model (Suchard et al. 2018). The MCMC (Markov Chain Monte Carlo) analysis was run $10^{7}$ with $10^{3}$ samples per generation. The first quarter of files were removed (burn-in), other three-quarter files were performed to construct the BI tree and measure posterior probability value. Phylogeny tree characteristics were visualized in Figtree v.1.4.4 (Rambaut 2019). Node on each branch represented the bootstrap (NJ/ML)/posterior probability (BI) value. Thirteen *Ompok* spp. consisting of 6 species were taken from GenBank for comparison purposes, namely *Ompok hypophthalmus* (MK473377 - MK473379), *Ompok eugeneiatus* (MK473374 and MK473376), *Ompok bimaculatus* (JX983415, JX983418, and JX260925), *Ompok pabo* (KX946739 and JN628926), *Ompok malabaricus*

(HQ009495), *Ompok pabda* (JX260929 and JX260930), and *Anabas testudineus* (MN640070) is used for the outgroup.

## RESULTS AND DISCUSSION

Nine out of all selais fish samples from Arut River were successfully amplified and sequenced using Fish F2 and Fish R2 primers (codes: LSA-1, LSA-2, LSA-5, LSA-6, LSA-7, LSA-8, LSA -9, LSA-10, LSA-11) and produce about 678-705 bp (226-235 amino acids). The one remaining sample yielded a poor sequence even though the DNA band was visible (Code: LSA-4) (Figure 3). The nine *COI* sequence data of selais fish have been registered in GenBank with accession number MZ634366-MZ634374.

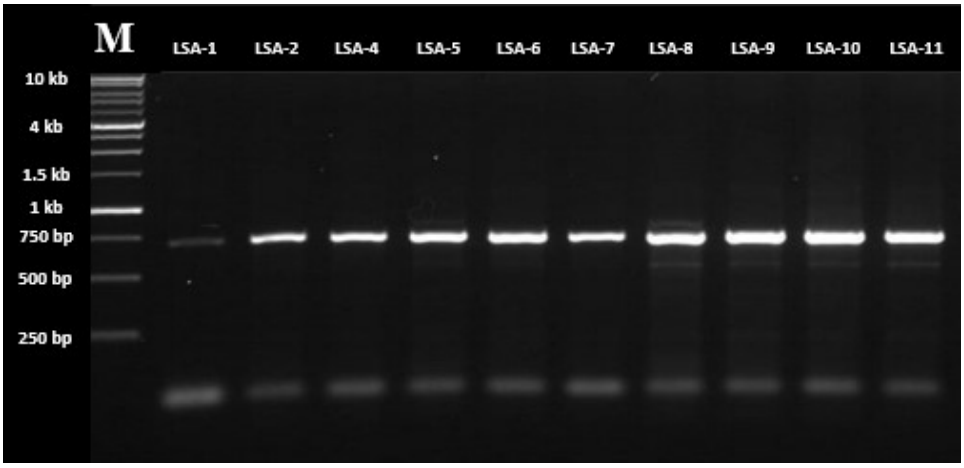


**Figure 3**. The result of *COI* mitochondrial gene amplification was visualized on the electropherogram. LSA was selais fish sample code and M was a marker.

The Identification Engine algorithm in BOLD and the BLAST algorithm in GenBank showed that these samples had percentage identity with *Ompok hypopthalmus* of 96.71% - 97.19% (BOLD) and 96.61% - 96.77% (GenBank). In particular, the *COI* gene sequence data of *Ompok hypophthalmus* in BOLD with a similarity value of 97.19% was still private data. This indicates that the *COI* gene sequence data has not been released to the public. Therefore, for comparison data, only the released *COI* sequence of *Ompok hypophthalmus* was analyzed. For intra-population level analysis, the *COI* gene sequences were aligned and resulted in 672bp (224 amino acids).

The mean nucleotide composition obtained was C = 30.65%, T = 26.64%, A = 24.40%, and G = 18.30% with a slightly higher amount of AT than the total CG. The CG content values not exceeding 50% were also detected in 6 species from 2 genera consisted of *Ompok pabda* (49.3%), *Ompok pabo* (46.4%), and *Ompok bimaculatus* (45.6%) (Malakar et al. 2012), *Kryptopterus apogon* (46.40%), *Kryptopterus micronema* (46.40%), and *Kryptopterus limpok* (46.40%) (Jusmaldi et al. 2017). This total can also be found for several marine fish species (Xu et al. 2021) and freshwater fish species (Arisuryanti et al. 2020b; Pandey et al. 2020). All samples showed identical sequences (only one haplotype) indicating no sequence variation within a population.

The three statistical methods of the phylogenetic tree formed almost similar tree topology, and the tree was only displayed using the NJ approach (Figure 4). For BI tree was constructed using the HKY (Hasegawa Kishino-Yano) with the gamma-distributed rate (+G) and invariant site (+I) as an ideal reference for substitution model under BIC in jModelTest. All samples from Arut River were grouped of only a clade (clade A) with a very strong bootstrap of 100/100 (for both NJ and ML) and posterior probability value of 1 (for BI). This indicates that the entire fish sample consisted of only one species, which was supported by 0% genetic distance among samples. This was confirmed by Roesma et al. (2020) that species possessing a genetic distance from 0% to 0.5% were still indicated as one identical species.

Furthermore, the samples (LSA) had a closer genetic relationship with *Ompok hypophthalmus* (MK473377, MK473378, and MK473379) in clade B compared to other *Ompok* groups. This was confirmed by quite a significant bootstrap value (100/98) and posterior probability (1) and the mean of genetic distance between clade A dan clade B was 3.6%. A previous study by Arisuryanti et al. (2020a) described that selais fish based on the *16S* mitochondrial gene were closer to genus *Kryptopterus* than genus *Ompok* with significant differences in genetic distance of 44.1% and 65.8%, respectively. This discovery has confirmed that the ambiguous name and dispels doubts of selais fish previously were still unclassified accurately.

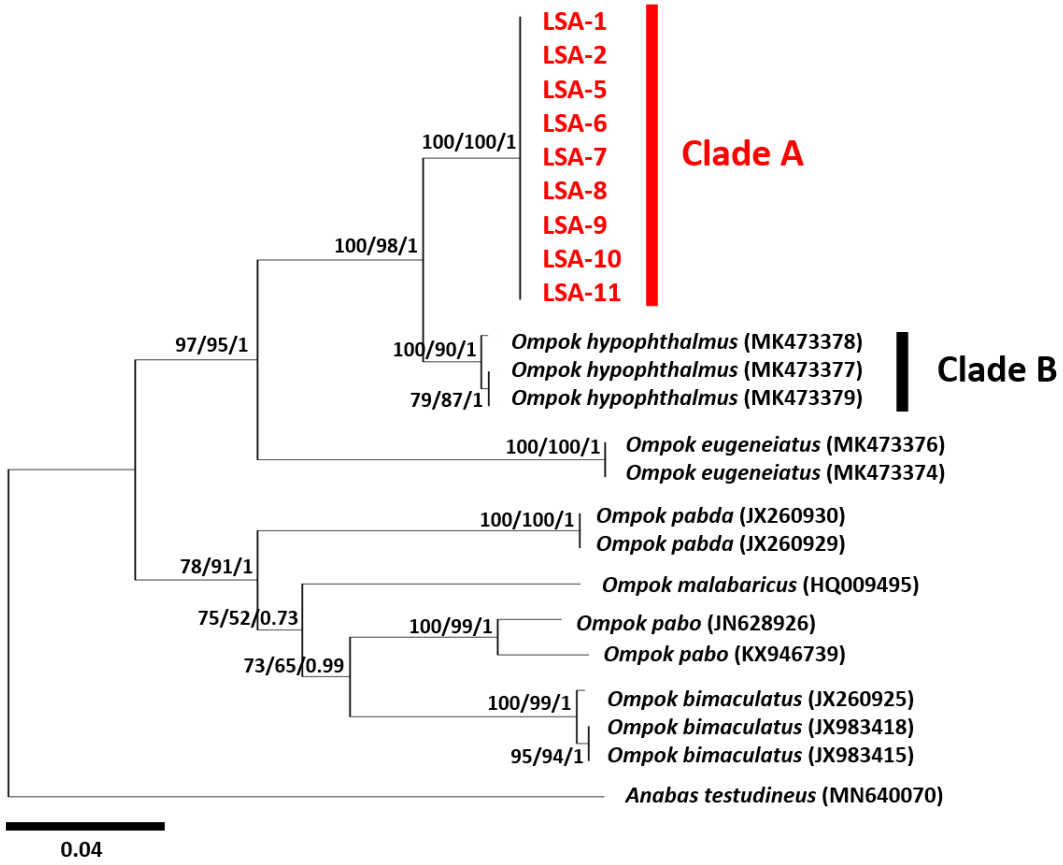


**Figure 4.** Phylogenetic tree reconstruction using three different statistics (NJ / ML / BI) based on *COI* mitochondrial gene. The tree was only displayed in the NJ approach. The number on nodes represented bootstrap (NJ/ML)/ posterior probability (BI).

For more detail, the intra-species analysis between selais fish and *Ompok hypophthalmus* (MK473377, MK473378, and MK473379) resulted in 633 bp-alignment sequences. Each nucleotide composition was presented in Table 1. There were no significant divergences in nucleotide composition between LSA* and *Ompok hypophthalmus* from the GenBank database. Total composition divergence of nucleotide LSA* was relatively similar from the 12 individuals with T=0%-0.47%, C= 0%-0.63%, A= 0%-0.32%, and G = 0%-0.47%. The AT composition for all samples was greater than the CG content but with the same difference of 0.79%.

The genetic distance for each sample was expressed in Table 2. The intra-species genetic distance ranged from 0% to 3.6%. The highest value was obtained between LSA* and MK473377, MK473378, and MK473379. The lowest percentage of genetic distance was between MK473377 and MK473379. Zemlak et al. (2009) stated that the species was still categorized as one species if the intra-species genetic distance threshold value was 3.5%. Meanwhile, the genetic distance between selais fish in this study and *Ompok hypophthalmus* from Indragiri River, Riau was 3.6%. However, samples in this study were still classified as *Ompok hypophthalmus* due to high similarity from BOLD database 97.19%, which means the genetic distance was still <3.5%. However, the mitochondrial *CO*I gene sequence has been in private data, which means that the *COI* sequence of *Ompok hypophthalmus* has been registered in BOLD but has not been released to the public (Table 4). Haplotype grouping between selais fish from Arut River and *Ompok hypophthalmus* from GenBank database was presented in Table 3.

**Table 1.** Percentage of nucleotide composition (%) based on *COI* mitochondrial gene between selais fish and *Ompok hypophthalmus* from GenBank database (*COI* fragment length = 633 bp).

| Accession Number | T(U) | C | A | G | A+T | C+G |
|---|---|---|---|---|---|---|
| LSA* | 26,57 | 30,66 | 24,69 | 18,08 | 51,26 | 41,08 |
| MK473377 | 26.10 | 31,29 | 24,37 | 18,24 | 50,47 | 49,53 |
| MK473378 | 26.10 | 31,29 | 24,37 | 18,24 | 50,47 | 49,53 |
| MK473379 | 26.10 | 31,29 | 24,37 | 18,24 | 50,47 | 49,53 |

*Mean

**Table 2.** Percentage of intra-species genetic distance (%) based on *COI* mitochondrial gene between Selais fish and *Ompok hypophthalmus* from GenBank database.

| Accession Number | LSA* | MK473377 | MK473378 | MK473379 |
|---|---|---|---|---|
| LSA* | | | | |
| MK473377 | 3.6 | | | |
| MK473378 | 3.6 | 0.3 | | |
| MK473379 | 3.6 | 0 | 0.3 | |

*Mean

**Table 3.** Haplotype grouping based on *COI* mitochondrial gene.

| Clade | Haplotype | Total individuals | Sample Code | Location |
|---|---|---|---|---|
| A | HapA | 9 | LSA-1 | This Study |
| | | | LSA-2 | This Study |
| | | | LSA-5 | This Study |
| | | | LSA-6 | This Study |
| | | | LSA-7 | This Study |
| | | | LSA-8 | This Study |
| | | | LSA-9 | This Study |
| | | | LSA-10 | This Study |
| | | | LSA-11 | This Study |
| B | HapB1 | 2 | MK473377 | Indragiri River |
| | | | MK473379 | Indragiri River |
| | HapB2 | 1 | MK473378 | Indragiri River |

The intra-species polymorphism sites among haplotypes were shown in Table 4. Of three haplotypes, there were 23 variable nucleotide sites (3.61%) with 22 informative parsimony sites (3.46%) and a singleton site (0.16%). Nucleotide diversity ($\pi$) and haplotype diversity (Hd) were $0.0142 \pm 0.00468$ and $0.439 \pm 0.158$ (Hd value $0<0.5$ low haplotype diversity and Hd $>0.5\leq1$ high haplotype diversity), consecutively. This indicates $\pi$ and Hd were low indexes. It is assumed that the samples in this study have a small population. The nucleotide divergence models in this sequence were fully substitutions with 22 sites (3.46%) represented transition and transversion was only one site (0.16%). Almost all base divergences were in the third position (20 sites / 3.14%), followed by the first codon position (3 sites / 0.47%), and no nucleotide divergences in the second codon position (0 sites / 0%). In addition, one nonsynonymous (from isoleucine to valine) was detected in the 145th codon.

The inter-haplotype mutation model was designed in Figure 5. Based on Figure 5, a very clear separation was depicted between haplotype A (HapA) and haplotype B ($HapB_1$ and $HapB_2$) due to many substitutions of the nucleotide arrangement. Between HapA and $HapB_1$ also HapA and $HapB_2$, there were 22point mutations. Intra-haplogroup B ($HapB_1$ and $HapB_2$) was inserted with only 2 mutation points. In general, the presence of intra-

**Table 4.** Intra-species polymorphism sites based on *COI* mitochondrial gene among haplotypes.

| Haplotype | Nucleotide Position | | | | | | | | | | | | | | | | | | | | | | | Codon Position | Total Sample (N) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 6 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 4 | 4 | 4 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 | 145 | |
| | 0 | 1 | 9 | 7 | 3 | 7 | 8 | 1 | 3 | 3 | 1 | 3 | 7 | 9 | 3 | 4 | 3 | 5 | 8 | 7 | 1 | 5 | 0 | | |
| HapA | T | T | T | G | C | G | A | A | A | C | T | A | T | A | G | C | T | A | A | C | G | T | G | I | **9** |
| $HapB_1$ | C | C | C | A | . | A | G | G | G | T | C | G | C | G | A | T | C | G | T | T | A | C | A | V | **2** |
| $HapB_2$ | C | C | C | A | T | A | G | G | G | . | C | G | C | G | A | T | C | G | T | T | A | C | A | V | **1** |

species genetic variation between selais fish from Arut River and *Ompok hypophthalmus* (3 haplotypes) was simplified in the Principal Coordinate Analysis (PCA) pattern in Figure 6.

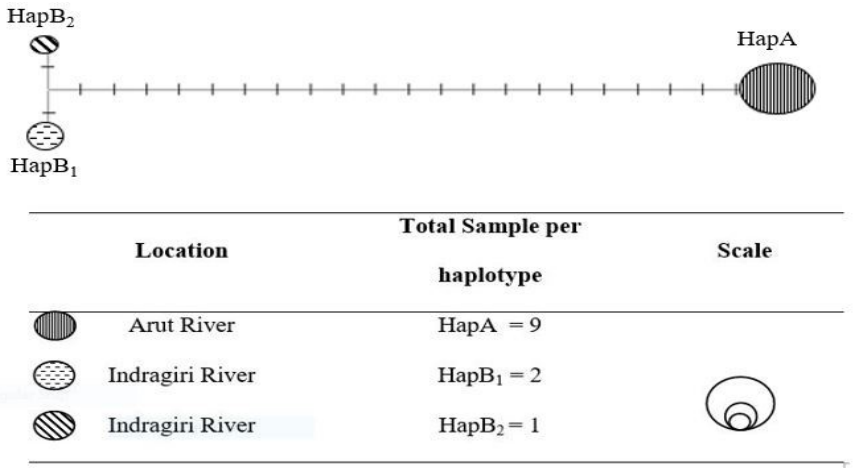


**Figure 5.** Median joining-haplotype network based on *COI* mitochondrial gene among haplotypes.

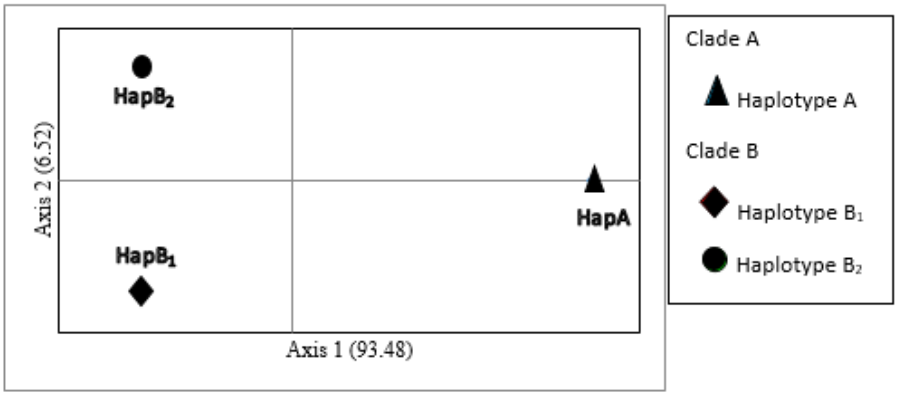


**Figure 6**. Principal Coordinates Analysis (PCA) based on *COI* mitochondrial gene among haplotypes.

## CONCLUSION

By way of conclusion, DNA barcoding using the partial *COI* mitochondrial gene was quite effective and acceptable for molecular identification, especially for morphologically indistinguishable species. Based on the phylogenetic tree construction (NJ/ML/BI), selais fish from Arut River had been confirmed as one single taxa *Ompok hypophthalmus* supported by a genetic distance value of 3.6%. The results of this study are also expected to be used as an entry point for the formulation of sustainable fisheries management and conservation strategies considering that the enormous potential of this important fish can provide maximum benefits in a sustainable manner if managed properly and responsibly.

## AUTHORS CONTRIBUTION

T.K. collected and analyzed the data and wrote the manuscript. T.A. designed the research and supervised all the processes.

## ACKNOWLEDGMENTS

We profoundly thanked the Head of the Laboratory of Genetics and Breeding, Faculty of Biology, Universitas Gadjah Mada who gave permission and facilitated for research. We also sincerely thanked to Field and Laboratory Work Team who had contributed during this study. This research has been supported by RTA-UGM 2020 funding with contract No. 2488/UN1.P.III/DIT-LIT/PT/2020

## CONFLICT OF INTEREST

The authors state that there is no conflict of interest. They were fully responsible for the writing of the manuscript.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66953
DOI: 10.22146/jtbb.66953

Research Article

# Diversity, Abundance, and Traditional Uses of Asteraceae Species in Mount Bisma, Dieng Plateau, Kejajar, Wonosobo, Central Java

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**Keywords:**
abundance
Asteraceae
Dieng Plateau
ethnobotany
species diversity
**Submitted:**
23 June 2021
**Accepted:**
24 September 2021
**Published:**
09 February 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT
Asteraceae is the largest and most diverse family of flowering plants which contains more than 20,000 species distributed in nearly all types of habitats all over the world. In mountainous regions such as Mount Bisma, it is estimated to have plenty and diverse member of the Asteraceae family, and used in the local community for various uses. This research aimed to understand the diversity, abundance, and uses of Asteraceae members that are found wild in Mount Bisma. Taxonomy and ecological data were gathered using an exploration method and purposive sampling method, from the point, a plot measured 3x3 m$^2$ was created to estimate the vegetation parameters in the mountain top and mountain valley area. Ethnobotanical data were gathered in Sikunang Village, a nearby village of Mount Bisma using a semi-structured interview and open-ended questions. Data were analysed descriptively and quantitatively using several indices such as Importance Value Index (IVI), Index of Cultural Significance (ICS), and index of Use Value (UV). The result showed that there were 18 species from two subfamilies that grew wild in both mountain top and valley of Mount Bisma. The highest importance value belonged to *Ageratina riparia,* which was scored in the mountain top and valley 71.00 and 91.53, respectively. Uses of Asteraceae in Sikunang were varies, ranging from being a side dish, medicine, firewood, souvenir, and other uses. *Galinsoga parviflora* and *Galinsoga quadriradiata* showed the highest ICS value of 41, whereas *Austroeupatorium inulifolium* scored the highest in UV of 1.8. The study presented high number of Asteraceae diversity and use. Thus, implies that Mount Bisma has vast unexplored biodiversity and locals around Mount Bisma have rich traditional knowledge.



## INTRODUCTION
Asteraceae Bercht. & J.Presl (1820); synonym Compositae Giseke (1763), *nom. cons.* is the largest and most diverse family of Angiospermae, composed of ca. 24,000 species from about 1,700 genera that are distributed in nearly all types of habitats all over the world except Antarctica, composing about 10% of recorded diversity of flowering plants (Funk et al. 2009; Tadesse 2014). Member of Asteraceae are undoubtedly widespread due to its highly adaptive characteristics and the specific feature of their flower; a cluster of numerous

tiny flowers (florets) called capitula attractive enough to attract a pollinator, self-pollinating mechanisms of the floret, specific type of fruit called achene that is light enough to be dispersed by wind, supported by the presence of pappus (Bhattacharyya 2016; Purnomo et al. 2016).

A high number of Asteraceae species was considered not proportional with the number of beneficial species from the family. Smaller families, e.g. Poaceae or Fabaceae contain more useful plants than Asteraceae (Funk et al. 2009). In general, members of Asteraceae already used for food (*Lactuca sativa* L., *Helianthus tuberosus* L.), medicine (*Artemisia absinthium* L., *Taraxacum officinale* Wigg.), insecticides (*Tanacetum cinerariifolium* (Trev.) Sch. Bip.), and ornamentals (*Chrysanthemum indicum* L.) (Simpson 2009). A high number of Asteraceae species presents in previous studies are generally acknowledged as a weed. The surrounding community managed to mow the weed because it disturbs the growth of cultivated plants. On the other hand, the surrounding community also finds benefits of Asteraceae species, such as food and medicine. Generally speaking, Asteraceae species are only known to be secondary material as food or medicine, not as the first choice. On the other hand, members of Asteraceae are also recognized as invasive in Indonesia, e.g. *Ageratum conyzoides* L., *Galinsoga parviflora* Cav., *Mikania micrantha* Kunth., and *Sonchus oleraceus* L. (Setyawati et al. 2015) and could affect the survival of native species. Traditional uses of Asteraceae in the local community in unexplored locations need to be researched in order to enrich knowledge on its uses.

Mount Bisma located in Dieng Plateau, Wonosobo, Central Java with a peak reached 2,365 masl, is a volcano, but the volcanic activity is decreased and leaving a wide caldera (Nurpratama et al. 2015). Recently, Mount Bisma is known to be located outside the potential geothermal energy sectors (Harijoko et al. 2016). Mount Bisma was not open for tourist attraction until 2019, thus the mountain has preserved the ecosystem of mountainous region biodiversity. Local communities on Mount Bisma, especially in Sikunang Village, are Javanese, male and female residents mostly work as a farmer, with potato and varieties of vegetables being main crops. Biodiversity and local knowledge of plants in Mount Bisma has not been reported before, especially for the Asteraceae family. Studies on Asteraceae were conducted by Sunarto et al. (2017) in Mount Lawu, Megawati et al. (2017) in Lore Lindu National Park, and Al Farishy & Salamah (2021) in Mount Halimun-Salak. All of the studies conducted before provided general information on Asteraceae diversity in mountainous regions. With the largest number of species, it is highly potential to find any of the beneficial species of Asteraceae. Wonosobo itself offers attractive natural tourism, with edelweiss, *Anaphalis longifolia*, being one of the Asteraceae species that is most attractive for tourists. Local archival research on ethnobotany around Mount Bisma is spoken between generations. There is no known archive of ethnobotany in communities around Mount Bisma. In addition, there is also no known research conducted in the surrounding communities as well. The research aimed to explore

the diversity, abundance, and traditional uses/knowledge about Asteraceae species in Mount Bisma. The result hopefully could present the newest record on the diversity, abundance, and local knowledge of Asteraceae for further research in the future.

## MATERIALS AND METHODS

### Materials

The materials used in this research were plant specimens collected from two research areas of Mount Bisma; Mountain Top (MT) and Mountain Valley (MV), 300 gr/m$^2$ acid-free mounting paper sized A3, alcohol 70%, ziplock plastic bag sized 40x30 cm, herbarium envelope, label, document-grade scotch tape, and PVA glue 90%. The tools used in this research were stationary, camera, board, cardboard, voice recorder, and GPS mobile.

### Methods

#### Study area and period

The research was carried out in Mount Bisma, administratively located in Sikunang Village, Kejajar, Wonosobo. The village is located at 7$^0$14'06" S and 109$^0$53'54" E, inhabited by Javanese people, who mainly talk Javanese language with a Banyumasan accent. Mostly, locals are moslem and work as a farmer. Field observation was carried out into two designated areas; Mountain Top (2,365.44 masl) and Mountain Valley (1,599.21 masl). The plot was carried out at two different heights because altitude influences the environment around the plants, limits the growth of certain species and their distribution. Mountain Top following the hiking trails, Mountain Valley started at the intersection between the local's farming area and forest of the valley. Map of the study area presented in Figure 1, red line for MT and blue line for MV. The research was conducted in February 2021 and March 2021.

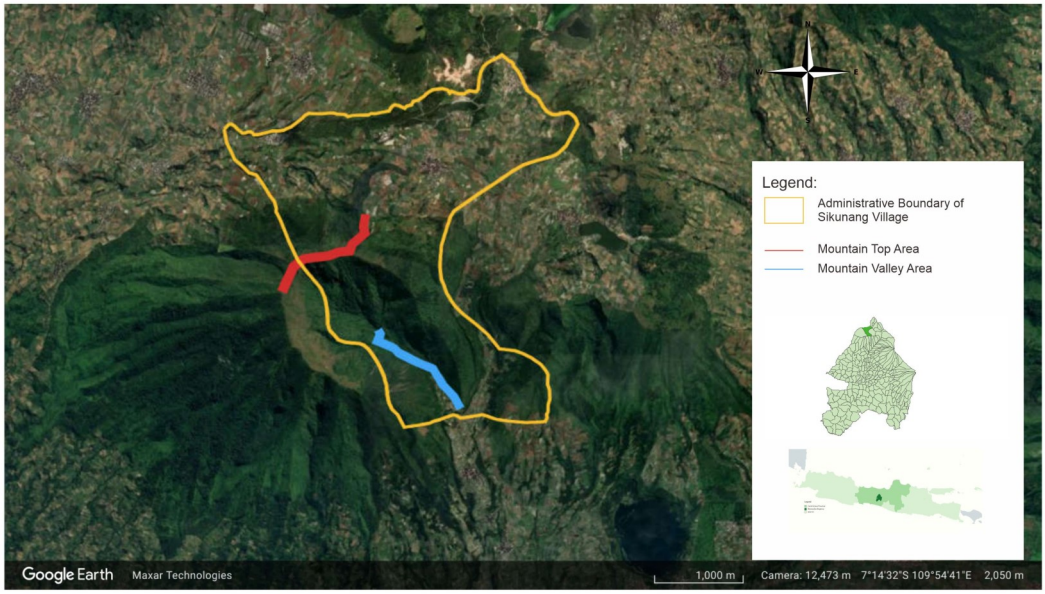


**Figure 1**. Map of study area showing Sikunang Village administrative boundary (yellow line), Kejajar, Wonosobo, Central Java. Source: Google Earth, 2021.

### Field observation and species identification

Field observation aimed to gather the taxonomy and ecological data. The exploration method was used to gather taxonomical data (Rugayah et al. 2004). Purposive sampling was used to find the location that represents Asteraceae diversity following the designated area, then from each point, a 3x3 $m^2$ plot was made to collect vegetational data, such as a number of species and frequency (Partomihardjo & Rahajoe 2004). A total of 9 plots and 12 plots were made in Mountain Top and Mountain Valley, respectively. Plant samples for each species were taken to make voucher specimens for further identification. The data then were brought to Laboratory of Plant Systematics, Faculty of Biology UGM for voucher specimen construction (Sardiwinata et al. 2008); identification was based on determination key from Flora of Java (Backer & Brink 1965), description and illustration from Mountain Flora of Java (van Steenis 2006).

### Interview

The village community survey was used to gather the informants, who are the residents of Sikunang Village, purposive snowball sampling was chosen to found informants with the village chief as a key informant (Silalahi 2016). The respondents are adults (more than 18 years old) from various professions. A total of 15 respondents, mostly by the suggestion of the Village Chief, were interviewed. Fifteen respondents are considered accurate because all of them were chosen by recommendation of a key informant so that all the informants know very well about plants around the village. A semi-structured interview with open-ended questions was performed to gather information from informants (Walujo 2004). The interview was equipped with pictures of Asteraceae species found in Mount Bisma.

### Data analysis

Asteraceae species were grouped by taxa, scientific names were rechecked based on Classification of Compositae (Funk et al. 2009) and Global Compositae Database (CWG 2021). The characteristics of invasiveness were determined based on the Guide to Invasive Plant Species in Indonesia (Setyawati et al. 2015). Ecological data in the form of relative density and relative frequency were further analysed by Importance Value Index (IVI) analysis (Barbour et al. 1987). The interview data were analysed to estimate the valuation of the index of cultural significance respective to the categorization of uses (ICS) (Turner 1988), along with the Use Value (UV) analysis (Walujo 2004; Cotton 1996) as general quantification on ethnobotanical data to represent how beneficial a species is for a community.

## RESULTS AND DISCUSSION

### Asteraceae Diversity

Study of species diversity on Asteraceae family in hiking trails (mountain top) and mountain valley of Mount Bisma was carried out. Based on Funk et al.

(2009), a total of 18 species under 16 genera, eight tribes, and two subfamilies were collected and identified. All species were considered wild. Based on the data obtained, out of the 18 species found in the research location, 17% were recorded only in Mountain Top (MT); 55% were recorded to be present in both locations (MT and MV); and 28% were recorded only in Mountain Valley (MV) (Table 1). The species found only in Mountain Top; *Anaphalis longifolia, Erigeron karvinkianus,* and *Leucanthemum vulgare* were known to inhabit slope ground and unshaded areas. *A. longifolia* was known to be typical to grow in the mountain region of Java (van Steenis 2006), the species also found in Mount Lawu and attract tourism activity (Sunarto et al. 2017).

**Table 1**. Diversity of Asteraceae in Mount Bisma.

| No. | Species | Subfamily | Tribe | Location Found | Specimen Voucher Number |
|---|---|---|---|---|---|
| 1 | *Artemisia vulgaris* L. | Asteroideae | Anthemideae | MV, MT | 13/BA/1, 30/BB/10, 64/BB/8, 68/BB/11 |
| 2 | *Leucanthemum vulgare* Lam. | Asteroideae | Anthemideae | MT | 41/BA/3 |
| 3 | *Dichrocephala integrifolia* (L.f.) Kuntze | Asteroideae | Astereae | MV, MT | 02/BA/1, 52/BA/1, 69/BB/11 |
| 4 | *Erigeron karvinskianus* DC. | Asteroideae | Astereae | MT | 14/BA/9, 15/BA/8, 46/BA/7 |
| 5 | *Erigeron sumatrensis* Retz. | Asteroideae | Astereae | MV, MT | 01/BA/1, 47/BA/2, 67/BB/10 |
| 6 | *Bidens pilosa* L. | Asteroideae | Coreopsideae | MV, MT | 04/BA/1, 09/BA/9, 10/BA/1, 12/BA/5, 33/BB/9, 36/BB/11, 42/BA/2, 62/BB/7 |
| 7 | *Ageratum conyzoides* L. | Asteroideae | Eupatorieae | MV | 73/BB/12 |
| 8 | *Ageratina riparia* (Reg.) R.M.King & H.Rob | Asteroideae | Eupatorieae | MV, MT | 05/BA/1, 07/BA/9, 08/BA/9, 16/BA/9, 24/BB/3, 25/BB/1, 26/BB/1, 39/BB/3, 48/BA/1, 53/BA/9, 54/BA/8, 57/BB/1, 58/BB/4, 59/BB/5, 60/BB/3 |
| 9 | *Austroeupatorium inulifolium* (Kunth.) R.M.King & H.Rob. | Asteroideae | Eupatorieae | MV, MT | 03/BA/7, 45/BA/2, 55/BB/1, 56/BB/2, 61/BB/6 |
| 10 | *Anaphalis longifolia* DC. | Asteroideae | Gnaphalieae | MT | 06/BA/5, 18/BA/9, 21/BA/9, 49/BA/13 |
| 11 | *Gnaphalium purpureum* L. | Asteroideae | Gnaphalieae | MV, MT | 37/BB/10, 51/BA/1 |
| 12 | *Acmella paniculata* (Wall ex. DC.) R.K.Jensen | Asteroideae | Heliantheae | MV | 32/BB/10, 63/BB/9 |
| 13 | *Tithonia diversifolia* (Hemsl.) A.Gray | Asteroideae | Heliantheae | MV | 40/BB/12 |
| 14 | *Galinsoga parviflora* Cav. | Asteroideae | Millerieae | MV, MT | 11/BA/1, 27/BB/9, 28/BB/9, 29/BB/10, 31/BB/10, 34/BB/10, 35/BB/11 |
| 15 | *Galinsoga quadriradiata* Ruiz. & Pav. | Asteroideae | Millerieae | MV | 65/BB/11 |

**Table 1**. Contd.

| No. | Species | Subfamily | Tribe | Location Found | Specimen Voucher Number |
|---|---|---|---|---|---|
| 16 | *Crassocephalum crepidioides* (Benth.) S. Moore | Asteroideae | Senecioneae | MV, MT | 19/BA/1, 22/BA/1, 38/BB/11, 71/BB/11 |
| 17 | *Sonchus oleraceus* L. | Cichorioideae | Cichorieae | MV, MT | 43/BA/3, 44/BA/4, 50/BA/1, 66/BB/10, 70/BB/12 |
| 18 | *Youngia japonica* (L.) DC. | Cichorioideae | Cichorieae | MV | 72/BB/12 |

Two subfamilies found wild in Mount Bisma were Asteroideae and Cichorioideae. There are informal categories in the Asteroideae subfamily, the Heliantheae allies. From the subfamilies, it was recognized that seven tribes were classified into Asteroideae (four tribes included in Heliantheae allies) and one tribe classified into Cichorioideae. The diversity of Asteraceae species found in Mount Bisma were similar to other research done in mountainous regions of Sulawesi and Mount Lawu (Megawati et al. 2017; Sunarto et al. 2017). Asteroideae leads in the number of species found because Asteroideae is the largest and most diverse subfamily of Asteraceae (Funk et al. 2009). Most species found in the research are considered invasive alien species (IAS), about 13 out of 18 are considered as IAS, four species known to be alien species, and only one species is recognized distributed naturally in Java. The majority of IAS and alien species are originated in New World (Tropical, North, or South America), the rest of them originated from Old World: *C. crepidioides* and *B. pilosa* (Africa); *A. vulgaris, L. vulgare, S. oleraceus* (Europe and Russia Far East); and two species originated in Asia: *D. integrifolia* and *Y. japonica* (Setyawati et al. 2015). The diversity of flower morphology of wild Asteraceae species found in Mount Bisma is presented in Figure 2.

## Asteraceae Abundance

The abundance of Asteraceae species found was determined by the value of the Importance Value Index (IVI). The highest IVI in both locations, MT and MV was obtained from species of *Ageratina riparia* with IVI 71.00% and 91.53%, respectively. The result showed that *A. riparia* was dominated the area with the most number of individuals and presented in the most study plot. *A. riparia* covered most of the forest floor in MT and MV. Figure 3 and figure 4 showed the IVI for each species found in MT and MV where the IVI does not distribute equally, two species (*A. riparia* and *Austroeupatorium inulifolium*) have prominent IVI compared to other species; statistically signs that the coverage of both species are considered high, meanwhile, the other species showed low IVI compared to the dominating species. *A. riparia* was recognized to be invasive alien species and dominate the forest floor of several mountainous areas of Java; the IVI scored 69.418% and 81.35% in Mount Lawu and Dieng Plateau (Mount Alang and Mount Klaras), respectively (Setyawati et al. 2015; Sunarto et al. 2017; Abdiyani 2008).


**Figure 2**. Species of Asteraceae found wild in Mount Bisma. A – H. Asteroideae (without Heliantheae alliance): Gnaphalieae: *Anaphalis longifolia, Gnaphalium purpureum*; Senecioneae: *Crassocephalum crepidioides*; Astereae: *Dichrocephala integrifolia, Erigeron karvinskianus, Erigeron sumatrensis*; Anthemideae: *Leucanthemum vulgare, Artemisia vulgaris.* I – P: Asteroideae (Heliantheae alliance): Coreopsideae: *Bidens pilosa*; Eupatorieae: *Ageratum conyzoides, Ageratina riparia, Austroeupatorium inulifolium*; Millerieae: *Galinsoga parviflora, Galinsoga quadriradiata*; Heliantheae: *Acmella paniculata, Tithonia diversifolia.* Q – R: Cichorioideae: Cichorieae: *Sonchus oleraceus*, *Youngia japonica.* Source: Personal Documentation, 2021.

The diversity and distribution at different altitudes are relatively different. In Mountain Valley, the area with lower altitude, the species richness counted was more than that of Mountain Top (14 species in MV, 12 species in MT). There is one species that is dominant in both locations, *Ageratina riparia*, IVI of MV is higher (91.53%) than in MT (71.00%), so the *A. riparia* is considered more dominant in MV than in MT. Even though had more number species, the dominance of *A. riparia* compared to the other species in MV made MV had lower diversity and distribution, which showed that in MT (higher altitude) it is considered more diverse and the species are distributed more evenly. Asteraceae found in higher altitudes, such as *Anaphalis longifolia* and *Erigeron sumatrensis* are observed to have special adaptation in terms of its morphological character, i.e. adapted to grow well in cliff/ sloping ground; have narrow leaves, drier stem, and strongly attached to the ground. In lower altitudes, the species generally have a wider and thinner leaf, grows perpendicular to the substrate, and wetter stem.

*Ageratina riparia* was known to be common in Java mountainous region (Purnomo et al. 2016). The invasiveness of *A. riparia* was categorized as high risk, *A. riparia* even had the highest Risk Index in Mount Papandayan. *A. riparia* has an uncommon feature of Asteraceae, that is the species could grow well in shaded areas. Coverage of *A. riparia* was very wide due to the fast-growing characteristics of the plant, even it is considered very fast compared to other invasive species. On the other hand, the species is also a productive

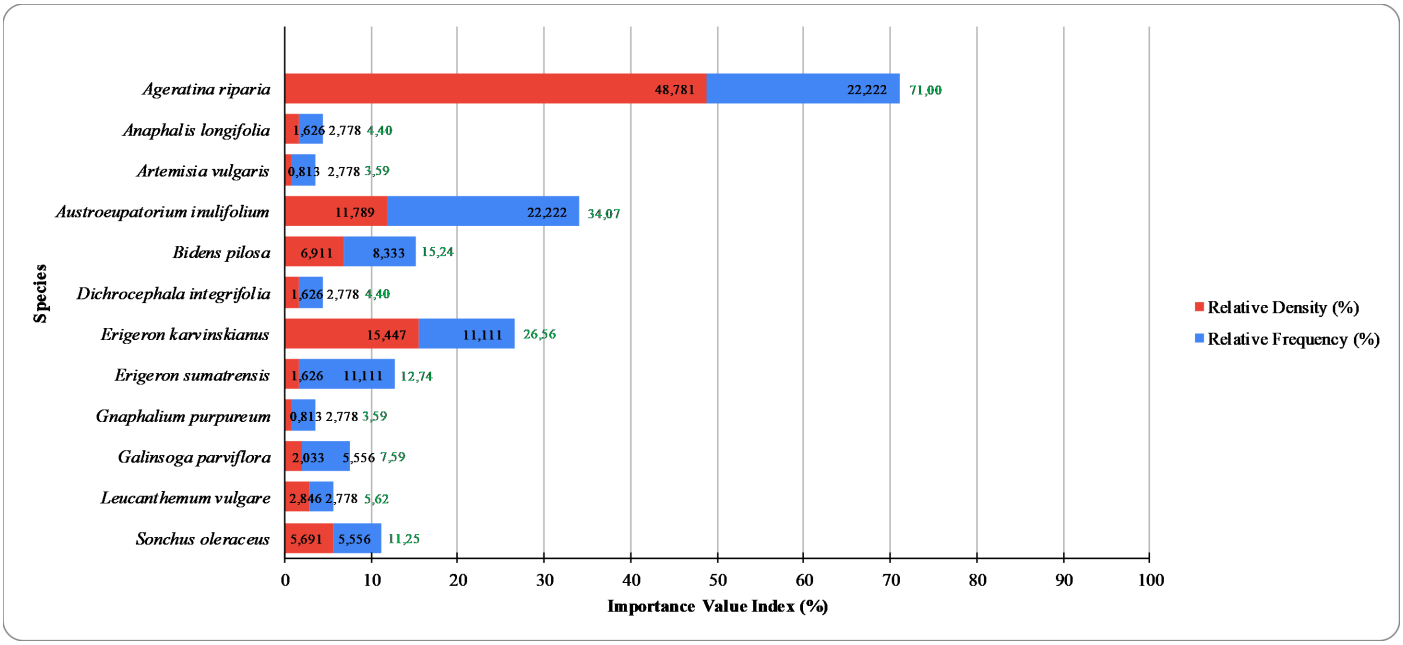


**Figure 3**. Importance Value Index (IVI) from Mountain Top (MT) Area. Number showed in green symbolized the summation of Relative Density and Relative Frequency (Importance Value Index).

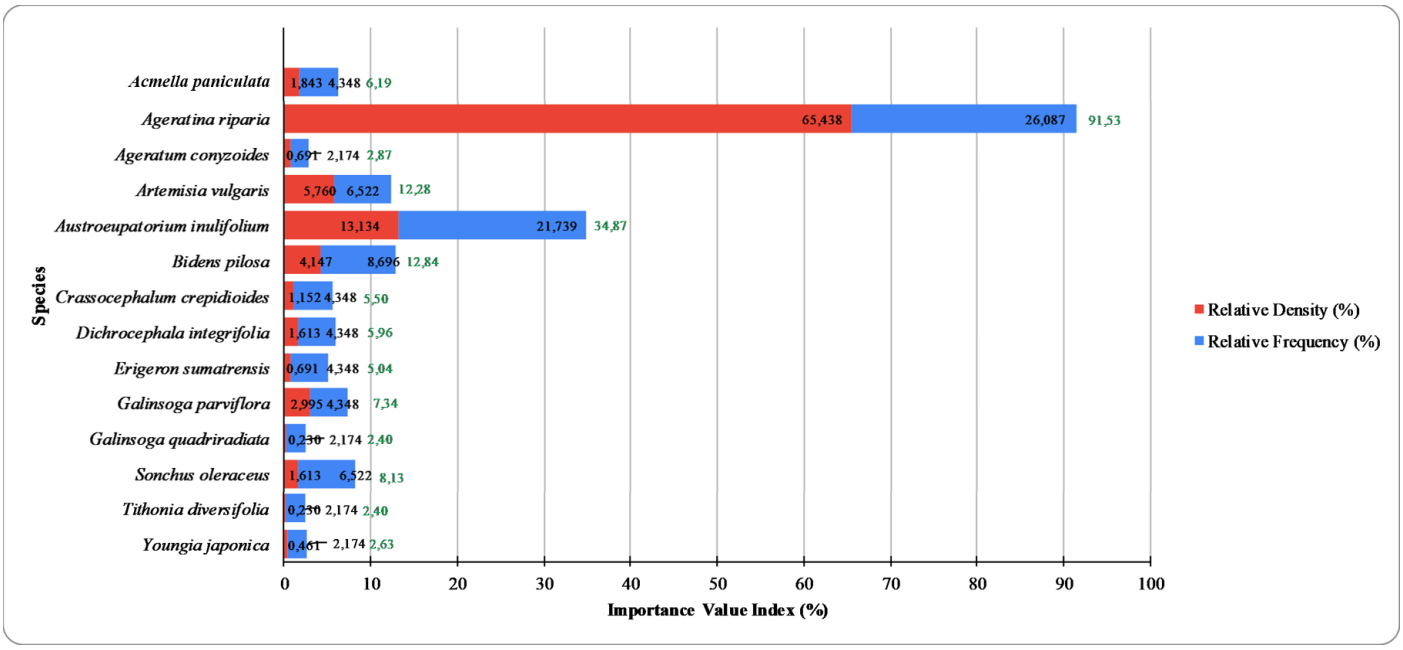


**Figure 4**. Importance Value Index (IVI) from Mountain Valley (MV) Area. Number showed in green symbolized the summation of Relative Density and Relative Frequency (Importance Value Index).

seeder (Nyuanti et al. 2020). A combination of the characteristics leads the species to invade an area, growing rapidly and dense to dominate the area so that the other species do not have enough space and resources to grow adequately.

## Asteraceae Uses

Locals of Sikunang Village, a nearby village of Mount Bisma were interviewed and the result showed that locals had vast knowledge of plant uses in their household; the uses from 17 out of 18 species of Asteraceae found were recognized by the locals. Locals in Sikunang Village perceive the

majority of Asteraceae species found as weed, disturbed their field. Several people also recognized species that grow in the upper part of Mount Bisma, inside the forest, and far from their field. However, locals not only mentioned the species as weeds. Local uses of the plant as told by the people and the valuation of ICS and UV are presented in Table 2.

Locals mentioned that several species could be utilized for consumption, medicine, daily needs, livestock forage, to be sold, etc. Even though locals perceive Asteraceae members in general as weeds, the utilization is a sign that locals have a well-understanding in plant uses. Locals in Sikunang Village maintain vegetation amid their mountainous environment, especially in Asteraceae members, make it possible for them to explore any utilization of plants. Plants mentioned used by locals have an opportunity as bioprospection in the future, because the plants are abundant and the benefits are varied, e.g. *G. parviflora* and *G. quadriradiata* as delicacies, *C. crepidioides* as cosmetics, *L. vulgare* as insecticides. Other bioprospection of Asteraceae members mentioned by locals is *S. oleraceus* (*gembos*) as rabbit forage. Some of the locals mentioned that rabbit which fed using a mixture of grass, rabbit pellets, and *gembos* produced better urine quality than rabbit which fed without mixture of *gembos*, that is highly beneficial as biofertilizer.

The quantification of ICS was based on the quality, intensity, and exclusivity of plant's uses (Turner 1988). The highest score of ICS was obtained by *mondrengan* or *G. parviflora* and *G. quadriradiata* with a score reached 41, that most locals told that the plant could be eaten just raw or cooked. *Sendura* (*A. longifolia*) scored second in ICS (36) because of the valuation of the flower, a rare plant with high demand in tourism attraction. *Gembos* (*S. oleraceus*) scored third highest of ICS (30) because of their utilization as food, high quality rabbit forage, even some medicinal properties. Meanwhile, *malenggo* (*A.inulifolium*) scored fourth highest of ICS (24) because it serves the daily needs of locals as easy-accessed firewood, livestock forage, and economic-importance fragrant flower. On the other hand, UV represents various types of uses known by the locals (Walujo 2004). *Malenggo* (*A. inulifolium*), *mondrengan* (*G. parviflora* and *G. quadriradiata*), *sendura* (*A. longifolia*), and *lengko* (*C. crepidioides*) relatively had high scores compared to other species found in Mount Bisma because various uses are recognized by most locals, meanwhile, the species which had a low score in UV means that the utilization is not as much as the other species or the utilization varies, but only known to the minority of locals (Cotton 1996). As mentioned in the beginning, most species are known as weeds and disturbing cultivated plants, therefore if locals do not utilize the plant for a specific activity, commonly seen plants will be used as livestock forage, e.g. *D. integrifolia* and *E. sumatrensis*. *G. purpureum* is an uncommon plant for locals; locals do not recognize the plant and therefore the utilization is unclear for locals. High UV or ICS value suggests that the species is more beneficial than other species. If the species is beneficial enough to be demanded by more people, the species tend to be cultivated by people, then it will preserve in

**Table 2**. Local name, traditional uses, ICS, and UV for each species of Asteraceae species found in Mount Bisma.

| No. | Species | Local Name | Traditional Uses | ICS* | UV** |
|---|---|---|---|---|---|
| 1 | *Galinsoga parviflora* Cav. | Mondrengan, Jarinten, Jangkungan | Young leaves eaten raw as "*lalapan"* or cooked to be *sayur bobor, sayur bening, oseng,* or *pecel*. Older leaves as rabbit forage. | 41 | 1.53 |
| 2 | *Galinsoga quadriradiata* Rez. & Pav. | | | | |
| 3 | *Anaphalis longifolia* DC. | Sendura | Flower kept as room freshner, dried as ornamental, insect repellent, or sold in nearby tourist attraction. | 36 | 1.40 |
| 4 | *Sonchus oleraceus* L. | Gembos | Young leaves having medicinal properties to cure hepatitis and other liver diseases, facilitating breast milk. Older leaves used as forage of rabbit. | 30 | 1.27 |
| 5 | *Austroeupatorium inulifolium* (Kunth.) R.X.King & H.Rob. | Malenggo, Maregol, Sembung, Krenyo, Maitan, Rikowot, Tembulungan, Wedangan | The wood used as firewood; leaves as forage for goat; fragrant flower as room freshner and mixed with sendura to sold in nearby tourist attraction. | 24 | 1.80 |
| 6 | *Crassocephalum crepidioides* (Benth.) S.Moore | Lengko, Cangklong, Menjangan | Young leaves eaten, having medicinal properties such as skin diseases, ulcer, and face mask; older leaves as forage of rabbit and guinea pig; mix of leaf and root as soporific or cure of vitamin B deficiency; flower as children's toy. | 18.5 | 1.40 |
| 7 | *Leucanthemum vulgare* Lam. | Jenu, Jenung, Pitrem | Flower used as mosquito repellant, tonic, poultice for sore, or used as ornamentals; root and stem used to strengthen soil, prevent weeds growing, as fish poison or insecticide. | 17.5 | 0.80 |
| 8 | *Ageratum conyzoides* L. | Bandotan, Rema, Seprah muda, Entut-entutan | Young leaves eaten (cooked, not usual), or used as forage of goat, leaves as poultice for wound or slices; stem latex as substitute of eucalyptus oil, could prevent bleeding. | 17 | 0.80 |
| 9 | *Erigeron karvinskianus* DC. | Lonte sore, Pitrem gunung, Jenu hitam, Kembang benik, Otot-ototan | Whole part of the plant used to prevent growth of other weeds or used as ornamentals; leaves and flower as poultice for sore; root used as additional materials in tonic. | 13.5 | 0.73 |
| 10 | *Artemisia vulgaris* L. | Sudamele, Sidamele, Ambril | Leaves used as insecticide and herbicide, root brewed as tonic. | 10.5 | 0.67 |
| 11 | *Acmella paniculata* (Wall. ex. DC.) R.K. Jensen | Suweng-suwengan, Suket jangkung, Bendotan | Flower in the form of earrings for children's toy; leaves as emergency toothpaste. | 7.5 | 0.40 |
| 12 | *Youngia japonica* (L.) DC. | Gembos kuning, Kenikir | Leaves as forage or eaten (not usual); flower as mosquito repellant and ornamentals. | 5.5 | 0.71 |
| 13 | *Ageratina riparia* (Reg.) R.M.King & H.Rob | Lakhar, Suket republik, Repeblik | Leaves used as subtitute of goat forage in dry season; stem and leaves fermented for 15 days as insecticide; root rotted as soil fertilizer. | 3 | 0.53 |
| 14 | *Bidens pilosa* L. | Kenul, Ketul, Ranjau, Trucukan, Puyengan | Leaves used as poultice/therapy of skin diseases (itch); flower as children's toy. | 2.5 | 0.13 |
| 15 | *Dichrocephala integrifolia* (L.f.) Kuntze | Rendeng, Semprang, Semprah | Livestock forage. | 1.5 | 0.07 |
| 16 | *Erigeron sumatrensis* Retz. | Jlantir | Alternative of livestock forage. | 1.5 | 0.20 |
| 17 | *Tithonia diversifolia* (Hemsl.) A. Gray | - | Flower used as poultice of ulcer. | 1.5 | 0.07 |
| 18 | *Gnaphalium purpureum* L. | - | - (no known uses) | 0 | 0.00 |

*ICS: Index of Cultural Significance, **UV: Use Value

the area. In Merapi-Merbabu Slopes, Boyolali there are two Asteraceae species found with relatively low UV; *Tagetes erecta* and *Lactuca sativa* with UV reached 0.1 and 0.07, respectively (Umartani & Nahdi 2021) Other research conducted in Cibodas Biosphere Reserve showed prominent ICS on two Asteraceae species; *Artemisia vulgaris* (ICS: 98.32) and *Bidens pilosa* (ICS: 67.81) (Handayani et al. 2021).

Table 3 showed the known and recorded uses of Asteraceae species found in Mount Bisma by scientific exploration. Traditional knowledge on the uses of each species is found to contribute and enrich the recorded uses of Asteraceae species from studies conducted before. Rich traditional knowledge and scientific exploration should be combined to find the best utilization for the species. On the other hand, in the discussion on Asteraceae diversity, it is known that most species were recognized as alien species. Locals play important role in the population control of alien species by utilizing the plant optimally (Al Farishy & Salamah 2021).

**Table 3**. Recorded uses of Asteraceae species found in Mount Bisma.

| No. | Species | Known Uses |
|---|---|---|
| 1 | *Galinsoga parviflora* Cav. | Young leaves as food, treated as wild vegetables. Contains minerals; older leaves as rabbit forage (Santosa et al. 2020) |
| 2 | *Galinsoga quadriradiata* Rez. & Pav. | |
| 3 | *Anaphalis longifolia* DC. | Flower sold as souvenir (Utomo & Heddy 2019) |
| 4 | *Sonchus oleraceus* L. | Edible (vegetables); treat anemia, liver infection, opium dependency, diurethic, bacteri infection (Jimoh et al. 2011; Setyawati et al. 2015) |
| 5 | *Austroeupatorium inulifolium* (Kunth.) R.X.King & H.Rob. | Phytotoxic, cytotoxic, and anti-fungal activity; leaveas as cure for cough and fever, sore, and regulating fertility (Chandrasiri et al. 2015; Quattrochi 2016) |
| 6 | *Crassocephalum crepidioides* (Benth.) S.Moore | Young leaves edible (vegetables), cure for gastrointestinal problem, wound or slice, prevent bleeding; rabbit forage (Quattrochi 2016; Dairo & Adanlawo 2007) |
| 7 | *Leucanthemum vulgare* Lam. | Ornamentals, mosquito repellent; leaves as food (Clements et al. 2004) |
| 8 | *Ageratum conyzoides* L. | Skin disease and wound cure; slices, burnt, ulcer (Syamsuhidayat & Hutapea 1991) |
| 9 | *Erigeron karvinskianus* DC. | Ornamentals, skin diseases (Quattrochi 2016; Sharmila et al. 2014) |
| 10 | *Artemisia vulgaris* L. | Menstruation cycle, miscarriage, dysentry, nosebleed, intestinal bleeding (Wijayakusuma et al. 1994) |
| 11 | *Acmella paniculata* (Wall. ex. DC.) R.K. Jensen | Emergency toothpaste, stomachaches, cure for fever (Quattrochi 2016; Setyawati et al. 2015) |
| 12 | *Youngia japonica* (L.) DC. | Consumed as food, larvicidal activity (Rojas-Sandoval 2020; (Liu et al. 2015) |
| 13 | *Ageratina riparia* (Reg.) R.M.King & H.Rob | Facilitate urination (Santosa et al. 2017) |
| 14 | *Bidens pilosa* L. | Cure for fever, rheumatic, toothache, throat disease, skin diseases, snake bites (Wijayakusuma et al. 1994; (Setyawati et al. 2015) |
| 15 | *Dichrocephala integrifolia* (L.f.) Kuntze | Analgesic, antibacterial, antiinflammation, cure for fever (Setyawati et al. 2015; Quattrochi 2016) |
| 16 | *Erigeron sumatrensis* Retz. | Wound poultice, headaches, vertigo, TBC, asthma, rheumatic, stomachaches (Silalahi et al. 2019) |
| 17 | *Tithonia diversifolia* (Hemsl.) A. Gray | Wound poultice, malaria, diarrhea, ornamentals, fever cure (Silalahi et al. 2019; Setyawati, et al., 2015) |
| 18 | *Gnaphalium purpureum* L. | unknown |

## CONCLUSION

Research in Mount Bisma discovered a total of 18 species of Asteraceae from two subfamilies and eight tribes, with the most abundant species was *Ageratina riparia,* dominating both the area observed due to its invasive nature. Locals of Sikunang Village recognized various uses of Asteraceae plants, such as consumption, livestock forage, medicine, sold in a tourist attraction, etc. *G. parviflora* and *G. quadriradiata*, known as *mondrengan*, scored the highest in ICS, that is 41. Meanwhile, *malenggo* (*A. inulifolium*), scored the highest in UV, which is 1.80. This study presented new information about Asteraceae species in Mount Bisma, implies that there are vast unexplored biodiversities in Mount Bisma and rich traditional knowledge on plant uses in its surrounding community. It needs further research in order to archive and preserve the biodiversity and the traditional knowledge.

## AUTHORS CONTRIBUTION

B.K. collected and analysed the data and wrote the manuscript, P designed and supervised the research and revised the manuscript, R.S.K. supervised, revised, and finalized the manuscript.

## ACKNOWLEDGMENTS

The author would like to thank Mr. Nur Amin, Chief of Sikunang Village for giving permission to conduct the research in Sikunang Village and local informants of Sikunang Village, Kejajar, Wonosobo for sharing their knowledge on the use of plants by participating in the survey. The manuscript is part of B.K. thesis under supervision of R.S.K.

## CONFLICT OF INTEREST

The authors state that there is no conflict of interest in this research.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66645
DOI: 10.22146/jtbb.66645

Research Article

# Morphological Characterization and Seed Germination Study of Wild Banana *Musa acuminata* var. *flava* (Ridl.) Nasution

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**Keywords:**
Conservation
Germination
Morphology
*Musa acuminata*
Seed
Wild banana
**Submitted:**
15 June 2021
**Accepted:**
04 September 2021
**Published:**
14 February 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT

Wild bananas provide important genetic materials for further banana improvement, therefore they need to be conserved and studied. This study aimed to describe morphological characteristics of plant and seed and also to study the seed germination of wild banana *M. acuminata* var. *flava* (Ridl.) Nasution. The morphological characteristics were observed descriptively by referring to the descriptor for banana. The internal and external morphology of the seeds were observed using a digital microscope. The germination testing was carried out by a completely randomized design, using fresh seeds extracted from a bunch of fruits with two ripeness levels *i.e.* fully-ripe (yellow peel) and under-ripe (green-yellow peel). The data resulted was then analyzed using an independent t-test. The results showed that *M. acuminata* var. *flava* is characterized as a perennial herb; pseudostem height ≥ 3 m; male bud like a top with prominent green-yellow bracts; fruit curved and tasted mild-sweet when ripe. The seed is angular with wrinkled surface, and dark brown-black color when ripe. The longitudinal section showed parts of the seeds comprising the seed coat, outer and inner integument, embryo, endosperm, chalazal mass, micropyle cap and channel. The seeds are classified as orthodox, with hypogeal type and gradual germination pattern. The seeds extracted from fully-ripe fruit germinated faster with higher germination percentage and growth variables (root number and plant height). Thus, it is suggested to use physiologically mature seeds (seeds from fully-ripe fruits) which should be separated from the seeds of under-ripe fruits to lower the heterogeneity.



## INTRODUCTION

Banana is an important cash crop worldwide; ranked next to rice, wheat, and maize. The monocotyledon Musaceae family includes three genera *i.e. Ensete*, *Musella* and *Musa* in which most edible bananas belong to the genus *Musa* (Perrier et al. 2009). It consists of approximately 70 wild species and 500 cultivars (Hakkinen & Vare 2008; Valmayor et al. 2002). In particular, in Indonesia, being part of the center of banana diversity; not less than 12 wild species and 325 cultivars have been found (Nasution & Yamada 2001). However, the evolution of wild seeded to seedless edible bananas is a complex pro-

cesses involving multiple stages and separated by time (over centuries and/or millennials and places) (Perrier et al. 2009; Langhe et al. 2009; Hapsari et al. 2018).

Most of the current banana cultivars were putatively the results of hybridization between two major wild banana species of *Musa acuminata* (A genome) and *M. balbisiana* (B genome); and few were of *M. schizocarpa* (S genome) and *M. textilis* (T genome) (Perrier et al. 2009; Langhe et al. 2009). The diversity of banana crop wild relatives (especially the putative progenitors) is important to ensure the future of modern bananas. Conservation and collection of crop wild relatives through seed is a suitable strategy to conserve germplasm in gene bank because they are the reservoir of traits and genes required to face the emerging abiotic and biotic stresses (Singh et al. 2021). Nonetheless, they have been under considerable threats due to habitat destruction, fragmentation and conversion of tropical forests, and other anthropogenic disturbances. Therefore, it is important to prioritize the collection, effective conservation, improving the availability, and providing related studies of wild bananas for use in further banana improvement (Heslop-Harrison 2011; Ford-Lloyd et al. 2011; Hapsari et al. 2020).

Two major wild species *M. acuminata* mostly grow in tropical rainforests in Southeast Asia, while *M. balbisiana* is native to monsoon climates in Southeast Asia and South Asia (Ploetz et al. 2007). In Indonesia, wild bananas can be found in Java, Borneo, Mollucas, Papua, Sulawesi and Sumatra (Sulistyaningsih et al. 2014). As an *ex-situ* conservation institution, Purwodadi Botanic Garden (PBG) which is located in Pasuruan (East Java) has been conserving various species of bananas, mostly from Eastern Indonesia. At least 103 accessions and 197 specimens, consisting of eight wild species and 95 cultivars, have been successfully collected (Hapsari 2014). Furthermore, recent publication reported that PBG has collected wild bananas comprised of three accessions of *Ensete glaucum*, three accessions of *M. balbisiana* and seven accessions of *M. acuminata* variety (Hapsari et al. 2020).

The wild species *M. acuminata* is considered to be the most important genetic material contributor for cultivated bananas (Martin et al. 2020). However, this species is thought to be a species complex; possible continuous variation and phenotypic plasticity due to environmental modifications and adaptations have made this species as a taxonomically difficult group. About fifteen varieties of *M. acuminata* have been stated by Nasution (1991) in the Memoirs of Tokyo University of Agriculture XXXII. The descriptions and key identification of *M. acuminata* varieties have been provided. However, the distinguishing characters among varieties remained confusing due to high variations, especially in the wild populations of Indonesia.

Wild banana *M. acuminata* var. *flava* is one of variety of *M. acuminata*, a status novus (STAT. NOV) or got a new rank given by Nasution (1991). It was firstly described by Ridley as *Musa flava* Ridl. (Trans. Linn. Soc. 2,3: 385-386, 1888-1894 et Ridl., F. Mal. Pen. 4: 294; Anon. Kew. Bull. 92: 249, 1894. – Type: Ridley s.n., Pulau Tijam, on Pahang River, Pahang (SING n.v.). Ac-

cording to Nasution (1991), it was generally characterized by a small to medium clump, tall and slender pseudostems, purplish brown blotching without wax. Leaf blade lanceolate, long petiole, purplish brown blotching, with erect margins. Inflorescence horizontal then pendulous, peduncle thinly pubescent, 10-12 hands per bunch, 12-21 fruits per hand. Male bud ovoid, greenish yellow or yellow in color. Fruits medium, pericarp thin, pulp yellowish. Seeds many, irregularly angular, not smooth, and black when ripe.

Related studies of wild bananas for use in further improvement of bananas are essential due to the recent global threat to cultivated bananas. Diploid wild banana produces fertile and viable seeds which are preferable as genetic material for breeding purposes because they provides more variability and any possible desired traits. Banana seeds may vary in size, shape and color, and also germination rate, depending on the species and varieties (Vineesh et al. 2015). Seed gene banks for bananas are applicable for seed-producing diploid wild *Musa* species, not for cultivar bananas. *Ex-situ* seed conservation of diploid wild banana, especially collected from wild population, brings impact on the seed collection quality (Sipen et al. 2011; Kallow et al. 2020). Furthermore ex-situ seeds conservation of wild bananas is constrained by critical knowledge gaps in their germination ecology, behavior, and also storage which need to be addressed (Kallow et al. 2020; Kallow et al. 2021).

The seed studies of some varieties of wild *M. acuminata* have been reported but still limited. One of wild bananas that has been studied its propagation method is *Musa acuminata* var. *sumatrana* (Roostika et al. 2019). Hence this study aimed to describe the plant and seed morphological characteristics of wild banana *M. acuminata* var. *flava* cultivated at PBG, and also to study the seed germination from two different fruit ripeness levels to evaluate the seed physiological maturity. A complete morphological observation on *M. acuminata* var. *flava* is important for better identification of this variety. The study of banana germination by seeds is still limited because vegetative propagation is more common in banana. Information on efficiency of seed germination is required for plant propagation and breeding programs. In addition, no previous study of seed germination particularly on *M. acuminata* var. *flava* is reported.

## MATERIALS AND METHODS

### Materials

Plant material used in this study is the wild banana species living collection of Purwodadi Botanic Garden *i.e. Musa acuminata* var. *flava* located at the nursery (previously located at plot XXIV.E.40-a). It was originated from wild populations in Krawak Protected Forest of Tuban, East Java (Lestarini et al. 2012). Morphological observation was conducted directly to the living collection in September 2020. A mature bunch of fruits was harvested from a single plant. The fruits were then divided in two categories *i.e.* fully-ripe ones with yellow peel and under-ripe ones with green-yellow peel (Figure 1). Later, the seeds

from both categories were extracted for further testing (September to November 2020).

A B

**Figure 1**. Fruit maturity categories: A. fully-ripe (yellow peel), B. under-ripe (green-yellow peel).

## Methods

### Morphological characterization

Plant morphological characterization was performed by referring to the banana descriptor (IPGRI-INIBAP/CIRAD 1996). All parts of the plant were documented using a digital camera, while the documentation of the seeds was carried out using a digital microscope (Dino-Lite AM3113T). External morphological characterization of seeds was conducted on quantitative characters (length, width, thickness and weight) and qualitative characters (shape, color and texture) while internal morphological characterization was conducted on seed coat, endosperm and other internal seed parts (Kallow et al. 2021).

### Seed moisture content and germination testing

1. Seed extraction. Seeds were hand-extracted from the ripe fruits in the laboratory, and the seeds were washed thoroughly using tap water and sieves, until all flesh was removed. Meanwhile the under-ripe fruits were left in the laboratory at room temperature to ripen until they began to yellow and soften and then seeds were extracted following the steps described above (Kallow et al. 2021). After extraction prior to air-drying, the seeds were soaked in water for a while in which the floating seeds were discarded and only the submerged seeds were used for further testing.
2. Measurement of seed moisture content. It was started by weighing the fresh weight of 25 seeds using an analytical scale (Mettler Toledo), then they were dried using a drying oven laboratory (Finco Inc Ovin 30) at 100°C for 18 hours and their dry weight was determined. Measurements were carried out in three replications for each ripeness level. Seed moisture content was calculated using the following formula:

$$\text{Seed moisture content} = \frac{\text{Fresh Weight} - \text{Dry Weight}}{\text{Fresh Weight}} \text{ x } 100\%$$

Furthermore, seed storage behavior was classified as referred to (Kallow et al. 2020). Seeds with moisture content of less than 22% are predicted to have orthodox storage behavior; seeds with higher moisture content (>40%) are predicted to be recalcitrant; while seeds in-between those limits (22% - 40%) are predicted to be intermediate.

3. Sowing the seeds. The seeds were sterilized before sowing by immersing them in a 10% chlorox solution (active substance NaOCl 5.25%) for 10 minutes. The experiment used a completely randomized design (CRD) with two treatments (two seed categories *i.e.* extracted from fully-ripe fruits and under-ripe fruits) and three replications for each treatment. The seeds were sown on plastic seed tray with moist straw paper media. After being sown, the seed trays were then covered with black plastic to keep them moist (regular watering was carried out when the media started to dry). Maintaining the air circulation was conducted by opening the plastic cover during observation in order to prevent the seeds from rotting.
4. Observation of germination. The seed germination variables, the number of seeds germinated, pattern and type of germination, were observed every day. Whereas to observe the growth variables *i.e.* plant height and number of roots; seven seedlings were taken randomly at 60 days after sowing (DAS). The percentage of seed germination was determined using the following formula (Sutopo 2010; Muschick et al. 2010; Darmayanti et al. 2017):

$$\text{Germination percentage} = \frac{\text{Number of seeds germinated}}{\text{Number of seeds sown}} \text{ x } 100\%$$

### Data Analysis

The morphological characterization was described qualitatively, while the seed moisture content, seed germination, and growth variables data were analyzed quantitatively. Quantitative data were compiled and analyzed using Microsoft Excel 2016 to figure out the data characteristics and distribution. Later, the data normality was tested using Shapiro-Wilk ensued by the homogeneity test using Lavene. Independent t-test was conducted using SPSS 16.0 to determine the statistical significant difference between the means of the two fruit ripeness levels on the seed moisture content, seed germination and growth variables.

## RESULTS AND DISCUSSION

### Plant morphological characteristics of *M. acuminata* var. *flava.*

The detailed morphological characterization of both the plant and the seeds of *M. acuminata* var. *flava* from this study `are presented below (Figure 2, 3 & 4). The plant habitus of *M. acuminata* var. *flava* is perennial herbaceous plant, leaf habit intermediate, small to medium clump, number of suckers 2-5, close to parent (vertical growth). ***Pseudostem*** height ≥ 3 m, slender to normal circumference, light green, shiny (not waxy), predominant underlying watery

green with purple pigmentation, milky sap, very little or no visible sign of wax on leaf sheaths. ***Petiole*** black-purple blotches, large blotches at petiole base, length 53-70 cm, width 2.5-3 cm, green, the third petiole canal leaf wide with erect margins (not clasping), margin width ≤ 1 cm and dried. ***Leaf blade*** length 185-205 cm, width 45-50 cm, upper surface green and shiny, lower surface medium green and dull, moderately waxy, the symmetric insertion point of blades on the petiole, very corrugated, green midrib dorsal surface, light green midrib ventral surface, green cigar leaf dorsal surface, leaves of water suckers without blotches. ***Peduncle*** length ≤ 30 cm, width ≤ 6 cm, green, slightly hairy. ***Bunch*** position horizontal, spiral to asymmetric shape, compact appearance. ***Rachis*** type present, horizontal position with some neutral flowers (one to few hands only, the stalk is bare below. ***Male bud*** present, like a top, diameter 6-7 cm, length 8-9 cm; ***bract*** base shape large shoulder, length 15.7 cm, width 7.5 cm, pointed old bracts overlap at the apex of bud, green-yellow external face, green-yellow internal face, color homogenous green-yellow, without discolored lines, lanceolate shape, lifting two or more at a time, revolute behaviour before falling, very few waxes, moderate grooving; bract scars on rachis very prominent. Female flowers not observed. ***Male flowers*** small, 17-18 flowers per hand; ***compound tepal*** cream, rust colored spots, yellow lobe; ***free tepal*** tinted with yellow, oblong, corrugated (several folding under apex), apex thread-like; ***anthers*** exserted, cream to yellow, 2.1 cm; ***filament*** cream, 1 cm; ***pollen*** brown/rusty brown; ***style*** cream, without pigmentation, same level exserted, straight; ***stigma*** pale orange; ***ovary*** cream, very little or no visible sign of pigmentation, male flower cream, two arrangement of ovules. ***Fruit*** biseriate, 10-12 hands per bunch, position curved upward, ≥17 fruits per hand, length 13 - 15 cm, 1.5-2.0 cm in diameter, curved (sharp curve) shape, rounded transverse section, bottle necked apex, without any floral relicts at apex; ***pedicel*** length 11-15 mm, width 5 to 10 mm, hairless surface, deciduous at maturity (fruits fall from hand); ***peel*** light green when immature, yellow when mature, two or less thickness, cracked at maturity; ***pulp*** cream before maturity, soft flesh texture, mild to sweet taste. ***Seeds*** numerous 20-170 seeds per fruit, wrinkled surface, angular (more or less pyramidal), dark brown to black when ripe.

The habitat of *M. acuminata* var *flava* is at open places, along the rivers or roads, 300-600 m above sea level (Nasution 1991), with distribution reported in Central Borneo and the Malay Peninsula. Even though the specimen examined in this study was originated from Krawak Protected Forest of Tuban (East Java) which considered far from its original type species, but the morphological characterization results matched very well to the description of *M. acuminata* var. *flava*, particularly for the green-yellow male bud color. There were some morphological variations observed, however it may possibly due to continuous variation and phenotypic plasticity of environmental modifications. It is locally named by East Javanese as the *pisang jantung kuning* (yellow bud banana), and they utilize its leaves for food wrapping. All plant parts are also used as fodder for wild animals and cattle (Hapsari 2014).

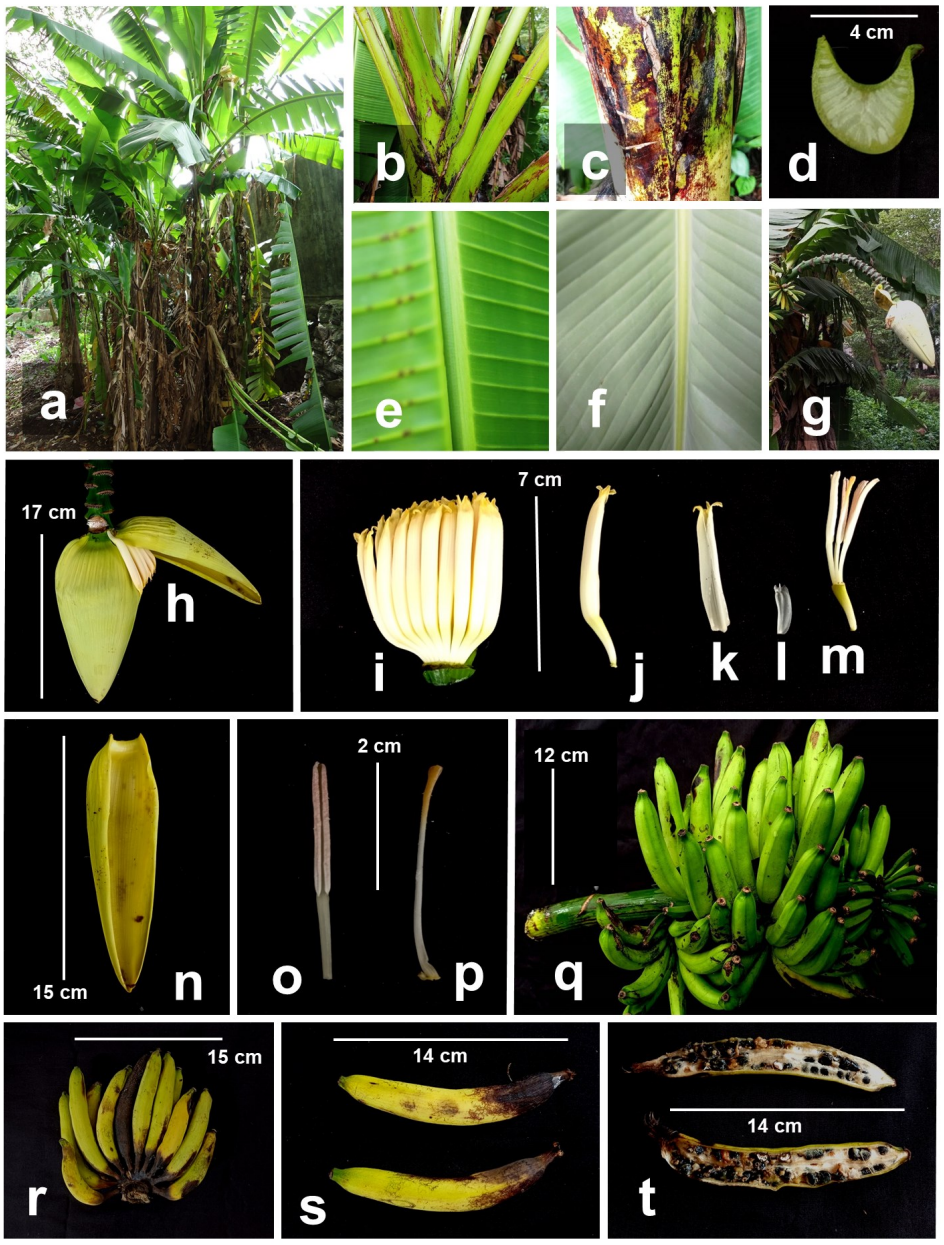


**Figure 2**. Plant morphological characteristics of *M. acuminata* var. *flava*. a. plant general appearance, b. petiole base arrangement, c. pseudostem blotches, d. cross-section of petiole canal, e. leaf adaxial surface, f. leaf abaxial surface, g. inflorescence, h. male bud, i. male flowers, j. ovary and compound tepal, k. compound tepal, l. free tepal, m. ovary, style and filament, n. male bract, o. filament and anther, p. style and stigma, q. a bunch of fruits, r. a hand of fruits, s. mature fruits, t. longitudinal section of fruit.

Morphological characteristic studies of *M. acuminata* var. *flava* are useful for determining morphological variations of wild bananas in Indonesia, although other studies have shown that various *M. acuminata* have been identified but currently do not have economic value for the community (Hastuti et al. 2019). Neglected wild bananas will be a threat to the existence and reduction of banana gene variation in nature. Conservation is a strategy to saving wild bananas before they become extinct. Wild bananas are very valuable for future breeding programs. One of the important activities in plant breeding is selecting phenotypes that have the desired morphological characters. Pheno-

type diversity can be influenced by environmental factors while morphological characters are expressions of genetic and environmental factors. The diversity of *M. acuminata* from Indonesia confirms that this species are genetically diverse (Poerba et al. 2019).

*Musa acuminata* var. *flava* is a diploid banana. Based on the results of morphological characterization, the fruits of *M. acuminata* var. *flava* are quite good in quality with 10-12 hands per bunch, curved position upwards, 17 fruits per hand, 13-15 cm in length and 1.5-2.0 cm in diameter. The strategy of crossing diploid banana with good agronomic qualities with a triploid banana that has disease-resistant will produce a diploid hybrid with agronomic advantages, such as resistance to pests and diseases (Pedraza et al. 2005). Conventional sexual hybridization is often applied in most cultivated banana. Banana breeding efforts are focused on increasing selected wild diploid, semi -partenocarpic and parthenocarpic male parents (Sipen et al. 2011).

## Seed morphological characteristics of *M. acuminata* var. *flava*

The seeds of *M. acuminata* var. *flava* weight was around 0.038 ± 0.001 g (mean ± standard deviation). The external morphological of the seeds comprised the seed coat (testa) and micropyle. They were angular in shape, dark brown to black in color, with size 2.19 ± 0.15 mm in diameter and 3.88 ± 0.10 mm in thickness (Figure 3a). The seed size is a plastic characteristic that can be altered within populations, individual plants, inflorescences and even in fruits due to environmental conditions in ripening, genetic factors, pollination rate, availability of nutrients, water, light and position of the fruit on the plant (Kaiser et al. 2016). Furthermore the seed had thick and hard coat with thickness of 0.46 ± 0.43 mm. The micropyle is located a the center of the seed, with size of 0.78 ± 0.03 mm (Figure 3b). The thick hard seed coat in wild bananas prevents the oxygen and water that are essential for germination from entering the seeds which subsequently leads to limiting factors of germination. However, it is still considered as water permeable, thus the process of water imbibition may occur (Kallow et al. 2021).

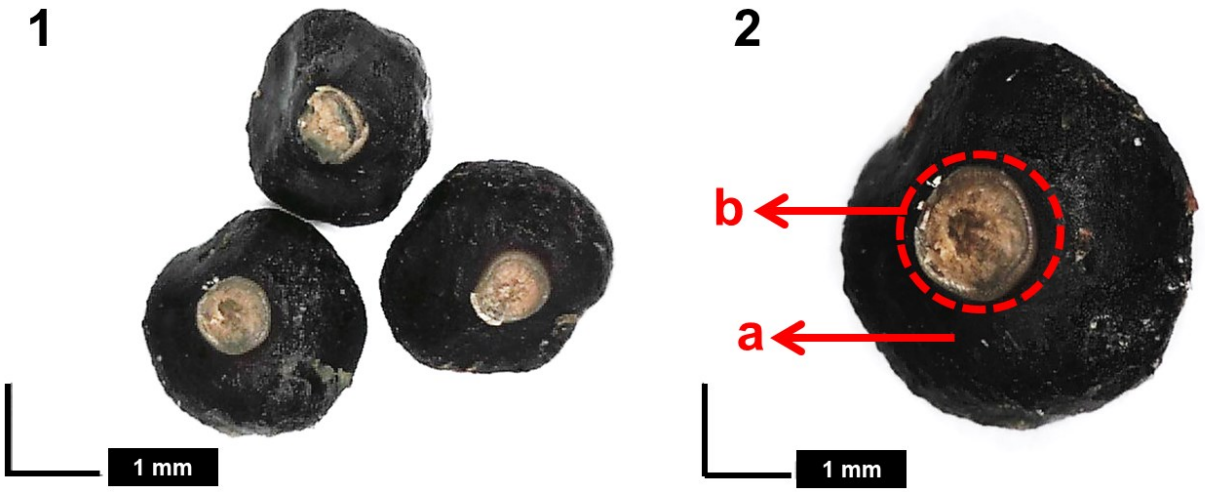


**Figure 3**. External seed morphology of *M. acuminata* var. *flava*. 1. seeds, 2a. seed coat, 2b. micropyle.

The longitudinal section of the seed showed the seed coat, outer and inner integument, embryo, endosperm, chalazal mass, micropyle cap and channel (Figure 4). Detail observation showed that the seed has two layers of integument (the outer multiple layers and the thin inner layer) which protect the seed during maturation, dispersal and dormancy (Silva et al. 2019). Furthermore, the seed has two chambers within the double-layered of the integument. The first chamber, which was larger, contained the embryo and endosperm. The embryo was small and undifferentiated, measuring 0.31 x 0.32 mm, and the embryonic axis extended to the micropyle collar. The embryo was surrounded by white flour-like (endosperm) served as food reserves for the embryo, with a size approximately of 1.04 x 0.35 mm. At the top of the embryo, there was the micropyle. The micropyle is where the shoot appears when seed germinated. The micropylar part of the seed coat develops into an operculum (a lid-like structure). During germination this lid is later displaced by the elongating radical-hypocotyl axis (Vineesh et al. 2015). In the second chamber, there was a brown chalazal mass measuring 2.65 x 1.57 mm, located at the basal part of the inner seed (Figure 4). The morphology and seed mass of *M. acuminata* var. *flava* observed in this study are in accordance to previous reports on other varieties and subspecies of *M. acuminata* (Puteh et al. 2011; Vineesh et al. 2015; Kallow et al. 2020) and also other wild *Musa* species (Burgos-Hernández et al. 2014; Bohra et al. 2020).

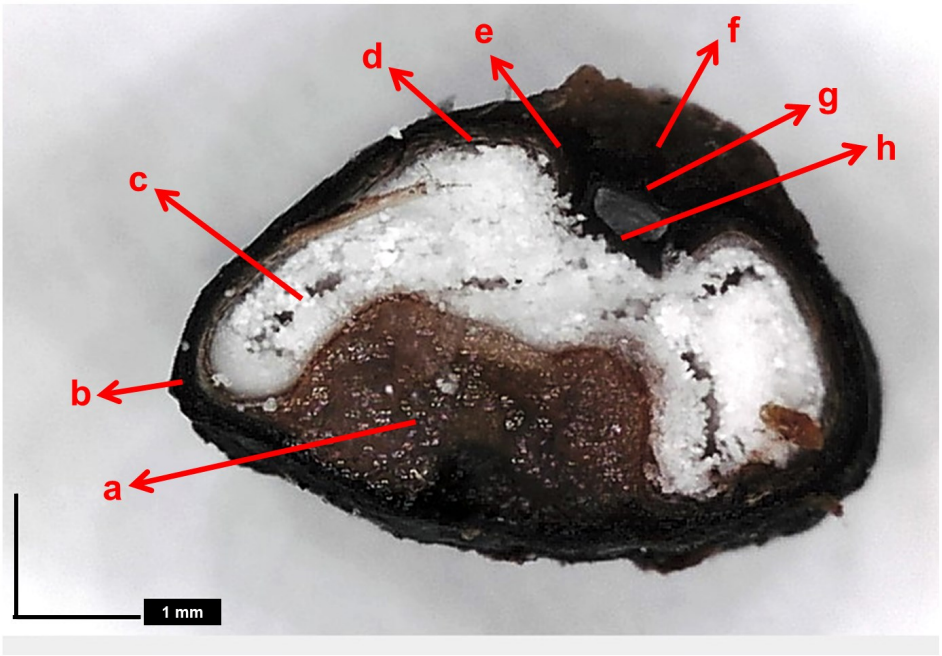


**Figure 4**. Longitudinal section of *M. acuminata* var. *flava* seed. a. chalazal mass, b. seed coat, c. endosperm, d. inner integument, e. outer integument, f. micropyle cap, g. micropyle channel, h. embryo.

Viability and quality of seeds can be determined by observing their morphological characters. Full mature of seeds are characterized by a darker color of the coat which indicated good seed development. The character of a healthy embryo is characterized by a compact mass in the seed (Figure 4H).

The morphological character of the embryo can be used as a measure of seed viability. Immature seeds show increased air space in the endosperm on drying since there are greater loss of structure during drying in embryos from less mature seeds (Kallow et al 2020). Full mature of seeds are estimated to have good seed viability (Figure 4). Some of their characteristics include compact embryo, white endosperm, darker seed coat and intact chalazal mass. Good quality seeds are characterized by successful germination approximately two weeks after being sown. Seeds with full maturity are characterized by a more powdery endosperm and are harvested from larger fruits with a softer flesh texture.

## Seed germination of *M. acuminata* var. *flava*

Fresh and dry weight of seeds from fully-ripe fruit was significantly lower than those from under-ripe fruit. However, the seed moisture content of both categories were not significantly different, but seeds from fully-ripe fruit were slightly higher (Table 1). The seeds moisture content of *M. acuminata* var. *flava* from this study were less than 22%, thus predicted to have orthodox storage behaviour. Wild banana seeds are generally known to be orthodox or long-term storage under very low moisture and sub-zero temperature. However, several studies have shown that some banana species have intermediate seeds, such as *Musa indandamanensis*, where the seed viability decreases over time, especially after three months of storage (Bohra et al. 2020). Meanwhile Kallow et al. (2020) reported that *M. balbisiana* is considered as orthodox and indicated that *M. acuminata* subsp. storage behavior was between orthodox and intermediate. To confirm the storage behavior of *M. acuminata* var. *flava* seeds, further studies are required.

Furthermore the seed germination study showed that seeds from fully-ripe fruit germinated faster with a higher germination percentage than seeds from under-ripe fruits (Table 1; Figure 5 & 6). Banana seeds are generally known to germinate after 20-21 DAS, either with special treatment or not (Burgos-Hernández et al. 2014). From this study, seeds from fully-ripe fruits initially germinated on the 15$^{th}$ DAS, while seeds from under-ripe fruit germinated three days later on the 18$^{th}$ DAS. More than 50% of seeds from fully-ripe fruits germinated (61.33% ± 2.32%), meanwhile the germination percentage of the seeds from under-ripe fruits were lower (37.33% ± 1.01%). Germination began with the appearance of white shoots through the micropyle, followed by the growth of radicles which then developed into fibrous roots. The roots and shoots showed their optimum growth on the 36$^{th}$ DAS. Seedlings from fully-ripe fruits also had significantly higher root numbers and plant height than seedlings from under-ripe fruits (Table 1). The first leaf of seedlings from the fully-ripe fruit appeared on the 43$^{rd}$ DAS, with the number of roots of 8.76 ± 0.36 on the 50$^{th}$ DAS.

Heterogeneity of seed maturity between and within bunches is considered as important factors for germination potential (Kallow et al. 2021). The level of fruit ripeness is related to physiological quality of seeds (Kaiser et al.

**Table 1**. Comparison of seed germination result of *M. acuminata* var. *flava* from fully-ripe fruits and under-ripe fruits at 50th DAS.

| Observed characters | | Seeds from fully-ripe fruits | Seeds from under-ripe fruits |
|---|---|---|---|
| Seed variable | Fresh weight of seeds (25 seeds) (g) | 0.94 ± 0.02[a] | 1.11 ± 0.38[b] |
| | Dry weight of seeds (25 seeds) (g) | 0.82 ± 0.44[a] | 0.97 ± 0.32[b] |
| | Moisture content of seeds (%) | 14.61±1.99[a] | 13.69±0.06[a] |
| Germination & growth variable | Germination percentage (%) | 61.33 ± 2.32 [a] | 37.33 ± 1.01 [a] |
| | Number of roots | 8.76 ± 0.36[a] | 6.62 ± 0.29[b] |
| | Plant height (cm) | 6.65 ± 1.07[a] | 3.74 ± 0.72[b] |

Note: The same letter in the same line shows no significant difference with independent t-test.

2016; Villa et al. 2019). The results of this study showed that with the advance of the ripening process, seeds extracted from fully-ripe fruits (yellow peel) generated seedlings with higher percentage and faster germination, also more vigorous in growth variables. The seeds from yellow fruits are considered to be fully developed (physiologically mature) compared to the seeds from green-yellow fruits (under-ripe). When the seeds were not completely mature, they could germinate, but did not result in seedlings as vigorous as those harvested at the appropriate ripening time. It was also observed from the lower amount of reserves deposited (endosperms) which may cause a limiting factor for the development of seedlings (Moiwend et al. 2015; Villa et al. 2019). The level of fruit maturity was also reported to affect *in vitro* seed germination percentage of *M. ornata* (Dayarani et al. 2014).

The seed germination type of *M. acuminata* var. *flava* was observed as hypogeal. In this type of germination, the cotyledons remain below the germination media due to rapid elongation of epicotyl (part of the stem above the cotyledon), while the hypocotyl (part of the stem below the cotyledon) remains the same in length. Then, the epicotyl pushes the plumule above the germination media, and followed by the formation of leaves. Most of monocots species considered to have hypogeal germination type (Tillich 2007), including wild banana in this study. In addition, the seeds germinated gradually over time if not simultaneously. It was started to germinate on the 15th DAS and continued until the 27th DAS in almost all replications (Figure 5 & 6).

In the wild, banana seeds buried in the soil might survive for years and germinate due to disturbances, especially after logging in the forest (Chin 1996). The germination may be stimulated by micro-climate changes in relation to disturbance, such as sunlight (due to the opening of forest canopy), moisture regimes, temperature, as well as changes in the ecological community such as predators and dispersers (Kallow et al. 2020). The germination rate is also affected by genetic factors, pollination rate, population size, pollinators availability and environmental conditions during fruit ripening (Kaiser et al. 2016; Fidalgo et al. 2019).

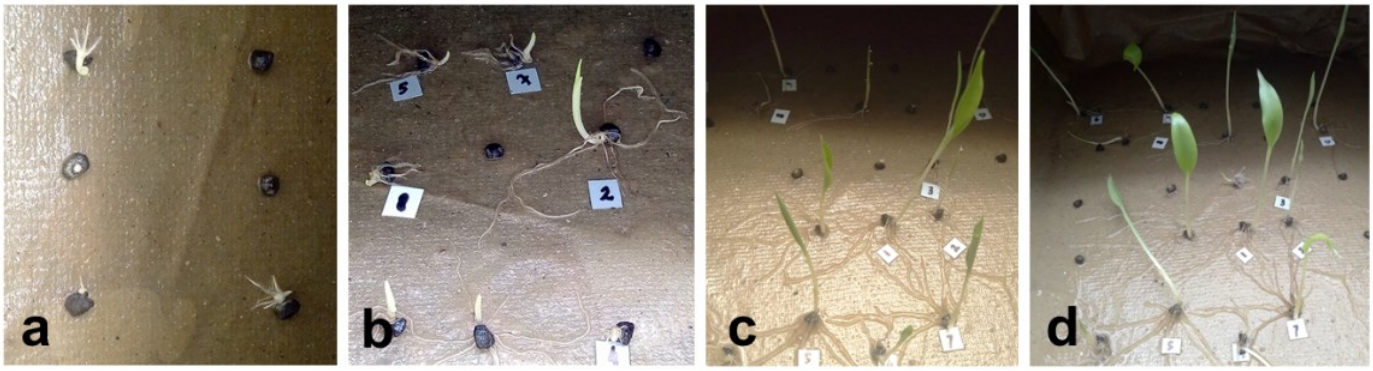


**Figure 5.** The germination of *M. acuminata* var. *flava* seeds extracted from fully-ripe fruits, a. 22nd DAS, b. 31st DAS, c. 43rd DAS, d. 50th DAS (DAS=Days After Sowing).

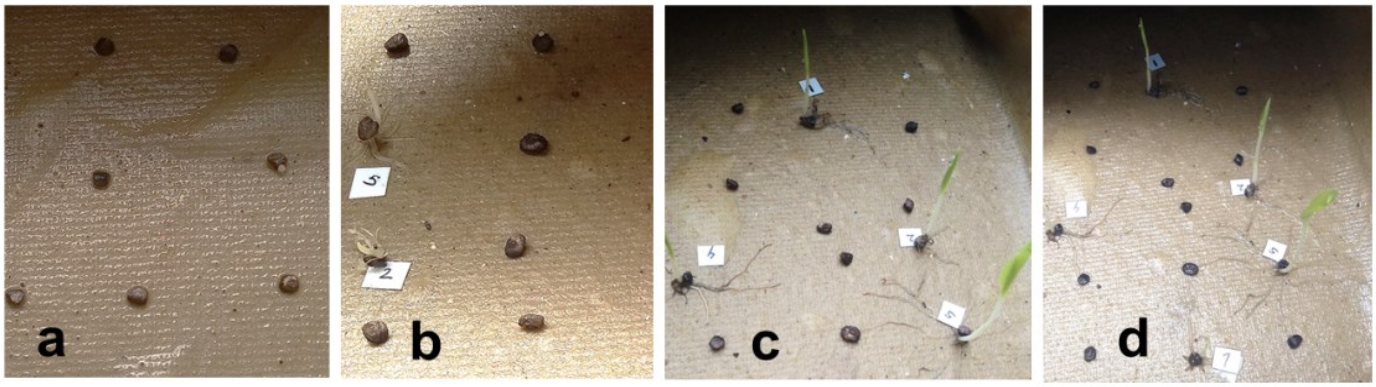


**Figure 6.** The germination of *M. acuminata* var. *flava* seeds extracted from under-ripe fruits. a. 22nd DAS, b. 31st DAS, c. 43rd DAS, d. 50th DAS (DAS=Days After Sowing).

## Implications for further seed conservation efforts and studies of wild bananas

A complete plant morphological characterization is necessary specifically on core collection to confirm the species identity. Because the morphological characteristics of wild bananas are very varied, especially at the infraspecific level, subspecies of *M. acuminata* is also difficult to identify. Therefore the collectors must prioritize to conduct the detail morphological characterization on site preferably using full descriptor for bananas (IPGRI-INIBAP 1996) or minimum descriptor (at least). Considering the time limit during the collecting mission or fieldwork, it is also important to take plenty of photographic documentation of various plant parts, populations and surrounding habitats for further identification and supporting information. Molecular analysis is also required for more advanced study to confirm genetic fidelity by taking leaf samples of each distinctive specimen or population (Hapsari et al. 2020). In addition, since wild bananas can be both propagated generatively and vegetatively, collectors should collect both seeds and suckers to complement each other for *ex-situ* conservation effort.

In seed conservation efforts, physical variables are generally adopted to harvest forest seeds, such as change in fruits and seeds color, size, odor, presence of predators, dispersers and dehiscence of fruit as indicators of ripening (Kaiser et al. 2016). Generally in wild bananas, seeds from fruits of basal

hands are produced and matured first, so that when harvested they are already in a more advanced state compared to the distal hands. The banana fruits from basal hands are faster in changing peel color to yellow and cracking, also the pulp may be softened and rotting with some aromatic odor (Hapsari 2014; Kallow et al. 2020). However, obtaining perfectly mature bunch of fruits during collecting missions is very challenging. Whereas, collecting mature fruits from field germplasm collections is more manageable in term of harvest time. Thus, if possible, initial survey should be conducted to manage the readiness of the population for seed collection. For seed collection management, it is suggested to apply different accession numbers of each bunch from different individual plants. Furthermore, to lower the heterogeneity in further studies (as highlighted from this study), the seeds from fully-ripe fruits (yellow peel) are suggested to be separated from under-ripe fruits (green, green-yellow peel) within a bunch, or fruits from basal hands are separated from distal hands (at least). It is not recommended to bulk or mix up the seeds within a bunch of wild banana fruits.

## CONCLUSION

The most prominent plant morphological characteristics of wild banana *M. acuminata var. flava* are the green-yellow male bud and bracts. The seeds are 20-170 seeds per fruit, wrinkled surface and dark brown-black when ripe. The seed had a thick and hard coat with a small embryo; and was classified as having orthodox storage behavior. Fresh seeds were germinated in two weeks after sowing, with hypogeal type and gradual germination pattern. The level of fruit ripeness was significantly affected the seed germination percentage, and growth variables *i.e.* plant height and number of roots. The seeds from fully-ripe fruit were considered more mature physiologically than the seeds from under-ripe fruits. When the seeds were not completely mature, they could germinate, but did not result in seedlings as vigorous as those harvested at the appropriate ripening time. For further conservation, storage, propagation, and related studies on seeds of wild bananas; it is suggested to clearly define and separate the seeds from the fully-ripe fruits out of the under-ripe fruits to lower the heterogeneity. Further studies on germination of periodically storage seeds of this species are required to confirm the behavior character of the storage. Germination studies by embryo culture method are also suggested in order to overcome the medium-low germination percentage of this species by conventional seed germination method.

## AUTHORS CONTRIBUTION

All authors contributed equally from conceptualization, writing, review and editing of the final manuscript.

## ACKNOWLEDGMENTS

The authors would like to acknowledge Millenium Seed Bank Partnership, Royal Botanic Gardens of Kew for funding this study. Sincere thanks were

also addressed to Dr. Dian Latifah for her supervision and support to the authors during the study.

## CONFLICT OF INTEREST

The authors state no conflict of interest from this manuscript.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb66231
DOI: 10.22146/jtbb.66231

Research Article

# Genetic Diversity of Elephant Foot Yam (*Amorphophallus paeoniifolius*) and Two Other Relatives from the Meratus Mountains of South Kalimantan, Indonesia

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**Keywords:**
*Amorphophallus*
genetic diversity
Meratus Mountains
phylogenetic relationship
**Submitted:**
31 May 2021
**Accepted:**
27 October 2021
**Published:**
23 February 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT
Elephant foot yam *(Amorphophallus paeoniifolius)* is a tuber crop with high economic value, so it is very prospective to be developed. This study aimed to characterize and determine the genetic diversity and relationship of *A. paeoniifolius* and two other relatives from the Meratus Mountains of South Kalimantan, Indonesia, using the *rbc*L marker. Eight samples of *A. paeoniifolius* and three other ones (outgroups), two of *A. muelleri* and one of *A. borneensis,* were used in the study. The genetic diversity was determined using the nucleotide diversity index ($\pi$), whereas the phylogenetic relationships were reconstructed using the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods. The results show that this germplasm has a high diversity at an inter-species level of 0.95% and a low at intra-species (0.33%). The phylogenetic analyses revealed that *Amorphophallus* from this region separated into different clades, three for NJ and one for ML. In this case, *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut is closely related to *A. paeoniifolius* var. *hortensis* from Marajai, Balangan. In conclusion, although *Amorphophallus* from the Meratus Mountains of South Kalimantan, Indonesia, shows a high diversity at an inter-species level, the phylogenetic analyses revealed a unique relationship. This finding is expected to be a reference in supporting efforts to conserve, cultivate, and utilize sustainable *Amorphophallus*, globally and locally, particularly for the Dayak Meratus community of the South Kalimantan, Indonesia.



## INTRODUCTION
The Meratus Mountains, which extend from the Hulu Sungai Tengah (HST) to the Tanah Laut (Tala) regencies of the South Kalimantan, Indonesia, is part of the world's mega-diversity that reserves abundant genetic resources. *Amorphophallus* is one of the local genetic resources of this region that have been underutilized by the local people, especially the Dayaks. For a long time, they only used this plant as the second food source after rice and sometimes as an offering food (*sesaji*) in the ritual ceremony for land clearing (King et al. 2017).

Nowadays, *Amorphophallus* is a type of tuber crop with high economic value in the global market, so the productivity of this cultivated plant should be increased to fulfil market demands (Mekkerdchoo et al. 2016). The tuber

of this plant is a source of glucomannan (a type of carbohydrate) and other substances, which can be used for the food and health industry (Mekkerdchoo et al. 2016). Even today, the tuber has been exported to other countries, especially Japan, with relatively large volumes, around 3,000 tons/ year. However, its export needs are often not met (Poerba et al. 2009). Hence, the opportunities for its cultivation are still very wide opened. In other words, various efforts, like preserving, maintaining, and developing this local crop, are very urgent to be conducted.

Genetic characterization is one of the essential tasks in supporting those programs, both the preservation, cultivation, and utilization of this plant. In brief, this activity is the one key in conservation and breeding programs (Malhotra et al. 2018). In general, conservation aims to ensure the continuing survival of species, habitats, and biological communities and interactions between species or species with their ecosystems. Besides, breeding can be used directly to preserve and utilize several genes with essential agronomic traits for future purposes (Acquaah 2017). In the Meratus Mountains of South Kalimantan, Indonesia, the characterization of these plants has been limited to only use morphological markers. Meanwhile these markers have certain limitations, some of which were also greatly influenced by environmental factors. In addition, the morphological characters are very inefficient and time-consuming due to the long period of the generative/flowering phase (Sunaryo 2015).

This study aimed to characterize and determine the genetic diversity and relationship of *Amorphophallus paeoniifolius* synonym to *A. campanulatus (*elephant foot yam) and two other relatives from the Meratus Mountains of South Kalimantan, Indonesia, using the *rbc*L marker. Conceptually, this marker its own advantages, mainly being the ability to distinguish germplasm with its very close genetic relationship (Wattoo et al. 2016). Besides, this marker is more accurate and reliable than others and has generated unbiased or unambiguous data (Lee et al. 2017; Singh et al. 2017). Thus, the results of our study are expected to be a reference in supporting efforts to conserve, cultivate, and utilize sustainable *Amorphophallus*, globally and locally, particularly for the Dayak Meratus community of the South Kalimantan, Indonesia.

## MATERIALS AND METHODS

### Plant materials

A total of eleven samples of *Amorphophallus* comprise of eight for *A. paeoniifolius* and three others, including two species as the outgroup, namely *A. muelleri* and *A. borneensis*, were used in this study (Table 1). All plant materials were collected by a purposive sampling method from seven locations of South Kalimantan, Indonesia (Figure 1).

### Molecular characterization

Molecular characterization was carried out in the Laboratory of Genetics and Molecular Biology, Faculty of Mathematics and Natural Sciences, University

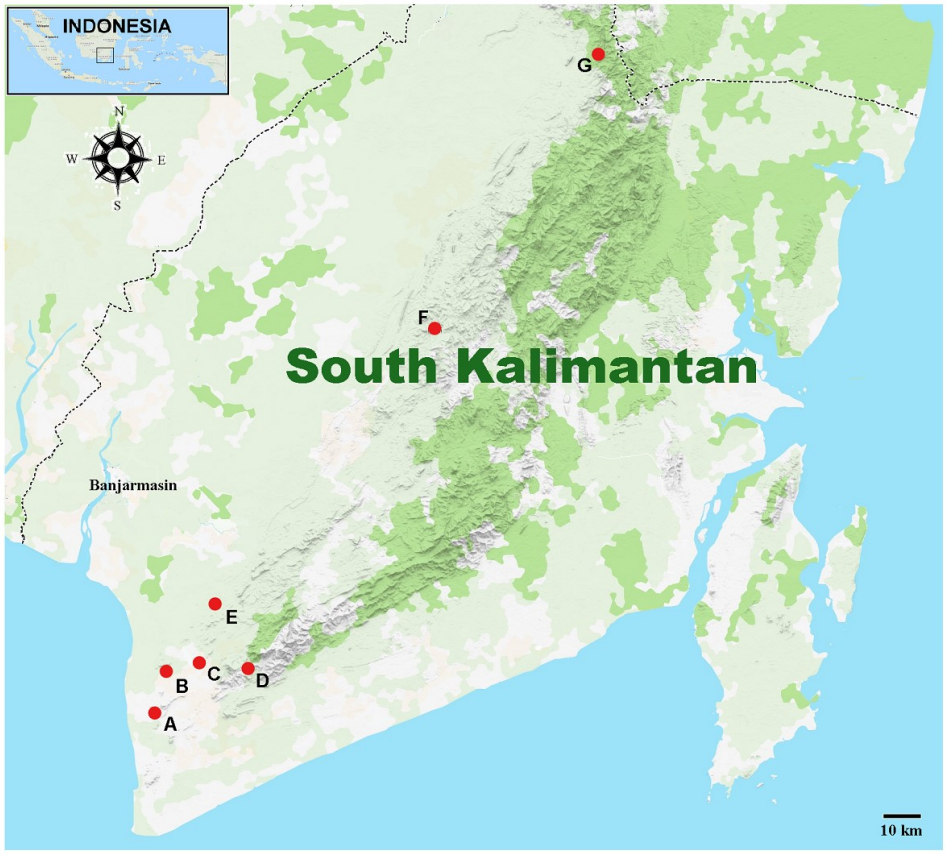


**Figure 1.** Map of South Kalimantan, Indonesia, showing seven sampling locations where the *Amorphophallus* were collected: A. Telaga, Takisung, Tanah Laut; B. Ranggang, Takisung, Tanah Laut; C. Angsau, Pelaihari, Tanah Laut; D. Sungai Bakar, Bajuin, Tanah Laut; E. Kait-Kait, Bati-Bati, Tanah Laut; F. Batung, Piani, Tapin; G. Marajai, Halong, Balangan.

**Table 1.** List of *Amorphophallus* samples used in this study and their origin.

| Species | Local Name | Origin (Village/District/ Regency) | Geographic Coordinate | Elevation (m-asl) |
|---|---|---|---|---|
| *A. paeoniifolius* var. *sylvestris* | *Bagang* | Sungai Bakar (upper), Bajuin, Tanah Laut | 3°77'95°S; 114°85'56°E | 93 |
| *A. paeoniifolius* var. *sylvestris* | *Bagang* | Sungai Bakar (lower), Bajuin, Tanah Laut | 3°77'53°S; 114°85'45°E | 64 |
| *A. paeoniifolius* var. *sylvestris* | *Bagang* | Kait-Kait, Bati-Bati, Tanah Laut | 3°58'42°S; 114°81'86°E | 41 |
| *A. paeoniifolius* var. *sylvestris* | *Bagang* | Ranggang, Takisung, Tanah Laut | 3°83'40°S; 114°69'29°E | 17 |
| *A. paeoniifolius* var. *hortensis* | *Suweg* | Batung, Piani, Tapin | 2°93'72°S; 115°40'90°E | 230 |
| *A. paeoniifolius* var. *hortensis* | *Suweg* | Telaga, Takisung, Tanah Laut | 3°83'40°S; 144°72'27°E | 24 |
| *A. paeoniifolius* var. *hortensis* | *Suweg* | Marajai, Halong, Balangan | 2°37'29°S; 115 ° 70'20 °E | 53 |
| *A. paeoniifolius* var. *hortensis* | *Suweg* | Angsau, Pelaihari, Tanah Laut | 3°79'55°S; 114°78'47°E | 24 |
| *A. muelleri* | *Porang* | Sungai Bakar (upper), Bajuin, Tanah Laut | 3°78'86°S; 114°85'74°E | 197 |
| *A. muelleri* | *Porang* | Sungai Bakar (lower), Bajuin, Tanah Laut | 3°77'40°S; 114°85'43°E | 60 |
| *A. borneensis* | *Maya* | Batung, Piani, Tapin | 2°93'73°S; 115°41'01°E | 242 |

of Lambung Mangkurat (ULM) and Laboratory of Molecular Biology, Agricultural Quarantine Agency (Class I) Banjarmasin, South Kalimantan. The activity began with DNA isolation from the leaves of all *Amorphophallus* samples of Meratus Mountain, South Kalimantan, which was successfully collected, using the DNAZol@Direct protocol (Molecular Research Center Inc., USA). DNA genomes were then quantified by UV-VIS spectrophotometry (NanoVue, GE Healthcare, UK). The DNAs were then amplified using the *rbc*L marker (Table 2) and a PCR machine (SimpliAMP, Applied Biosystem, USA).

The total volume of PCR reactions used in the study was 25 µL, consisting of 2 µL of 20 ng genomic DNA (templates), 1 µL of 0.2 µmol for each primer, and 22 µL of PCR mix (MyTaq HS Red Mix, Bioline, UK). The PCR reaction was carried out following the instructions of Mursyidin et al. (2021), with the following conditions: (1) initial denaturation, 94˚C for 5 min; (2) denaturation, 94˚C for 30 sec; (3) annealing, 48˚C for 30 sec; (4) extension, 72˚C for 45 sec; and (5) final extension, 72˚C for 7 min.

The amplified DNAs were separated by gel electrophoresis with 2% agarose and a 1X TBE buffer solution. After electrophoresis, the gel was stained with GelRed (Biotium, USA). Furthermore, DNA fragments in the gel were observed on UV transilluminator light and documented using a digital camera. DNA fragments that were amplified then sent to 1st Base Ltd., Malaysia, for purification and sequencing bidirectionally using the Sanger method.

## Data analysis

The partial sequences of *rbc*L of *A. paeoniifolius* and two other relatives (outgroup), were edited, assembled, and analyzed using the software of MEGA-X (Kumar et al. 2018). The gapped regions in the alignment were excluded from subsequent analysis unless some positions included nucleotide diversity (Mursyidin et al. 2018). The genetic diversity was determined using the nucleotide diversity index ($\pi$) with three-level categories, i.e., low (0.1 - 0.4), medium (0.5 - 0.7), and high (0.8-2.00) (Nei & Li 1979; Nei 1987). For phylogenetic analysis, multiple sequence alignments of sequences were performed with Clustal X ver. 2.0 (Larkin et al. 2007). The phylogenetic relationships were reconstructed using the Maximum Likelihood (ML) and Neighbor-Joining (NJ) methods on the program of MEGA-X (Kumar et al. 2018). In these analyses, the bootstrap method with 1,000 replicates and the substitution model of Kimura 2-Parameter was applied to reconstruct and evaluate the phylogenetic trees (Felsenstein 1985; Kumar et al. 2018).

**Table 2.** Primers were used in this study.

| Primer | Position | Sequence (5'-3') | Annealing (°C) | Target (bp) |
|---|---|---|---|---|
| *rbc*L | *Forward*<br>*Reverse* | ATGTCACCACAAACAGAGACTAAAGC<br>GTAAAATCAAGTCCACCRCG | 48 | 600 |

Source: Gholave et al. (2017).

## RESULTS AND DISCUSSION

### Results

The partial sequences of the *rbc*L region of *A. paeoniifolius* and two other species originating from the Meratus Mountains of South Kalimantan, Indonesia, were successfully sequenced and aligned. The results show that this germplasm has a different length of *rbc*L, ranging between 542 and 607 bp (Table 3). At the inter-species level, *A. paeoniifolius* has the *rbc*L ranging between 574 and 605 bp. The polymorphic sites and the rate of the substitutional matrix were shown in detail in Table 4 and 5, respectively. Following Table 3 & 4, the partial sequences of *rbc*L of *Amorphophallus* have 24 polymorphic loci, i.e., nine parsimony-informative and 15 singleton sites. Furthermore, these sequences have shown different substitutional rates, 57.51 for transitional mutation and 26.49 for transversional ones (Table 3). Regarding genetic diversity, *A. paeoniifolius* has lower genetic diversity (0.33%) than at the intra-species level (0.95%) (Table 3).

**Table 3.** Characteristics of the *rbc*L sequences of *A. paeoniifolius* and two other species (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia, including its genetic (nucleotide) diversity.

| Parameter | Intra-species | Inter-species |
|---|---|---|
| Range of sequence length (bp) | 574-605 | 542-607 |
| Number of polymorphic sites (*S*) | 9 | 24 |
| Substitution-transition rates (%) | 33.32 | 57.51 |
| Substitution-transversion rates (%) | 66.68 | 26.49 |
| Transition/transversion bias value (*R*) | 0.50 | 1.35 |
| GC content (%) | 41.68 | 41.88 |
| Maximum likelihood value (*In*L) | -912.08 | -1021.29 |
| Nucleotide diversity (p%) | 0.33 | 0.95 |

The phylogenetic analyses showed that *Amorphophallus* from the Meratus Mountains of South Kalimantan, Indonesia, was separated into different clades, three for Neighbor-Joining (NJ) and one for Maximum Likelihood (ML) (Figure 2). In this case, *A. paeoniifolius* is generally far separated from two other *Amorphophallus* species (outgroup), namely *A. muelleri* and *A. borneensis*.

The genetic distance analysis (Table 6) revealed that *A. paeoniifolius* var. *hortensis* from Pelaihari, Tanah Laut has a close relationship with similar germplasm from Takisung, Tanah Laut region. Similarly, *A. paeoniifolius* var. *sylvestris* from Takisung, Tanah Laut is also closely related to *A. paeoniifolius* var. *hortensis* from Pelaihari and Takisung, Tanah Laut. In contrast, a far relative relationship showed by *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut with *A. paeoniifolius* var. *hortensis* from Marajai,

**Table 4.** Polymorphic sites of the *rbc*L sequences of *A. paeoniifolius* and two other species from the Meratus Mountain of South Kalimantan, Indonesia.

| Name of Samples | Nucleotide Positions | | | | | | | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 1 | 1 | 3 | 3 | 3 | 4 | 8 | 1 | 1 | 2 | 2 | 2 | 2 | 3 | 4 | 4 | 5 | 5 | 5 | 5 | 5 | 5 | 5 | 6 |
| | 1 | 4 | 4 | 5 | 7 | 3 | 6 | 5 | 4 | 5 | 6 | 7 | 8 | 1 | 7 | 9 | 6 | 7 | 1 | 4 | 6 | 8 | 6 | 9 | 9 | 0 | 7 |
| *A. paeoniifolius* var. *sylvestris* (lower Bajuin, Tanah Laut) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | - |
| *A. paeoniifolius* var. *hortensis* (Piani, Tapin) | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | A | T |
| *A. paeoniifolius* var. *hortensis* (Pelaihari, Tanah Laut) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | A | A |
| *A. paeoniifolius* var. *hortensis* (Takisung, Tanah Laut) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | A | A |
| *A. paeoniifolius* var. *sylvestris* (Bati-Bati, Tanah Laut) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | G | T | - | - | - | - |
| *A. paeoniifolius* var. *sylvestris* (upper Bajuin, Tanah Laut) | - | C | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | A | A |
| *A. paeoniifolius* var. *sylvestris* (Takisung, Tanah Laut) | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | A | A |
| *A. paeoniifolius* var. *hortensis* (Marajai, Balangan) | - | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | . | T | T | A | A |
| *A. muelleri* (upper Bajuin, Tanah Laut)* | - | . | . | . | . | . | G | . | . | . | T | G | G | C | C | A | T | . | . | . | . | . | T | A | A |
| *A. muelleri* (lower Bajuin, Tanah Laut)* | - | . | . | . | . | . | G | . | . | . | T | G | G | C | C | A | T | . | . | . | . | . | T | A | A |
| *A. borneensis* (Piani, Tapin)* | - | - | A | A | G | C | . | C | G | G | . | C | G | . | C | . | . | T | . | - | - | - | - | - | - |
| Consensus | T | A | G | T | T | T | T | T | A | A | C | T | A | T | T | G | C | G | A | G | C | A | T | - | |

Notes. * Outgroup; Yellow highlight = parsimony informative site; Green highlight = singleton sites.

Balangan, at a distance coefficient of 0.0053 (Table 6). At the inter-species level, *A. paeoniifolius* var. *sylvestris* from Takisung, Tanah Laut, was closely related to *A. muelleri* from lower Bajuin, Tanah Laut (0.0133). A far relative relationship (0.0207) showed by *A. paeoniifolius* var. *sylvestris* (Bati-Bati, Tanah Laut) with *A. muelleri* (lower Bajuin, Tanah Laut).

**Table 5.** Maximum likelihood estimates of substitution matrix of the *rbc*L sequences of *A. paeoniifolius* and two other species from the Meratus Mountain of South Kalimantan, Indonesia.

| From\To | Intra-species | | | | Inter-species | | | |
|---|---|---|---|---|---|---|---|---|
| | **A** | **T** | **C** | **G** | **A** | **T** | **C** | **G** |
| A | **-** | *8.33* | *8.33* | **8.33** | - | *5.31* | *5.31* | **14.38** |
| T | *8.33* | - | **8.33** | *8.33* | *5.31* | - | **14.38** | *5.31* |
| C | *8.33* | **8.33** | - | *8.33* | *5.31* | **14.38** | - | *5.31* |
| G | **8.33** | *8.33* | *8.33* | - | **14.38** | *5.31* | *5.31* | - |

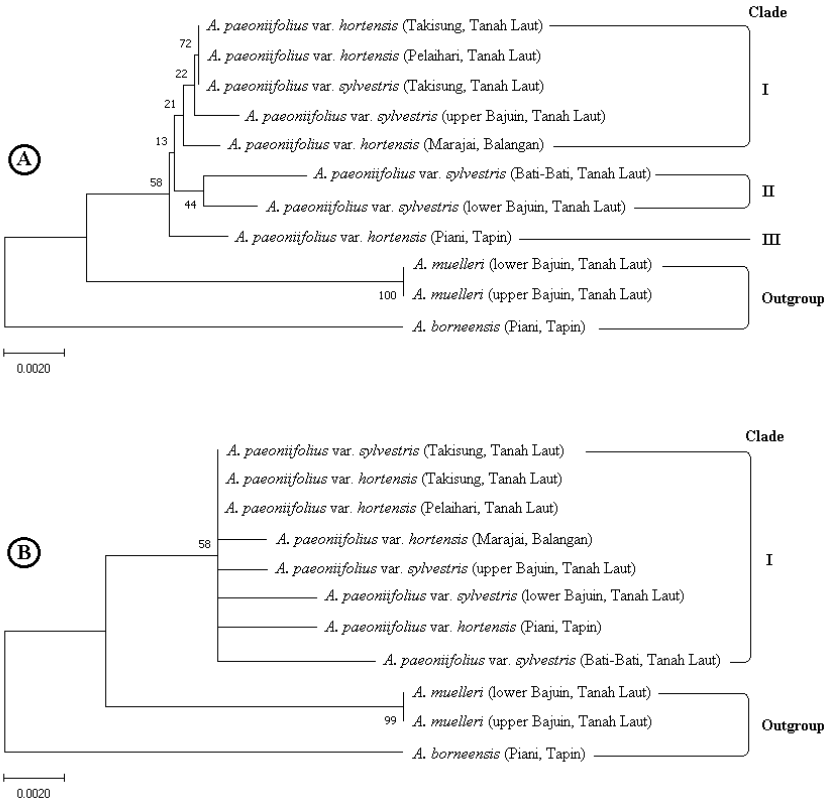


**Figure 2.** Phylogenetic relationship of elephant foot yam (*A. paeoniifolius*) and two other relatives (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia, based on the NJ (A) and ML (B) methods.

**Table 6.** The genetic distance of *A. paeoniifolius* and two other species (outgroup) from the Meratus Mountains of South Kalimantan, Indonesia.

| Code | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9* | 10* | 11* |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | | | | | | | | | | | |
| 2 | 0.0050 | | | | | | | | | | |
| 3 | 0.0033 | 0.0033 | | | | | | | | | |
| 4 | 0.0033 | 0.0033 | 0.0000 | | | | | | | | |
| 5 | 0.0052 | 0.0070 | 0.0052 | 0.0052 | | | | | | | |
| 6 | 0.0050 | 0.0033 | 0.0017 | 0.0017 | 0.0070 | | | | | | |
| 7 | 0.0033 | 0.0033 | 0.0000 | 0.0000 | 0.0052 | 0.0017 | | | | | |
| 8 | 0.0050 | 0.0033 | 0.0017 | 0.0017 | 0.0053 | 0.0033 | 0.0017 | | | | |
| 9* | 0.0168 | 0.0151 | 0.0134 | 0.0134 | 0.0195 | 0.0151 | 0.0134 | 0.0151 | | | |
| 10* | 0.0168 | 0.0151 | 0.0134 | 0.0134 | 0.0195 | 0.0151 | 0.0133 | 0.0151 | 0.0000 | | |
| 11* | 0.0206 | 0.0206 | 0.0206 | 0.0206 | 0.0207 | 0.0206 | 0.0206 | 0.0206 | 0.0263 | 0.0263 | |

Notes. * Outgroup; Green highlight = closely related or identic; Yellow highlight = distant related

1 = *A. paeoniifolius* var. *sylvestris* (lower Bajuin, Tanah Laut);
2 = *A. paeoniifolius* var. *hortensis* (Piani, Tapin);
3 = *A. paeoniifolius* var. *hortensis* (Pelaihari, Tanah Laut);
4 = *A. paeoniifolius* var. *hortensis* (Takisung, Tanah Laut);
5 = *A. paeoniifolius* var. *sylvestris* (Bati-Bati, Tanah Laut);
6 = *A. paeoniifolius* var. *sylvestris* (upper Bajuin, Tanah Laut);
7 = *A. paeoniifolius* var. *sylvestris* (Takisung, Tanah Laut);
8 = *A. paeoniifolius* var. *hortensis* (Marajai, Balangan);
9 = *A. muelleri* (upper Bajuin, Tanah Laut);
10 = *A. muelleri* (lower Bajuin, Tanah Laut);
11 = *A. borneensis* (Piani, Tapin).

## Discussion

The *rbc*L is a functional gene in the chloroplast genome that is involved mainly in plant photosynthesis and encodes the ribulose-1, 5-bisphosphate carboxylase/oxygenase, or Rubisco (Liu et al. 2012). This gene is in the large single-copy region of the chloroplast genome and shows high homology among different plant germplasm (Dong et al. 2013). Singh and Banerjee (2018) reported that this gene has an intergenic spacer with 600-800 nucleotides. Following CBOL (2009), the *rbc*L gene includes approximately 1,400 nucleotides coding for the large subunit protein, and the length varies slightly among flowering plants (Angiosperm).

In this study, the *rbc*L sequences of *Amorphophallus* were recorded with different lengths, ranging between 542 and 607 bp (Table 3). Specifically, in *A. paeoniifolius* population, the *rbc*L length ranging between 574 and 605 bp. Compared to other studies, *A. paeoniifolius* has a different one, both in partial and complete sequences. For example, in *A. paeoniifolius*, Grob et al. (2002) and Gao et al. (2017) reported the total *rbc*L region of 1,479 bp and 1,391 bp, respectively. In *A. paeoniifolius* var. *campanulatus,* a partial *rbc*L sequence length of 636 bp (Dean et al. 2018). In *A. muelleri*, Grob et al. (2002) reported that this region is around 1,441 bp, while in *A. borneensis*, it is 1,453 bp (Sedayu et al. 2010).

Following Table 3, there are a different number of polymorphic sites (*S*), mutation rates (especially substitutions), and the number of transition/transversion bias values (*R*) on the *Amorphophallus*, both at the intra- and inter-species levels. In general, the number of polymorphic sites, substitution-transition rates, and a transition/transversion bias value are relatively higher in the intra-species than the inter-species level, except for substitution-transversion rates (Table 3). However, at the inter-species level, the mutation rates of transitional substitutions are higher than transversion for each nucleotide probability. At the intra-species level, both substitutions (transition and transversion) are equal (Table 5).

According to Stoltzfus and Norris (2015), a transition/transversion bias is described for the difference ratio, which is the effect of a complex function of sequence divergence degree. Generally, transitions are more often found in most sequences than transversions (Aloqalaa et al. 2019). Hence, these phenomenon are common in molecular evolution (Stoltzfus & Norris 2015). However, this underlying phenomenon is not universal, as observed in grasshopper (*Podisma pedestris*) and two types of metazoans, namely *Drosophila* and the Mammalian (Keller et al. 2007).

Conceptually, mutation, both substitution and indel, tends to cause changes in the biochemical properties of protein products (Keller et al. 2007). According to Ripley (2013), the mutation is permanent changes inherited in the genes or nucleotide sequences (genome) of an organism, and it can affect a single nucleotide (point mutation) or some that are close to each other (segmental mutation). This phenomenon is closely related to nucleotide-based evolutionary changes or genetic diversity emerge (Nei 2007). Thus,

this event is an initial step in establishing a primary population for natural selection and an integral part of evolution and genetic diversity (Govindaraj et al. 2015).

Regarding genetic diversity, *Amorphophallus* from this region shows a higher diversity in inter-species level (0.95%) than intra-species (0.33%) (Table 3). According to Bhandari et al. (2017), this is a normal phenomenon. The higher the taxonomical hierarchy is, the higher of genetic diversity among different communities of species occurs. However, this is the opposite for the lower taxonomical one. In this context, therefore, genetic diversity is referred to the diversity present within different genotypes of the same species. Bhandari et al. (2017) defined genetic diversity as the variation of heritable characteristics present in a population of the same species.

The phylogenetic analysis revealed that *Amorphophallus* from South Kalimantan formed different clades, three for NJ and one for ML. However, in this case, the *rbc*L region could not resolve this germplasm, particularly *A. paeoniifolius* (intra-specific level) well into two varieties, namely *sylvestris* and *hortensis*. Such cases are also reported by several researchers, e.g., Ude et al. (2019) in yam (*Dioscorea*), Dong et al. (2011) in *Pterygiella*, and Chandrasekara et al. (2021) in *Cinnamomum* accessions. According to Newmaster et al. (2006), this may be corresponding with the lower nucleotide substitution rates of this gene used. Hence, using others or combines two or more DNA barcoding markers are recommended to be conducted.

However, information on genetic diversity and its relationships is valuable in supporting the breeding and conservation programs in the future, particularly for parental selection and the development of novel superior cultivars (Acquaah 2017).

## CONCLUSION

Based on the *rbc*L marker, *Amorphophallus* from the Meratus Mountain of South Kalimantan, Indonesia, has high diversity, particularly at an inter-species level and low at intra-species one. The phylogenetic analyses revealed that this germplasm is separated into three main clades, both for NJ and ML, where *A. paeoniifolius* var. *sylvestris* from Bati-Bati, Tanah Laut has closely related to *A. paeoniifolius* var. *hortensis* from Marajai, Balangan. This information may be used as a reference in supporting the conservation and breeding efforts of *Amorphophallus*, both locally and globally.

## AUTHORS CONTRIBUTION

DHM and BZ conceived and designed the experiments; MAH did the fieldwork and performed the experiments; DHM and MAH analyzed the data and wrote the manuscript; DHM and BZ reviewed the manuscript internally.

## ACKNOWLEDGMENTS

Thank you very much to Nico and fellow students who are members of the "Molecular Biology" Study Group, Study Program of Biology, Faculty of

Mathematics and Natural Sciences, University of Lambung Mangkurat, for helping with the sample collection. We would also like to thank the Director of the Agricultural Quarantine Agency (Class I) Banjarmasin, South Kalimantan, who helped with the PCR analysis.

## CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb69425
DOI: 10.22146/jtbb.69425

Research Article

# The Influence of Agrochemicals on Macroinvertebrate Community Structure in Various Agricultural Rivers in Jember Regency

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**Keywords:**
composition
ecological indices
ecotoxicity
environment health
freshwater ecosystem
**Submitted:**
28 September 2021
**Accepted:**
20 December 2021
**Published:**
09 March 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT

The intensive use of agrochemicals in agricultural areas of Jember's Regency presents a potential threat to the freshwater ecosystem's community. The use of the benthic macroinvertebrates community may provide a key to monitor the extent of agrochemical impact to maintain valuable ecosystem services. Macroinvertebrates community structure and environmental factors were studied from September–December 2020 in Jember Regency by comparing three different types of agricultural rivers (organic, semi-organic, and conventional). Five community indices (taxa, individuals, Simpson dominancy index, Margalef species richness, and Shannon diversity index) were used to compare the macroinvertebrates community structure between sites. Using community composition and physicochemical properties (bare sediment, width, depth, water current, pH, conductivity, dissolved oxygen (DO), and temperature), we generated CCA triplot and correlogram plot to investigate the grouping and the correlation between variables and sites. Results on macroinvertebrate composition showed the importance of using sensitive taxa-group and community indices as an indicator of environmental changes. The family of Tipulidae, Naididae, Cysticidae, and Nereididae demonstrated relation to semi-organic agricultural rivers. Temperature and water current correlate to the presence of clean water indicator species such as Philorheitridae and Chironomidae, as observed in organic agricultural rivers. Conventional and semi-organic agricultural rivers were grouped and largely contributed by the 5 families including Ampullariidae, Pachychillidae, Baetidae, Enchytraidae, and Gomphidae. Correlogram plot suggests a complex interaction between macroinvertebrate community and environmental variables. It can be concluded that the intensive use of agrochemicals may lead to a detrimental change toward the diminished quality of freshwater community and environment.



## INTRODUCTION

Agricultural sectors in Jember's Regency of East Java serve as a backbone of the economy by primarily producing crop commodities including rice. With over 86.685 ha (26.32 %) of total land being used as paddy fields (Dinas Pertanian Dan Ketahanan Pangan Jawa Timur 2013), the use of pesticides and fertilizers to increase crop production were inevitably high. This raises a concern about the detrimental effects of pesticides and fertilizers on the organ-

ism, ecosystem, and environment. Agricultural and pesticide utilization has been reported to negatively impact the freshwater ecosystem leading to environmental contamination (Chimwanza et al. 2006; Wu et al. 2018; Stoyanova & Harizanova 2019). Moreover, high erosion (because of the loss of vegetation), steeply plain fields, high rate of rainfall, and ineffective farming methods may lead to the deterioration of water reservoirs in soil. Thus, the pesticides and other contaminants could flow along with the organic materials via water runoff because of rain plash erosion and decrease of natural vegetation. Pesticides and fertilizers could represent a significant stressor in the freshwater ecosystem. However, linking the degree of exposure to the observed impact could be challenging, mainly due to the complex interaction within the ecosystem (Jasem 2011).

The community of macroinvertebrates in the freshwater river is generally considered as a bioindicator of environmental changes due to its sensitivity to organic pollution (Ghaly & Ramakrishnan 2015), habitat degradation (Wu et al. 2018), and pesticide contamination (Berenzen et al. 2005). The distribution, species richness, and population of the macroinvertebrate community may reflect the changes in the freshwater ecosystem due to its specificity to the agrochemicals. In this study, we used the community structures of benthic macroinvertebrates as primers to investigate the potential influence of pesticide runoff in conventional, semi-organic, and organic agricultural types in Jember Regency. To the best of our knowledge, there has never been a thorough analysis using multiple ecological indices to investigate the pattern of the impact on community structures of macroinvertebrates in various agricultural rivers in Jember. We aim to evaluate the influence of the degree of pesticides and fertilizers on the macroinvertebrate community, while also analyze the interacting factors that significantly correlate to the structures of its community.

## MATERIALS AND METHODS

### Study area and sampling time

The study was conducted on 3 rivers located within various agricultural types, i.e., conventional (K), semi-organic (SO), and organic (O) which divided into 9 subsampling sites (K1, K2, K3 for conventional type; SO1, SO2, SO3, for semi-organic type; and O1, O2, O3 for organic type) in Panti district (-8.616667 S, 113.5767 E) and Sumber Jambe district (-8,12259 S; 113,63308 E) (Figure 1).

The conventional type was managed using agrochemical fertilizers and pesticides, the semi-organic type was utilized by agrochemical fungicides as pest control, whereas the organic type was managed by organic fertilizer as a source of nutrients (Table 1).

These areas were used for paddy crops, the biggest crop commodity in East Java (Fadil 2017; BPS Provinsi Jawa Timur 2021). The observed rivers were directly connected to agricultural areas and channeling the paddy fields in the seeding period. The observed rice fields were irrigated by the adjacent

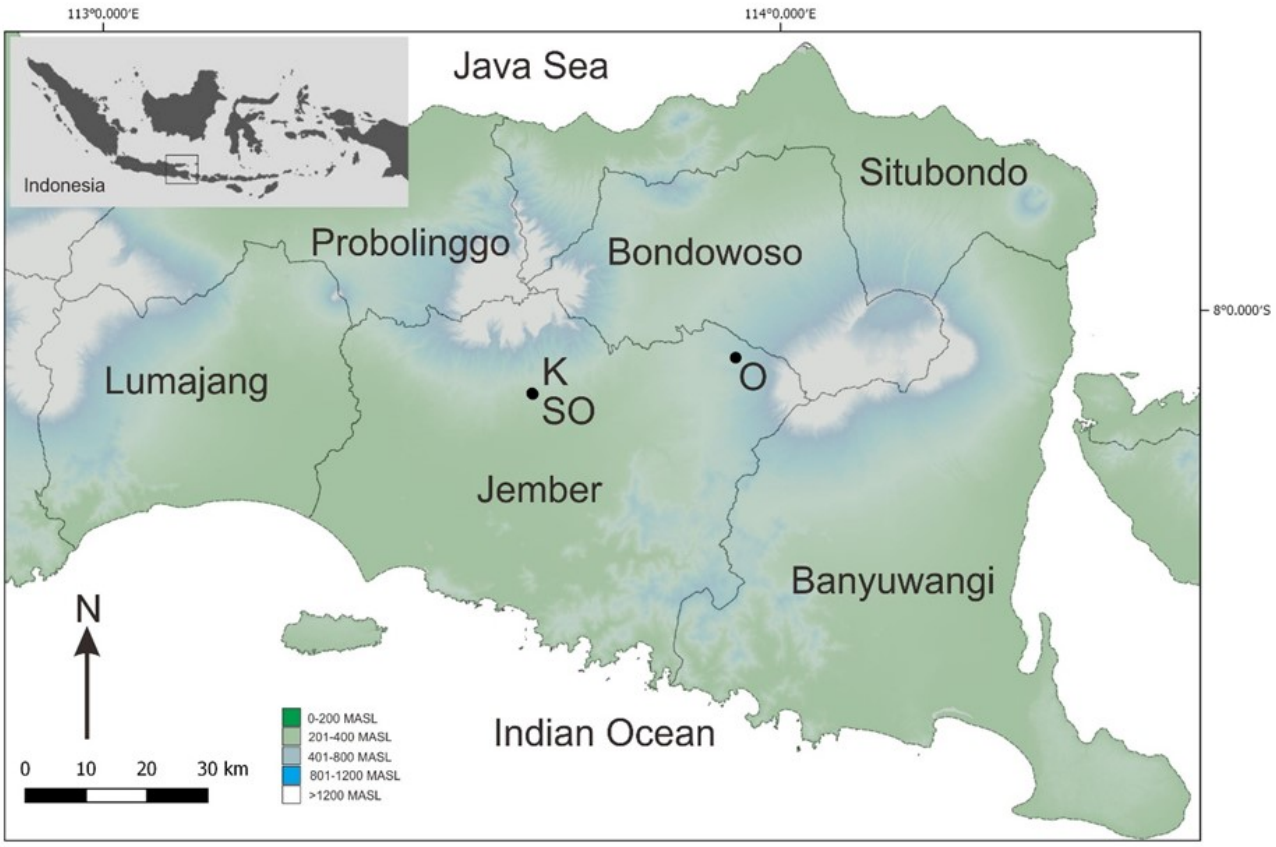


**Figure 1.** Geographical map showing the study area of 3 agricultural types in Jember Regency along with their corresponding altitude. Abbreviations are as follows: (K) conventional, (SO) semi-organic, and (O) organic.

| Sites | Types | Fertilizer | | Pesticide | | Fungicide | |
|---|---|---|---|---|---|---|---|
| | | Brand | Application | Brand – Active compound | Application | Brand – Active compound | Application |
| K1–K3 | Conventional | Phonska© | 16.66 kg/ha; every 2 weeks (week-5 toweek -9) | Fenite© – Emamectin benzoate | 200 ml/ season (June–August) | Zole© – difenoconazole | 200 ml/ season (June –August) |
| SO1–SO3 | Semi-organic | Organic | 125 kg/ha; every 2 weeks, (week-3 to week-9) | – | – | Zole© – difenoconazole | 200 ml/ season (May–July) |
| O1–O3 | Organic | Organic | 222.22 kg/ha; every 2 weeks, (week-3 to week-9) | – | – | – | – |

upstream rivers throughout the year. Using 1x1 meter plots for each agricultural type, preliminary vegetation analysis covering the seedling type (height = 0–1.5 m) by important value index (Odum & Barrett 1971), revealed a various types of plant species that may support and represent various benthic communities (Table 2). The plot locations were chosen randomly by considering the distance to the river bank. The yearly precipitation is 250 $mm^3$ which can reaching up to 349 $mm^3$ in the rainy period (BPS Jember 2021). The study was conducted from September–October 2020.

**Table 2.** List of plant species and important value index (IVI) values for each type of agricultural types. Abbreviations are as follows: (K) conventional, (SO) semi-organic, and (O) organic.

| Plant species | Agricultural type | | |
|---|---|---|---|
| | K | SO | O |
| *Acmella paniculata* | – | – | 8.19 |
| *Ageratum conyzoides* | 18.84 | – | – |
| *Althenanthera philoxiroides* | 17.23 | 32.59 | – |
| *Chromolaena odorata* | 25.92 | – | – |
| *Clinopodium vulgare* | – | – | 94.56 |
| *Coffea canephora* | – | – | 21.48 |
| *Cyperus iria* | – | 36.02 | – |
| *Eleusine indica* | – | 36.02 | – |
| *Emilia sonchifolia* | – | – | 26.10 |
| *Euchinochloa colona* | – | 135.16 | 23.79 |
| *Euphorbia hirta* | 12.28 | – | – |
| *Gomphrena sp.* | 17.23 | – | – |
| *Ipomoea lacunosa* | 12.28 | – | – |
| *Kyllinga brevifolia* | 34.87 | – | 26.10 |
| *Micania micrantha* | – | – | 23.02 |
| *Oryza sativa* | 23.51 | 32.51 | 29.95 |
| *Oxalis barrelieri* | 18.84 | – | 19.95 |
| *Paspalum conjugatum* | 86.42 | – | – |
| *Phyllanthus niruri* | 24.45 | 27.71 | – |
| *Setaria plicata* | – | – | 26.87 |
| *Zea mays* | 8.14 | – | – |

## Survey and data collection

The subsampling sites were chosen to represent the best overall diversity of benthic macroinvertebrates. The distance for each subsampling site within each of the agricultural types is up to 100 m, where we address (i) habitat characteristics, (ii) physicochemical properties, and (iii) macroinvertebrate species. The selected habitats were agricultural areas with various degree of agrochemicals exposure (Table 1). Each subsampling site represents the habitat in the inlet (denoted by 1), middle (denoted by 2), and outlet (denoted by 3) flows. Physicochemical properties including bare sediment, width, depth, water current, pH, conductivity, dissolved oxygen (DO), and temperature were measured.

Bare sediment was estimated by using 1x1 meter plot to observe the composition and proportion of sediment. The width of the river was measured using a ribbon meter stretched to each of the river edges. The depth of the river was measured using a rope with a weighted load. Water currents were estimated based on the buoy traveling time within a certain distance which then converted into velocity (m/s). The estimation of pH, conductivity, DO, and the temperature was based on Aquacombo HM3070 (Transintrument). We collected the samples using a hand net square with a frame size of 25 x 40 cm and a mesh size of 500 μm. For each species ob-

tained in each subsampling site, we collected the voucher specimens and preserved them in bottles filled with 70% ethanol for identification purposes in the laboratory. The physicochemical properties and samples were collected 3 times within the same subsampling sites.

## Identification and analyses

The collected samples were rinsed off and cleaned from debris. Samples were sorted out and placed in a plain-colored container using a brush. We identified the samples up to the family level using literature and identification books (Lehmkuhl 1979; Gooderham & Tsyrlin 2002). The data were tabulated in Microsoft Excel 2017.

Five community indices (taxa, individuals, Simpson dominancy index, Margalef species richness, and Shannon diversity index) were used to compare the macroinvertebrates' community structure between sites. For the Simpson dominancy index (Brower et al. 1997) we used the equation as follows,

$$Id = \frac{\Sigma n_i(n_i-1)}{N(N-1)} \quad \ldots(1)$$

where Id is the Simpson dominancy index, $n_i$ is the individual number of particular species-i, and N is the total individual number of all species found. For the Margalef species richness (Farris 1976) we used the equation as follows,

$$\mathrm{Dmg} = \frac{S-1}{ln\ N} \quad \ldots(2)$$

where Dmg is the Margalef index, S is the total number of species and N is the total number of individuals. For the Shannon-Wiener diversity index (Odum & Barrett 1971) we used the equation as follows,

$$H = - \Sigma n_i . N^{-1} . \ln(ni.N^{-1}) \ldots(3)$$

where H is the Shannon-Wiener index, $n_i$ is the individual number of particular species-i, and N is the total individual number of all species found.

The relative abundance is estimated and represented in Bubble Plot using ggplot2 R-packages (Wickham 2016) analyzed in R v.3.4.1 (R Core Team 2013). Community structures were analyzed based on taxa, individuals, Simpson dominancy index, Margalef species richness, and Shannon diversity index using PAST software (Dasgupta 2013). Moreover, to see the variations and grouping of subsampling sites using macroinvertebrate community composition and physicochemical properties, a Canonical Correspondence Analysis (CCA) triplot was analyzed using PAST software (Dasgupta 2013). Apart from that, the correlation matrix using the results on community indices (i.e., taxa, individuals, Simpson dominancy index, Margalef species richness, and Shannon diversity index) and physicochemical factors (i.e., water currents, depth, pH, bare sediments, temperature, width, conductivity) were estimated using Pearson correlation and interpreted using Correlogram plot using corrplot R-packages (Levy 2021) analyzed in R v.3.4.1 (R Core Team 2013).

## RESULTS AND DISCUSSION

### Results

#### Environmental variables

Bare sediments were varied from 17–83.32 % throughout various agricultural rivers. The width and depth of the river correspond with the water current variables. Most of the rivers have strong water currents capable to sweep away the litter, except in K2, K3, SO2, and SO3 which show slow currents. Various agricultural rivers were buffered within a pH of 6.94–7.58. Low conductivity was detected in SO2 and SO3 (35.02–37.07 µmhos/cm). The dissolved oxygen in all the observed sites ranged from 7.16–8.4 mg/L. Temperature ranges from 23.88–29.43 °C. The environmental variables in each of the three agricultural rivers are shown in Table 3.

#### Macroinvertebrate composition

Three agricultural rivers were characterized by the abundance of benthic taxa. In conventional type, K1 and K2 were dominated by Ampullariidae (40.5–62 %) except K3 which concurrently dominated by Pyralidae (25.3 %) and Ampullariidae (27.5 %). In Semi-organic type, SO1 was dominated by Naididae (72.4 %) followed by Chironomidae (10.3 %). SO2 was mostly contributed by Pachychillidae (16 %), Naididae (20 %), and Gecarcunidae (24 %). SO3 was dominated by Pachychillidae (43 %), followed by Naididae (29 %), Cordulidae (14 %), and Lumbriculidae (14 %). Organic type shows a relatively stable abundance of taxa with O1 mostly dominated by Simuliidae (58 %); O2 mostly contributed by Hirudinidae (26 %) and Philorheithridae (22.0 %); and O3 dominated by Ampullariidae (27%) and Chironomidae (20%). The relative abundance of macroinvertebrates in each of the three agricultural rivers is shown in Table 4 and the Bubble Plot (Figure 2).

**Table 3.** Mean (± SE) of environmental variables in each of three agricultural rivers in Jember Regency. This data was used for further Canonical correspondence analysis (CCA) and Correlation matrix.

| Parameter | Agricultural type | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Conventional | | | Semi-organic | | | Organic | | |
| | K1 | K2 | K3 | SO1 | SO2 | SO3 | O1 | O2 | O3 |
| Bare sediments (%) | 28.32 ± 2.87 | 75 ± 5 | 25 ± 5 | 17 ± 2.89 | 71.67 ± 10.39 | 83.32 ± 2.89 | 36.67 ± 5.76 | 73.32 ± 5.76 | 73.32 ± 5.76 |
| Width (m) | 2.05 ± 0.80 | 0.52 ± 0.06 | 2.38 ± 0.34 | 2 ± 0.77 | 0.62 ± 0.08 | 0.6 ± 0.80 | 0.41 ± 0.08 | 0.72 ± 0.09 | 0.59 ± 0.22 |
| Depth (m) | 0.21 ± 0.06 | 0.35 ± 0.14 | 0.37 ± 0.11 | 1 ± 0.19 | 0.52 ± 0.056 | 0.34 ± 0.06 | 0.23 ± 0.05 | 0.47 ± 0.09 | 0.66 ± 0.11 |
| Water current (m/s) | 0.11 ± 0.07 | 0.05 ± 0.02 | 0.07 ± 0.03 | 0.1 ± 0.09 | 0.05 ± 0.01 | 0.03 ± 0.09 | 0.22 ± 0.06 | 0.14 ± 0.03 | 0.15 ± 0.04 |
| pH | 7.53 ± 0.01 | 7.33 ± 0.10 | 7.48 ± 0.01 | 7.53 ± 0.01 | 7.1 ± 0.006 | 6.94 ±0,04 | 7.58 ± 0.04 | 7.22 ± 0.05 | 7.49 ± 0.09 |
| Conductivity (µmhos/cm) | 169.8 ± 0.3 | 163.6 ± 0.55 | 160.67 ± 0.65 | 169.8 ± 0.87 | 35.02 ± 0.81 | 37.07 ± 3.48 | 93.42 ± 7.74 | 129 ± 0.25 | 129.25 ± 0.59 |
| Dissolved oxygen (mg/L) | 7.76 ± 2.3 | 7.45 ± 0.96 | 7.83 ± 3.27 | 7.16 ± 1.97 | 7.73 ± 1.36 | 7.51 ± 5.24 | 7.9 ± 4.75 | 8.4 ± 1.07 | 7.98 ± 4.64 |
| Temperature (°C) | 23.88 ± 0.38 | 25.21 ± 0.37 | 24.72 ± 0.33 | 23.88 ± 0.38 | 28.05 ± 0.11 | 28.4 ± 0.14 | 29.43 ± 1.33 | 25.36 ± 1.40 | 25.21 ± 0.71 |

**Table 4.** Relative abundance (%) of macroinvertebrates in each of three agricultural rivers in Jember Regency.

| Order | Family | Agricultural type | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | K1 | K2 | K3 | SO1 | SO2 | SO3 | O1 | O2 | O3 |
| **Araneae** | Tetragnathidae | – | – | 1.1 | – | – | – | 0.4 | – | – |
| **Architaenioglossa** | Ampullariidae | 40.5 | 62.0 | 27.5 | – | 12.0 | 29.0 | 2.7 | 13.0 | 27.0 |
| **Arhynchobdellida** | Erpobdellidae | – | 4.0 | – | – | – | – | – | – | – |
| | Hirudinidae | 0.8 | – | – | – | – | – | – | 26.0 | – |
| **Basommatophora** | Planorbidae | 0.8 | 6.0 | – | – | – | – | 0.4 | 4.4 | 3.0 |
| **Caenogastropoda** | Pachychilidae | 1.6 | 2.0 | 1.1 | – | 16.0 | 43.0 | – | 11.0 | 2.4 |
| **Coleoptera** | Dytiscidae | – | – | – | 1.72 | – | – | – | – | – |
| **Diptera** | Ceratopogonidae | 12.7 | – | – | – | – | – | – | – | 1.8 |
| | Chaoboridae | – | – | – | – | – | – | – | 1.1 | – |
| | Chironomidae | – | 6.0 | 1.1 | 10.3 | – | – | 15.0 | 2.2 | 20.0 |
| | Empididae | – | – | – | 8.6 | – | – | – | – | – |
| | Psychodidae | – | 2.0 | – | – | – | – | – | – | – |
| | Simuliidae | – | – | – | – | – | – | 58.0 | 2.2 | – |
| | Tipulidae | – | – | 1.1 | 5.1 | – | – | – | 3.3 | 0.6 |
| **Decapoda** | Gecarcinucidae | – | – | – | – | 24.0 | – | – | – | – |
| **Ephemeroptera** | Baetidae | 1.6 | – | – | – | 12.0 | – | – | – | – |
| | Caenidae | – | – | 16.5 | – | – | – | – | 8.9 | 7.8 |
| | Leptophlebiidae | 5.6 | – | – | – | – | – | 6.5 | – | 7.8 |
| **Geophilomorpha** | Geophilidae | – | – | – | – | – | – | – | – | 0.6 |
| **Haplotaxida** | Enchytraeidae | – | 16.0 | – | – | 4.0 | – | – | – | – |
| | Naididae | – | – | – | 72.4 | 20.0 | – | – | – | – |
| **Hemiptera** | Coreidae | – | – | – | – | – | – | 0.4 | – | – |
| | Corixidae | – | – | – | – | – | – | 0.4 | – | 0.6 |
| | Mesovelliidae | 0.8 | – | – | – | – | – | – | – | – |
| **Lepidoptera** | Pyralidae | 3.1 | – | 25.3 | – | 8.0 | – | – | – | – |
| **Littorinimorpha** | Pomatiopsidae | – | – | – | – | 4.0 | – | – | – | – |
| | Hidrobiidae | – | – | – | – | – | – | – | 3.3 | – |
| **Lumbriculida** | Lumbriculidae | – | – | – | – | – | 14.0 | – | – | – |
| **Moniligastrida** | Moniligastridae | – | 2.0 | – | – | – | – | 1.1 | 1.1 | 4.8 |
| **Neotaenioglossa** | Thiaridae | – | – | – | – | – | – | – | 1.1 | – |
| **Odonata** | Telephlebiidae | – | – | 1.1 | – | – | – | – | – | – |
| | Corduliidae | – | – | – | – | – | 14.0 | – | – | – |
| | Gomphidae | 3.1 | – | – | – | – | – | – | – | – |
| | Petaluridae | – | – | 1.1 | – | – | – | – | – | – |
| | Platycnemididae | – | – | – | – | – | – | – | – | 0.6 |
| **Phyllodocida** | Nereididae | – | – | – | 1.72 | – | – | – | – | – |
| **Sphaeriida** | Sphaeriidae | – | – | – | – | – | – | – | – | 0.6 |
| **Trichoptera** | Hydrobiosidae | – | – | 17.6 | – | – | – | – | – | – |
| | Hydropsychidae | 0.8 | – | – | – | – | – | 6.1 | – | 3.6 |
| | Lepidostomatidae | – | – | – | – | – | – | 5.0 | – | – |
| | Philorheithridae | 4.8 | – | 6.6 | – | – | – | 4.2 | 22.0 | 19.0 |
| | Polycentropodidae | 23.8 | – | – | – | – | – | – | – | – |

## Variations in macroinvertebrate grouping

The CCA triplot shows a site grouping based on the macroinvertebrate community composition and environmental variables (Figure 3). Several families demonstrate the specific relation to SO1 including a family of Tipulidae, Naididae, Cysticidae, and Nereididae. Within Nereididae, *Tubifex* sp. was encountered at the highest abundance. For the environmental variables, the river depth was the primary factor to group SO1 from other sites. SO2 was like SO1 but was mostly contributed by Tipulidae, Pomatiopsidae, and Corduliidae, and environmental variable of river width. Other environmental variables including temperature and water current correlate to the presence of clean water macroinvertebrate indicators such as Philorheitridae and Chironomidae, as observed in organic type (i.e., O1, O2, O3). Apart from that, conventional type (i.e., K1, K2, K3) and semi-organic type (i.e., SO3) were grouped and largely contributed by 5 families, i.e., Ampullariidae, Pachychillidae, Baetidae, Enchytraidae, and Gomphidae.

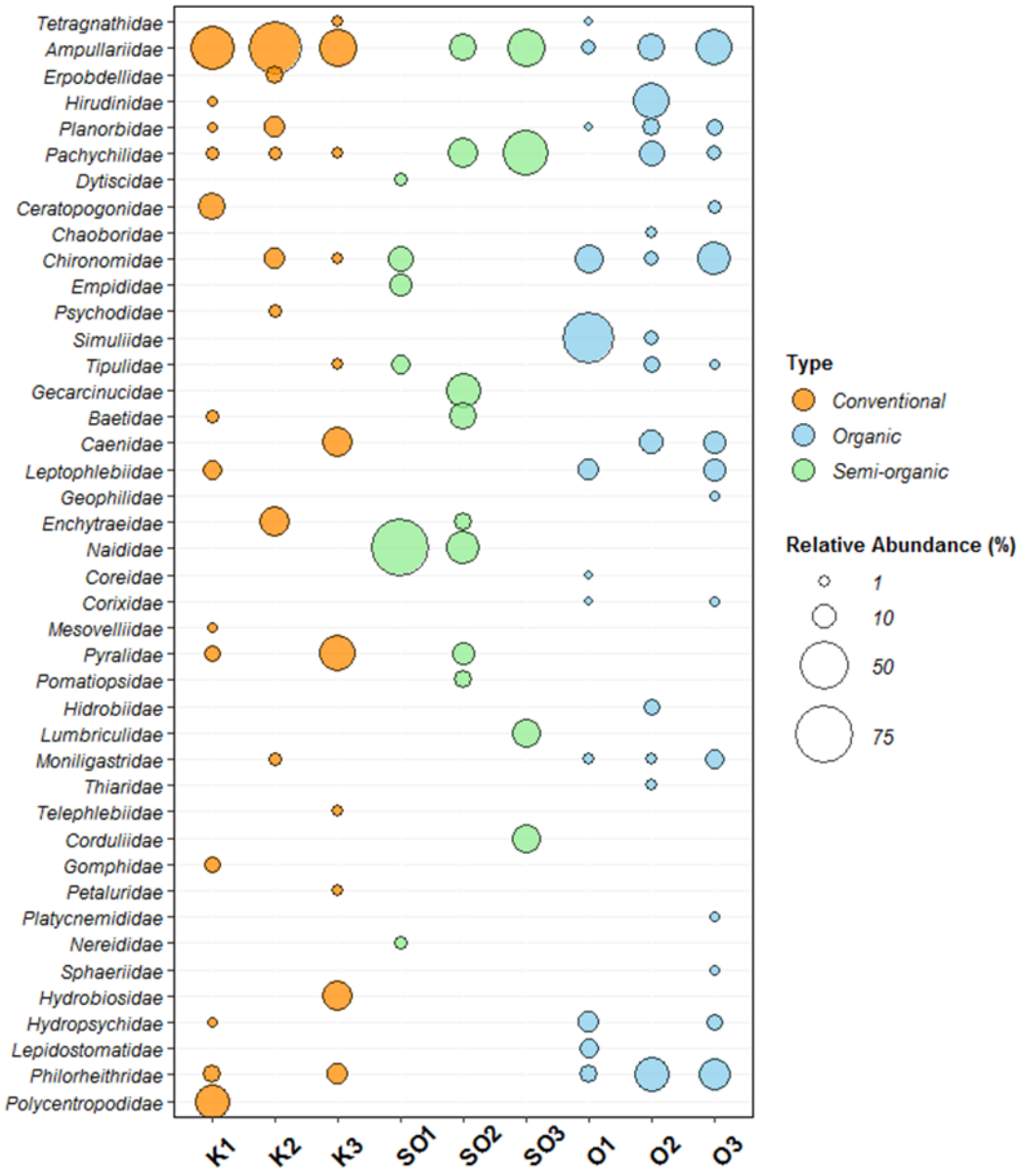


**Figure 2.** Bubble plot depicting relative abundance (%) of macroinvertebrates in each of three agricultural rivers in Jember Regency.

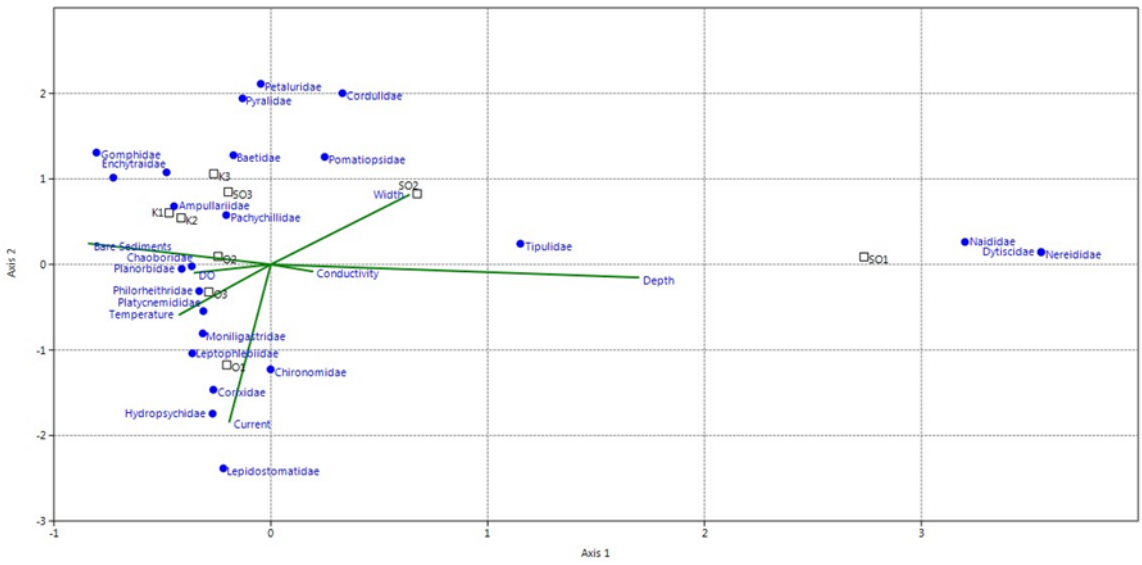


**Figure 3**. Canonical Correspondence Analysis (CCA) triplot showing the site grouping based on macroinvertebrate community composition and environmental variables from three agricultural rivers in Jember Regency

## Ecological indices and correlation with environmental variables

The total individual in organic type was relatively higher among other agricultural types with the highest individual were in O1, followed by O3 and K1. The number of taxa in organic type was found to be higher than other agricultural types with the highest taxa richness in O3, followed by O2 and K1. The total individual and taxa richness is further confirmed in the Shannon diversity index and Margalef species richness shows the highest in O2, suggesting a stable diversity and integrated taxa. Semi-organic type of SO2 also has a high diversity of taxa, although the total individual was low. Simpson dominancy index shows that all agricultural type has high dominancy especially for K2, SO1, and O1. Six graphs showing the individual, taxa, Shannon diversity index, Margalef species richness, and Simpson dominancy index in each of three agricultural rivers in Jember Regency are shown in Figure 4.

Further investigation on the driver of community structure based on the correlation between community indices (Figure 4) and environmental variables (Table 3) showed that each type of agricultural river shows various patterns and correlations (Figure 5). In the conventional river, bare sediment is negatively correlated with all of the indices and factors. Depth is also negatively correlated with individuals, taxa, and Margalef species richness, with the addition of temperature that is negatively correlated with the Shannon diversity index. On the contrary, width, dissolved oxygen, and pH are positively correlated with all the indices and factors. In addition, water current is positively correlated with all the community indices and factors, except for the Simpson dominancy index.

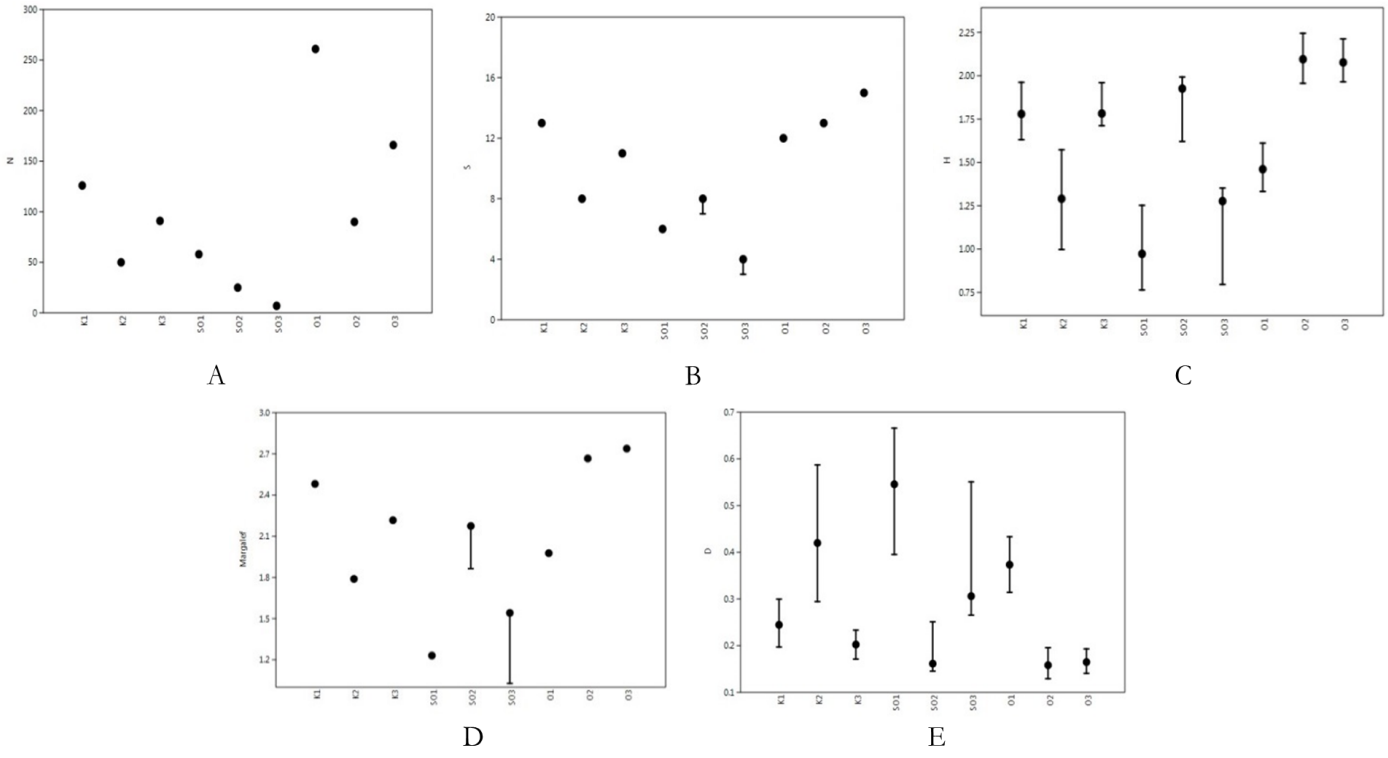


**Figure 4.** Graphs showing five ecological indices including: A) individual, B) taxa, C) Shannon diversity index, D) Margalef species richness, and E) Simpson dominancy index of macroinvertebrate in each of agricultural rivers in Jember Regency.

In the semi-organic river, width and conductivity are negatively correlated with Shannon diversity index, Simpson dominancy index, and Margalef species richness. Besides, water current, depth, and pH are negatively correlated with the Simpson dominancy index. Dissolved oxygen is negatively correlated with taxa and individuals, while bare sediments and temperature are negatively correlated with individuals. Out of 5 indices and factors, all the abiotic factors are positively correlated with individuals and the Simpson dominancy index only.

In the organic river, water current, temperature, and pH are negatively correlated with almost all the community indices and factors. In contrast, width is positively correlated with all the community indices and factors, whereas water current, temperature, bare sediments, conductivity, and depth, are positively correlated with several community indices and factors. The correlogram across the various agricultural river is shown in Figure 5.

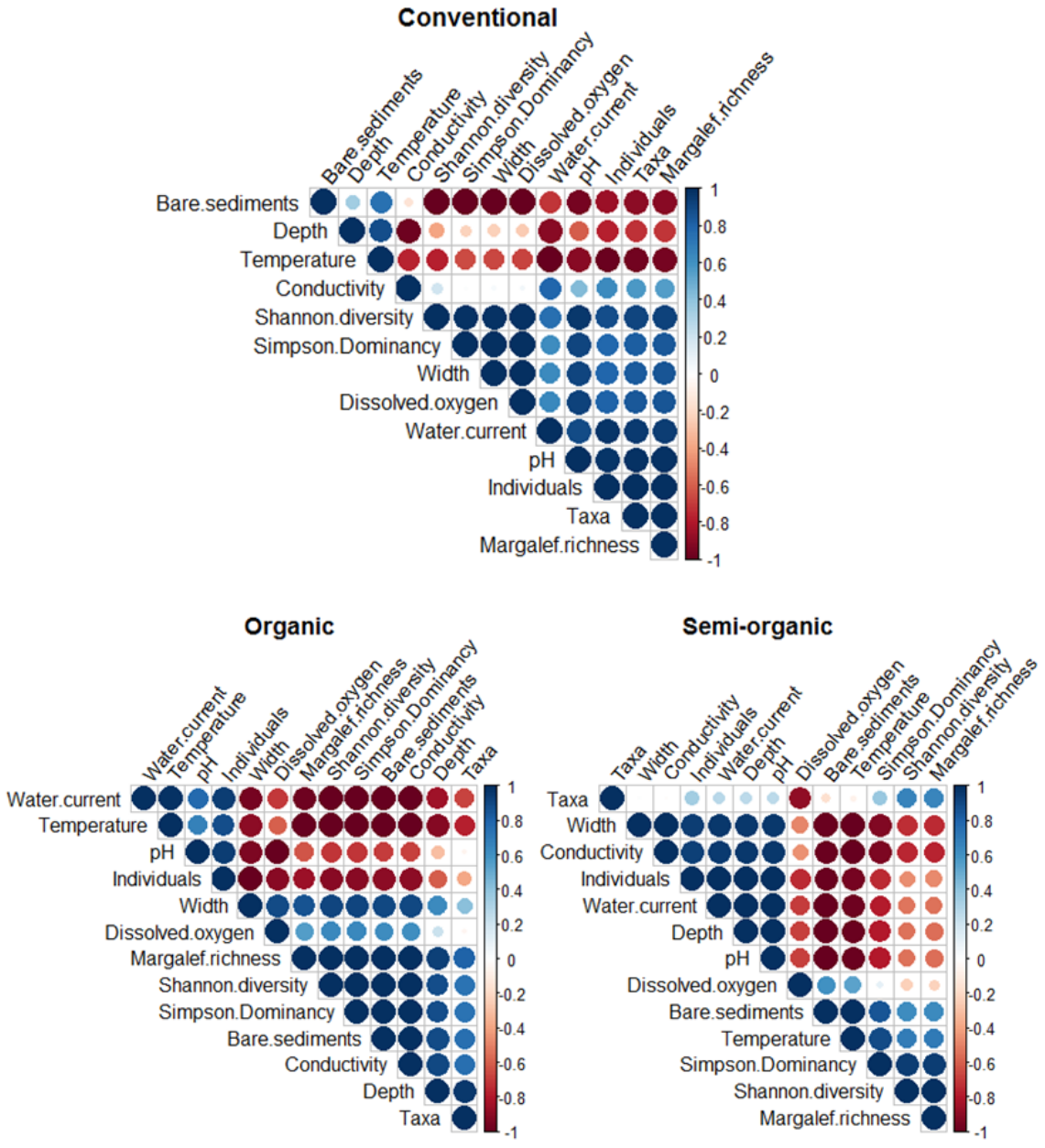


**Figure 5.** Correlation matrix shown in a Correlogram plot using community indices and environmental variables of macroinvertebrates analyzed for each type of agricultural river in Jember Regency.

## Discussion

Based on relative abundance (Table 3; Figure 2), it is revealed that the agrochemical exposure may potentially suppress the macroinvertebrate abundance in conventional and semi-organic types, except for the family of Ampullariidae and Pachychilidae. The macroinvertebrate composition in organic type showed the dominance of specific taxa, although a high abundance of Ampullariidae across three types of agricultural rivers was still observed. In organic type, the dominance of this family was compensated by a higher diversity of macroinvertebrate taxa compare to the conventional type or semi-organic type. As the CCA triplot suggests (Figure 3), the absence of several taxa in the macroinvertebrate community might be influenced by the decrease of dissolved oxygen produced from the roots of the nearby vegetations. Roots may provide an oxygen supply through the decomposition of organic substances. Moreover, the dissolved oxygen from the re-aeration process is difficult to occur in a river with gentle wind and slow-streamed water, as observed in conventional and semi-organic rivers (Table 2). These factors resulting in the decreased of macroinvertebrate composition but invites the tolerant macroinvertebrate which can survive in hypoxic conditions. In accordance with our results, Toft et al. (2003) report the increase of tolerant macroinvertebrate in hypoxic and low dissolved oxygen condition; De Marco et al. (2001) report the increase of macroinvertebrate density which is related to the increase of temperature, conductivity, and neutral pH; and De Neiff & Carignan (1997) report that *Eichhornia crassipes* occurrence has a positive correlation with the dissolved oxygen concentration and conductivity, but negatively correlated with temperature and turbidity.

Based on community indices, it showed that intensive pesticide utilization in conventional type (i.e., K2) significantly decreased the macroinvertebrate diversity (Figure 4C). It might be due to the utilization of inorganic fertilizers, pesticides, fungicides, or other agrochemicals. As previously reported, inorganic fertilizer used in conventional type (i.e., Phonska®) was responsible to cause blooming algae (Ghaly & Ramakrishnan 2015) and water drinking contamination (Follett & Hatfield 2001). The semi-organic type shows different influence patterns. The low diversity of macroinvertebrate taxa in SO1 (Figure 4C) is likely due to the close distance to adjacent conventional rivers, vulnerable to the agrochemical exposure indicated by low dissolved oxygen and high conductivity (Table 2). Nonetheless, it shows the increase of macroinvertebrate diversity especially in SO2 (Figure 4C).

It was previously reported that reducing the use of agrochemicals may increase macroinvertebrates and the quality of other soil biotic (Berenzen et al. 2005; Musonge et al. 2020). Still, the use of active substance Zole© (difenoconazole; triazole group fungicides) in conventional and semi-organic types could decrease the macroinvertebrate diversity evidenced by the absence of some clean water species indicator in organic type (Table 3). It might be due to the properties of difenoconazole having stable photochemistry and a low biodegradability rate. The previous study shows that these fun-

gicide groups caused physiological and cytotoxic effects in ciliate protozoa, *Tetrahymena pyriformis*, based on the observation of morphology, behavior, and regeneration time (Maurya et al. 2019). In SO3, the reduced quality in ecological indices is likely due to the close distance to the adjacent conventional rivers, like SO1. The organic type shows the highest abundance mainly contributed by the Ampullariidae family. Within this family, the Golden apple snail (*Pomacea canaliculata*) has wide toleration across various agricultural types. The high abundance of this species demonstrate that it could fastly reproduce, has a low number of natural predators, and could suppress other species population by occupying the same ecological niche (Estebenet & Martín 2002; Yusa et al. 2006; Joshi et al. 2017), ultimately known as invasive species in Asia (Naylor 1996; Pallett 2016; Joshi et al. 2017). The location of the organic type was far isolated from agrochemicals exposure resulting in a high diversity of macroinvertebrates (Bickham et al. 2000), especially in O2 and O3. It can be concluded that the impact on the macroinvertebrate community was less detrimental in organic type than other sites with intensive agrochemical exposure (Kartikasari 2013).

Based on the correlation matrix (Figure 5), it can be observed that width and water current are concurrently involved in increasing the community indices and factors (positively correlated) across each type of agricultural type. Interestingly, dissolved oxygen and pH are observed to influence the community indices and factors in conventional type (positively correlated), but not observed in semi-organic and organic types (negatively correlated). Correlation matrix among the community indices and factors, and abiotic factors shows that some of the factors did correlate well to each other, and some of the others did not, suggesting a complex abiotic response to taxa variations.

## CONCLUSION

Based on our results on macroinvertebrate composition, it is suggested that it is important to use sensitive taxa-group and community indices as an indicator of environmental changes. Several families, i.e., Tipulidae, Naididae, Cysticidae, and Nereididae demonstrated relation to semi-organic type. For environmental variables, i.e., temperature and water current, may correlate to the presence of clean water indicator species such as Philorheitridae and Chironomidae, as observed in organic agricultural rivers. Conventional and semi-organic agricultural rivers were grouped and largely contributed by the 5 families including: Ampullariidae, Pachychillidae, Baetidae, Enchytraidae, and Gomphidae. Complex interaction between macroinvertebrate community and environmental variables were found by Correlogram plot. It can be concluded that the intensive use of agrochemicals may lead to a deterioration of environmental services and quality of freshwater community and environment.

## AUTHORS CONTRIBUTION

A.S.K. collected, analyzed the data, and wrote the manuscript. L.S.. analyzed the data and wrote the manuscript. H.P. designed the research and supervised all the process.

## ACKNOWLEDGMENTS

We express our gratitude to Mr. Yusnan and Tani Jaya II Farmers Group who have assisted in the field. We thank the University of Jember for the *Penelitian Dosen Pemula* (PDP) Internal Grant to support this research.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the content and there is no financial interest to report. We declare that the manuscript is original work, and is not under review at any other publication.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb62239
DOI: 10.22146/jtbb.62239

Research Article

# Biostratigraphy and Paleobathimetry Microfossil Foraminifera in the Sentolo Formation on the Jambon Line, Bantul Regency, Special Region of Yogyakarta Province

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**Keywords:**
biostratigraphy
foraminifera
paleobathimetry
Sentolo Formation
**Submitted:**
15 December 2020
**Accepted:**
21 August 2021
**Published:**
14 March 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT
Foraminifera microfossils can be used to determine the age of rocks and the depositional environment of an area. The research location is part of Sentolo Formation. Our stratigraphic data located on the Jambon section, Bantul Regency, Special Region of Yogyakarta Province. The appearance of the research area is in the form of well-exposed and ideal cliffs and the lithology of the formation has the potential for rock content rich in foraminifera microfossils. This is the reason for the microfossil analysis of planktic and benthic foraminifera in the study area. The purpose of this study is to determine the age and depositional environment. The research method was carried out by measuring the stratigraphic sections, sampling, and doing paleontological analysis based on planktic and benthic foraminifera. The results showed that the biostratigraphy can be divided into *Globigerina venezuelana* Zone (N18) & the *Globorotalia plesiotumida* Zone (N19), as well as the paleobathimetry, belongs to upper - lower bathyal.



## INTRODUCTION
Foraminifera is unicellular organisms from the phylum Protozoa with pseudopods (pseudopodia). The Sentolo Formation is one of the formations in Bantul Regency with rock constituents in the form of agglomerates and marl at the bottom and gradually turning into limestone. The rocks are layered and abundant in planktic and benthic foraminifera microfossils (Rahardjo et al. 1995).

In non-taxonomic classification (based on habitat) foraminifera can be divided into two, there are planktonic foraminifera and benthic foraminifera. Planktic foraminifera lives on the surface of sea waters while benthic foraminifera live on the suitable substrate. Planktic foraminifera can be used for dating while benthic foraminifera fossils can be used to determine biofacies of the ancient depositional environment.

The Jambon route is in Jambon, Argosari Village, Sedayu District, Bantul Regency, Yogyakarta Special Region Province. This line is the upper part

of the Sentolo Formation. The path appearance is in the form of well-exposed cliffs and is ideal for microfossil analysis for planktonic and benthic foraminifera. This encourages the author to research because from the foraminifera microfossil analysis, the abundance of foraminifera can be seen as well as determining the age and depositional environment in the Jambon Line.

## MATERIALS AND METHODS

### Materials

The sampling location is in Jambon, Argosari Village, Sedayu District, Bantul Regency, Yogyakarta Special Region Province. The samples were prepared and analysed at the Palaeontology Laboratory, Department of Geological Engineering, Faculty of Engineering, Universitas Gadjah Mada. The tools used in the field are geological hammers, tape meters, markers, and chest boards while the materials used are HCl, zip lock, labels, and stationery. The tools used in the laboratory are plastic bottles, porcelain pounder, filter/mesh (sizes 991, 850, 351, 175 millimetres), heater (oven) while the materials used are a solution of soapy water, blue methyl solution, and watered.

### Methods

This research was carried out in several stages, such as the creation of stratigraphic columns, collection, and grouping of rock samples, rock sample preparation, identification, and analysis of foraminifera.

#### Making Stratigraphic Columns

The stratigraphy on the Jambon Line has measured a scale of 1:10 in 2 dimensions on the stratigraphic column form. Retrieval of stratigraphic measurement data using the Jacob Staff method generates data stratigraphic measurements ± 10.8 meters thick. Written information about the sedimentary structure, rock composition, rock characteristics including colour, strike/dip of layer, and thickness of the rock (Figure 6).

#### Rock Sampling and Grouping

Sediment sampling uses a continuous sampling technique. Each sediment sample to be taken is dripped with 0.1 M HCl solution to determine the presence of carbonate content in the rock. If it is bubbly, 1 kg of rock is taken. Rock samples were taken as much as 1 chunk per meter of the total thickness of the rock. Each sample taken is then stored in a separate zip lock and described the sample code and sample location and date of the collection so that it can be systematically arranged.

#### Rock Sample Preparation

The sample preparation process serves to separate the microfossils that are in the sediment from other materials that cover the microfossils. The fresh samples were crushed and weighed about ± 500 grams, then the samples were

cleaned with a detergent solution for 15 minutes. After that, the samples were washed using a mesh filter under running water to remove the sludge. After cleaning, the samples were dried in the sun until the samples were dry. The filter that has been used is then immersed in a blue methyl solution so that it can be seen if there is mixing of fossils in the next sample. The dry sample obtained and ready for observation weighs about ± 100 g.

### Identification and Analysis of Foraminifera Data

Foraminifera samples were placed on foraminiferal slides for identification using a binocular microscope and a maximum of 300 individuals were taken in each sample (Hallock et al. 2003). The identification of benthic foraminifera used references from Barker (1960), Jones (1994), Holbourn et al. (2013) as well as for determining the depth range of their habitat for each bathymetric zone. Identification of planktic foraminifera using references Postuma (1971) and Bolli et al. (1985) as well as for determining age. From the identification of fossils, the data obtained are recorded in the fossil list table and distribution chart. The identification results were used for biostratigraphy and paleobathimetry analysis.

## RESULTS AND DISCUSSION

The Jambon route is part of the Sentolo Formation which has quite good outcrops. The measured stratigraphic thickness of the Jambon Line is 10.8 m which is composed of 3 rock facies, that are tuffa-calcareous sandstone, calcareous siltstone, and grainstone (Figure 1). From the measured stratigraphy, 10 samples of rocks were systematically taken from the old layer (bottom) to the younger layer (top). The name of the sample refers to the research location which is in Jambon (JBN). The distribution and abundance of planktic and benthic foraminifera species can be seen in Figure 2 and Figure 5.

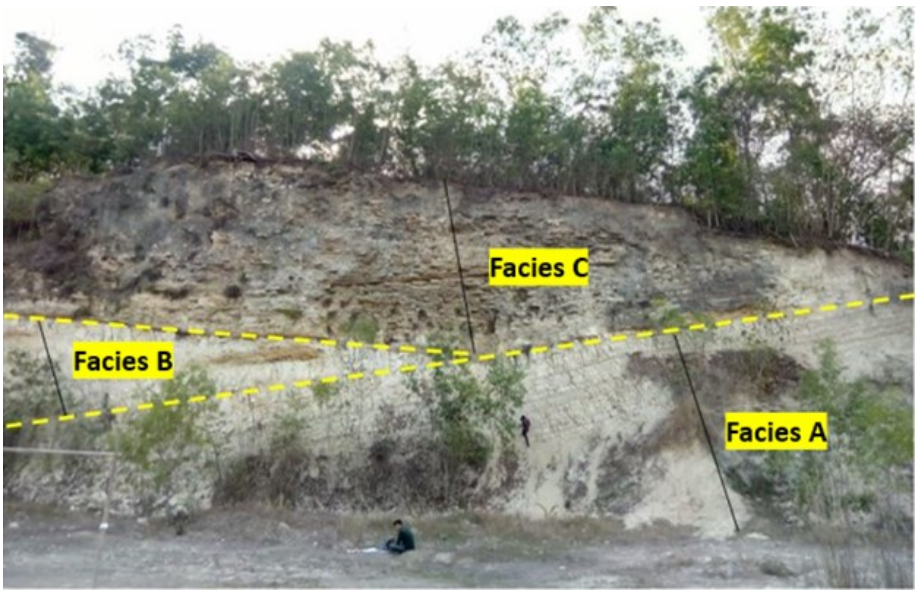


**Figure 1.** Outcrops of tuffa-calcareous sandstone, calcareous siltstone, and grainstone in the Jambon area. There are three facies, that are Facies A (tuffa-calcareous sandstone), Facies B (calcareous siltstone), and Facies C (grainstone).

**Planktic Foraminifera Biostratigraphy (Age Determination)**

| Facies | Fossil Type | PLANKTONIC FORAMINIFERA | | | | | | | | | | | | | | | Abundance | Foraminifera Biodatum | Foraminifera Biozonation | | Age |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Species | *Globigerina praebulloides* | ***Globogerina venezuelana*** | *Globigerinoides altiaperturus* | *Globigerinoides obliquus* | *Globigerinoides primordius* | *Globigerinoides quadrilobatus* | *Globigerinoides sacculifer* | *Globigerinoides trilobus immaturus* | *Globoquadrina altispira* | ***Globorotalia plesiotumida*** | *Globorotalia siakensis* | *Globorotalia tumida tumida* | *Orbulina bilobata* | *Orbulina universa* | *Sphaerodinellopsis seminulina seminulina* | | | This Study (2020) | Blow (1969) | |
| | Samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | | | | | |
| Grainstone | JBN 010 | - | - | 4 | - | - | 5 | - | 2 | 7 | 39 | - | - | - | 29 | - | 86 | | | | |
| | JBN 009 | - | - | 5 | - | - | 3 | - | - | 23 | 30 | - | - | - | 41 | 8 | 110 | | | | |
| | JBN 008 | - | - | 4 | - | 1 | 3 | 2 | 2 | 19 | 20 | - | 1 | 1 | 39 | 5 | 97 | | *Globorotalia plesiotumida* | N19 | Late Miocen - Early Pliocene |
| | JBN 007 | - | - | 19 | - | 4 | 10 | 2 | - | 32 | 51 | - | 3 | - | 73 | 3 | 197 | | | | |
| | JBN 006 | 2 | - | - | 2 | 5 | 26 | - | 1 | 63 | 25 | - | - | 5 | 53 | - | 182 | | | | |
| Calcareous Siltstone | JBN 005 | - | - | 26 | 8 | 8 | 2 | 4 | 2 | 49 | 72 | - | 3 | 5 | 98 | 9 | 286 | | | | |
| | JBN 004 | 1 | 3 | 30 | - | 14 | 4 | 13 | 1 | 37 | 78 | - | - | 3 | 89 | 15 | 288 | *Globogerina venezuelana* | *Globogerina venezuelana* | N18 | |
| Tuffa-calcareous Sandstone | JBN 003 | - | - | 7 | 1 | 9 | 15 | 9 | - | 57 | 75 | - | 9 | 1 | 87 | 6 | 276 | | | | |
| | JBN 002 | - | 2 | - | 4 | - | 41 | 15 | - | 39 | 68 | 1 | 10 | 10 | 87 | - | 277 | | | | |
| | JBN 001 | - | 5 | 4 | 18 | 2 | 14 | - | 3 | 47 | 62 | 1 | 16 | 15 | 100 | - | 287 | | | | |

**Figure 2.** Distribution chart and biozonation Jambon line from planktic foraminifera.

The biostratigraphy reconstruction in the study area was carried out using the planktic foraminifera zoning method. The number of planktic foraminifera found in all samples was 2086 individuals. From the total sample, 15 species of planktic foraminifera were identified (Figure 2). In general, the Sentolo Formation in the study area can be divided into 2 zones, the *Globigerina venezuelana* Zone (N18) and the *Globorotalia plesiotumida* Zone (N19).

*Globigerina venezuelana* (N18) was used as bio datum to delimit the end of zone N18 and the beginning of zone N19. The initial (lower) boundary datum in this zone was not found but the final (upper) boundary datum was found in the JBN 004 sample, which is the end of the emergence of *Globigerina venezuelana*. From measured stratigraphic data, this zone has a thickness of about 4 meters. This sample also found some reworked fossils. The reworked fossils found in the form of species were *Globigerina praebulloides*, *Globigerinoides altiaperturus*, *Globigerinoides primordius*, *Globigerinoides altiaperturus*, and *Globorotalia siakensis*. *Globigerina venezuelana* is thought to have experienced a severe extinction caused by unsuitable environmental conditions. It is marked with no emergence of this species in samples JBN 005 to JBN 010. The morphological appearance of *Globigerina venezuelana* can be seen in Figure 3.

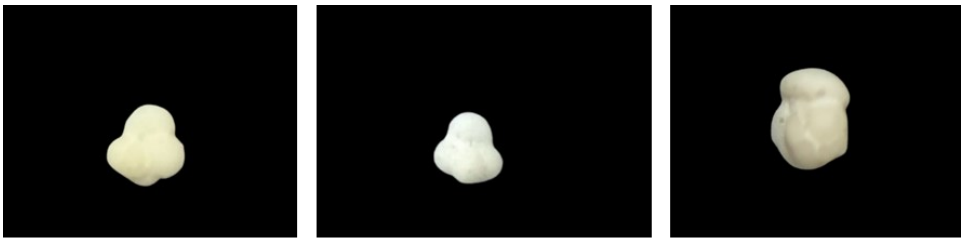


**Figure 3.** *Globigerina venezuelana*: (a) Dorsal, (b) Ventral, (c) Peripheral. (1 mm line scale).

*Globorotalia plesiotumida* (N19) was used as bio datum aged N19. The baseline (lower) and final boundary (upper) datum of this species was not found because this species appeared continuously from samples JBN 001 to JBN 010. From measured stratigraphic data, this zone has a thickness of about 6.8 meters. This sample also found some reworked fossils. The reworked fossils found in the form of species were *Globigerina praebulloides*, *Globigerinoides altiaperturus*, *Globigerinoides primordius*, and *Globigerinoides altiaperturus*. The morphological appearance of *Globorotalia plesiotumida* can be seen in Figure 4.

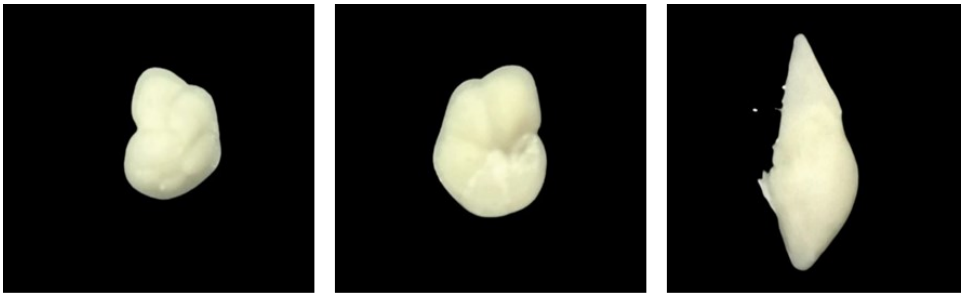


**Figure 4**. *Globorotalia plesiotumida*: (a) Dorsal, (b) Ventral, (c) Peripheral. (1 mm line scale).

The depositional environment of the study area was obtained based on the content of benthic foraminifera in the sample. The number of benthic foraminifera found in all samples was 197 individuals. From the total sample, 20 species of benthic foraminifera were identified (Figure 5). For the classification of the depositional environment, P / B ratio is used (Murray 1976 & Boersma 1983 in Valchev 2003). Also, a species analysis of benthic foraminifera was carried out using an overlapping method of bathymetry on the fossil forms (Figure 5). The P/B ratio of benthic foraminifera in Jambon line is shown in Table 1.

**Biofacies Foraminifera Benthic (Determination of the Precipitation Environment)**

| Facies | Fossil Type | BENTHIC FORAMINIFERA | | | | | | | | | | | | | | | | | | | | Abundance | Paleobathimetry | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Species | *Bolivinita quadrilatera* | *Bricalina subspinescens* | *Cibicides kuelleribergi* | *Cibicidoides wuellerstrofi* | *Dentalina filiformis* | *Elphidium crispum* | *Fissurina bradi* | *Logena sulcata* | *Lenticulina convergens* | *Marsipella cylindrica* | *Melonis affinis* | *Melonis pompiloides* | *Nodosaria sp.* | *Praeoglobulimina pupoides* | *Procerolagena gracilima* | *Pyrgo murrina* | *Rectuvigerina multicostata* | *Sphaeroidina bulloides* | *Stilostomella paleocenica* | *Uvigerina hispida* | | Transition | Inner Neritic | Middle Neritic | Outer Neritic | Upper Bathyal | Lower Bathyal | Abyssal |
| | Samples | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | | | | | | | | |
| Grainstone | JBN 010 | - | - | 3 | - | - | 7 | - | - | - | - | - | - | 2 | - | 9 | - | - | - | - | - | 21 | | | | | | ● | |
| | JBN 009 | - | - | 3 | - | - | - | - | - | - | 2 | - | 1 | - | - | 7 | - | - | - | - | - | 13 | | | | | | ● | |
| | JBN 008 | - | - | 2 | 3 | - | 1 | - | - | 2 | - | - | 1 | - | - | 7 | - | - | - | - | - | 16 | | | | | | ● | |
| | JBN 007 | - | - | 4 | - | - | 1 | 5 | - | 5 | 1 | - | - | - | - | 7 | 2 | - | - | - | - | 25 | | | | | | ● | |
| | JBN 006 | - | - | 9 | 1 | - | - | - | - | 3 | 1 | - | 3 | - | - | 14 | 4 | - | - | - | 1 | 36 | | | | | | ● | |
| Calcareous Siltstone | JBN 005 | 4 | - | - | 1 | - | - | - | - | - | 1 | - | 1 | - | - | 3 | 2 | - | - | 1 | 1 | 14 | | | | | | ● | |
| | JBN 004 | - | - | 1 | 1 | - | - | - | - | - | - | - | 2 | 1 | - | - | 2 | 1 | 2 | - | 2 | 12 | | | | | ● | | |
| Tuffa-calcareous Sandstone | JBN 003 | - | 3 | - | 1 | 1 | - | - | - | - | 1 | 1 | 5 | 2 | 1 | 2 | 1 | - | 1 | 2 | 3 | 24 | | | | | | ● | |
| | JBN 002 | - | - | - | 6 | - | - | 2 | 1 | - | - | - | - | - | - | - | 3 | 3 | - | 8 | - | 23 | | | | | ● | | |
| | JBN 001 | - | - | - | 2 | - | - | - | - | - | - | 1 | 2 | - | - | 5 | 1 | - | 1 | - | 1 | 13 | | | | | | ● | |

**Figure 5.** Distribution chart and paleobathimetry of Jambon Line benthic foraminifera species.

**Table 1.** P/B Ratio of Benthic Foraminifera in Jambon Line.

| Samples | Number of Individual Foraminifera | | | P/B Ratio | |
|---|---|---|---|---|---|
| | Planktic | Benthic | Total | Percentage | Information |
| JBN 010 | 86 | 21 | 107 | 80.37 | Upper bathyal |
| JBN 009 | 110 | 13 | 123 | 89.43 | Upper bathyal |
| JBN 008 | 97 | 16 | 113 | 85.84 | Upper bathyal |
| JBN 007 | 197 | 25 | 222 | 88.74 | Upper bathyal |
| JBN 006 | 182 | 36 | 218 | 83.49 | Upper bathyal |
| JBN 005 | 286 | 14 | 300 | 95.33 | Lower bathyal |
| JBN 004 | 288 | 12 | 300 | 96.00 | Lower bathyal |
| JBN 003 | 276 | 24 | 300 | 92.00 | Lower bathyal |
| JBN 002 | 277 | 23 | 300 | 92.33 | Lower bathyal |
| JBN 001 | 287 | 13 | 300 | 95.67 | Lower bathyal |

The results of the analysis of this benthic foraminifera species have the same depositional environment results as the P / B ratio, which is in bathyal. Based on the P / B ratio, it is found that rock samples JBN 001 - JBN 005 show the lower bathyal environment, and JBN 006 - JBN 010 shows the upper bathyal environment. Benthic foraminifera analysis with the overlap method shows that the JBN 001 sample is in the lower bathyal, JBN 002 is in the upper bathyal, JBN 003 is in the lower bathyal, JBN 004 is in the upper bathyal, and JBN 005 - JBN 010 is in the lower bathyal. This difference indicates sea-level fluctuation in the research path.

*Cibicidoides wuellerstrofi* is a species that reflects the deep-sea environment with active currents (Singh & Rai 2011). The JBN 004 sample contains the species *Nodosaria* sp. as an indicator of the shallow environment that is present along with the species *Cibicidoides wuellerstrofi* (Figure 5). The JBN 002 sample also containing the *Fissurina bradii* species which was present along with the *Cibicidoides wuellerstrofi* species. This indicates a downslope. This condition is supported by the older sample, JBN 003 (tuffa-calcareous sandstone) which has a grain size that is coarser than the sample JBN 004 (calcareous siltstone). The grain size that smooths upwards to the JBN 005 sample characterizes the energy that was initially high then weakened. Shallow benthonic foraminifera can be transferred to the deep ocean when energy is high.

## CONCLUSION

Based on the planktic foraminifera assemblage, the stratigraphic range of the Jambon line in the study area appeared as the Late Miocene to the Early Pliocene, which is between N18 - N19. The research area was divided into 2 zones, the *Globigerina venezuelana* zone (N18) and the *Globorotalia plesiotumida* zone (N19). The depositional environment of the Sentolo Formation based on the content of benthic foraminifera shows that the research area is in the Bathyal Zone (200 - 2000 m) which changes from lower to upper bathyal.

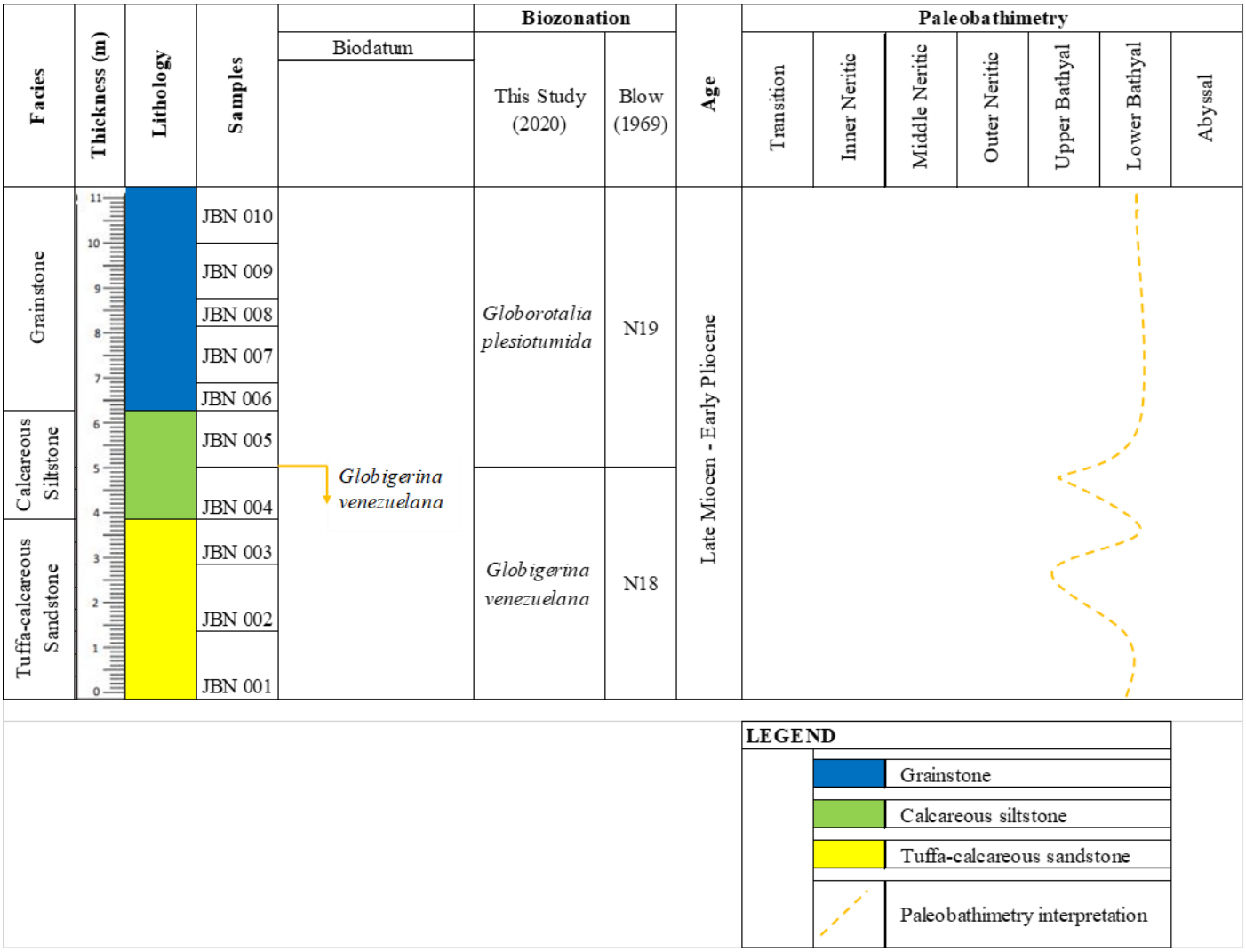


**Figure 6.** Stratigraphic column, biostratigraphy zones, and paleobathimetry of Jambon line.

## AUTHORS CONTRIBUTION

C.F.R. collected and analysed the data and wrote the manuscript, D.S.Y and D.H.B. designed the research and supervised all the process.

## ACKNOWLEDGMENTS

Thank you to the team which helped us in the completion of this study especially the palaeontology laboratory assistant.

## CONFLICT OF INTEREST

There is no conflict of interest regarding the research or the research funding.

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## Erratum

Due to a technical error with the typing of one word in the previous title, this manuscript has changed its title to be "Biostratigraphy and Paleobathimetry Microfossil Foraminifera in the Sentolo Formation on the Jambon Line, Bantul Regency, Special Region of Yogyakarta Province".

The editorial team of Journal of Tropical Biodiversity and Biotechnology would like to apologize for the inconvenience caused by this oversight.

Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb68650
DOI: 10.22146/jtbb.68650

Research Article

# Growth of Kaffir Lime (*Citrus hystrix* DC) Cell Line Derived from Seed Explant After Yeast Elicitation Using Pure and Technical Grade Yeast

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**Keywords:**
cell line
elicitation
kaffir lime
*S. cerevisiae*
technical grade yeast
**Submitted:**
24 August 2021
**Accepted:**
11 December 2021
**Published:**
18 March 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT

The addition of elicitors in kaffir lime (*Citrus hystrix* DC.) culture is one of strategies for obtaining and increasing the production of secondary metabolites. *Saccharomyces cerevisiae* is one of the elicitors that can be used to increase secondary metabolites such as terpenoids. However, in its use, the pure cultures of *S. cerevisiae* are expensive. Therefore, the first objective of this study was to analyze the ability of technical grade (commercial baker's yeast) to be used as an elicitor and measure the growth of kaffir lime cell line after being elicited by pure and technical grade (commercial baker's yeast). The second objective is to determine the best time to subculture kaffir lime cell line after elicitation. We observed the morphology and measured the growth curve of pure and technical grade yeast until the 4th subculture generation. Furthermore, we used both grades of yeast for elicitation. Kaffir lime cell suspension was treated with 10 ppm pure grade or 5 ppm and 10 ppm technical grade yeast for 4 days. After elicitation, kaffir lime cell lines were subcultured and their growth was analyzed. The result showed that the morphology and growth curve of technical grade until 4th subculture generations was similar to the pure grade. On the other hand, after elicitation using pure and technical grade yeast and being subcultured, the growth of the elicitated kaffir lime cell line had the same pattern as the control group, but the cell density of the control group was higher than the elicitated group. The initial stationary phase of kaffir lime cell line was on the 17th day which is the best time to subculture. The subculturing process is important to maintain the viability of the kaffir lime cell line.



## INTRODUCTION

Kaffir lime (*Citrus hystrix* DC.) leaf extract has the potential as an anticancer (Tunjung et al. 2015). However, the use of the extract from nature has several obstacles such as overexploitation of kaffir lime leaf and the production of

bioactive compounds that are strongly influenced by environmental conditions. One method for producing bioactive compounds can be done by using tissue culture techniques (Bourgaud et al. 2001). Unfortunately, the type and level of bioactive compounds in kaffir lime callus extract were less than in the leaf extract. Therefore, kaffir lime cell culture requires an elicitor to increase the production of bioactive compounds.

Elicitor is a biotic or abiotic compound that induces the synthesis of other specific compounds that are used for defense mechanisms in plants (Murthy et al. 2014; Ramirez-Estrada et al. 2016). Biotic elicitors consist of living organisms such as fungi, bacteria, and herbivores. Inorganic compounds such as heavy metals, pesticides, detergents, or physical factors (e.g. cold shock, UV light, and high pressure) are examples of abiotic elicitors. These biotic and abiotic elicitors trigger the enzymatic activities in plant stress responses (Gueven 2003; Ramirez-Estrada et al. 2016).

*S. cerevisiae* is a yeast that is able to increase the number of terpenoid compounds in some plants. Treatment by *S. cerevisiae* with a concentration of 1.5% for 72 h was able to increase the number of gymnemic acid by 9.3 fold - in the suspension culture of *Gymnema sylvestre* (Chodisetti et al. 2013). Furthermore, *S. cerevisiae* increase ajmalicine content in the cell aggregate culture of *Catharanthus roseus*. The number of ajmalicine was increased to 25.288 ± 0.102 jig/g DW after being treated with *S. cerevisiae* with a concentration of 0.5% for 24 h (Ratnasari et al. 2001). Moreover, according to Pereira et al. (2007), the production of triterpenes was increased after the addition of *S. cerevisiae* to the cell suspension culture of *Tabernaemontana catharinensis*.

Furthermore, the use of *S. cerevisiae* as an elicitor has several advantages such as it is easy to grow, has a short life cycle, can grow at low pH, and is safe for health because it is non-toxic (Sitinjak et al. 2000). On the other hand, in its use, the pure culture of *S. cerevisiae* has several disadvantages including not being sold freely, impractical, difficult to carry from one place to another because it must be in aseptic conditions, and expensive. Hence, we need another type of yeast elicitor that has the same ability as *S. cerevisiae*. Commercial baker's yeast consists of *S. cerevisiae* and has several advantages, including easy to find, easy to use, easy to carry because it is in powder form, and has an affordable price. However, no scientific report about commercial baker's yeast can be used as an elicitor.

Kaffir lime (*C. hystryx*) shows the potential to be used as a traditional medicine for several diseases such as cancer. In our previous study, we found that terpenoids are detected in the first subculture 35 days (control group) and callus preserves in 4°C of kaffir lime callus from seed explants. The type of terpenoids were squalene and geranyl acetate, whereas geranyl linalool was found in kaffir lime callus preserve in 4°C with alginate encapsulation. Furthermore, some compounds that act as anti-cancer were also detected in preserved callus such as lauric acid, palmitic acid, stearic acid, 1-decanol, undecylenic acid, oleic acid, 2H pyran-2-one, octadecane, 1-hexcosanol, hexane, methane, dodecane, tetracosane, 2 decenoid acid, and 3-dodecane

(Fajarina et al. 2021). Because of its capability to synthesize secondary metabolites especially terpenoids, kaffir lime callus, or callus cell suspension can be used as raw materials for traditional medicine. The number of terpenoid compounds that have the potential of anti-cancer needs to be increased. One method that can be used to increase the production of secondary metabolites is using elicitation in cell suspension culture.

Cell suspension culture contains a population of cells with a fast growth rate. Good culture conditions make the reproduction of cell suspension cultures suitable for increasing secondary metabolites production (Moscatiello et al. 2013). During incubation, the cell suspension culture was shaken using a shaker to make the single cells could divide and increase the aeration. Agitation or shaking in cell suspension cultures can increase the aeration to maintain cell viability during the incubation period (Dwimahyani 2007). Our previous study Damayanti et al. (2020) succeeded in optimizing the growth of kaffir lime suspension cells in MS liquid media with a concentration of 2 ppm 2,4-D addition. This condition was suitable for yeast elicitation. On the other hand, the synthesis of compounds that have the potential as an anti-cancer by callus or cell suspension needs to be preserved for long-term and large-scale use. So that, we need a method to maintain these compounds. A previous study reported that the cell suspension subculture method can stabilize alkaloids. Alkaloid production from two cell lines of *Tabernaemontana divaricata* cell suspension culture showed a maximum amount in the 4$^{th}$ subculture after changing it with the same medium and stabilized on a higher level than found in the original cell lines (Sierra et al. 1992).

The first objective of this study was to analyze the ability of technical grade (commercial baker's yeast) to be used as an elicitor and measure the growth of kaffir lime cell line after being elicited by pure grade (pure culture of *S. cerevisiae)* and technical grade (commercial baker's yeast). The second objective is to determine the best time to subculture kaffir lime cell line after elicitation.

## MATERIALS AND METHODS

### Materials

Kaffir lime fruit was obtained from kaffir lime orchards in Kaliduren Village, Candirejo, Borobudur, Magelang Regency, Central Java. We used fresh fruit, and the diameter of the fruit was approximately 5-6 cm, the length and width of the seed were approximately 0.7 - 1 cm and 0.3 – 0.5 cm, respectively. The pure cultures of *S. cerevisiae* were taken from the Center Studies for Food and Nutrition of Universitas Gadjah Mada whereas commercial baker's yeast powder was bought from a supermarket.

## Yeast Observation

### Culture medium

Pure cultures of *S. cerevisiae* and technical grade were grown and subcultured once a week on Peptone Glucose Yeast (PGY) extract solid media. Pure and technical grade subculture was carried out four times to obtain the fourth subculture (G4).

### Morphological observation

Pure grade and technical grade culture were transferred into PGY liquid media for 24 hours. After 24 hours, 1 ml of yeast was taken from the liquid medium for morphological analysis using a binocular microscope at 100x magnification.

### Growth measurement

One use of one week old pure cultures of *S. Cerevisiae* and technical grade / commercial baker yeast were inoculated into 10 ml of PGY liquid medium then they were incubated at room temperature without being shaken. Cells were harvested at 2 h to 24 h time intervals to determine growth curves. The cell number was calculated using a spectrophotometer at a wavelength of 660 nm, with 3 replicates.

## Growth of Kaffir Lime Cell Suspension

### Medium Preparation and sterilization of explant

The tissue culture method was conducted according to Damayanti et al. (2020). The basal medium is Murashige and Skoog (MS) (Damayanti et al. 2020) containing myo-inositol, it is added with sucrose (30 g/l), agar (8 g/l), and 2 ppm concentration of 2,4-D and distilled water. The medium pH was adjusted to 5,8 using 1 N HCl or 1 N KOH. Then, the medium was sterilized using the autoclave at 121$^{o}$C with pressure 1 atm for 15 min.

Kaffir lime seeds were removed from the fruit then sterilized using sodium hypochlorite 5.25% for 5 min. The seeds were washed with distilled water 2 times for 5 min each. After sterilization, the seed explants were transferred to sterile petri dishes lined with filter paper.

### Callus Induction

This protocol was referred to Damayanti et al. (2020). The seed explants were grown on an MS solid medium containing 2 ppm of 2,4-D. Seeds were maintained and stored in the incubation room under dark conditions (25$^{o}$C) until they reached the stationary phase (G0), and then 1$^{st}$ subcultures (G1) were done every 25-30 days.

### Cell Suspension Establishment

This method is according to a previous study (Damayanti et al. 2020). Kaffir lime callus G1 at 25-30 days (early stationary phase) was transferred to 50 ml MS containing 2 ppm 2,4-D for 21 days and shaken at 100 rpm. After 21

days, homogenates were separated from the medium and used as an inoculum for the establishment of suspension culture. Homogenates contain a single cell population, explant debris, and dead cells. To distinguish single cells and dead, we analyze the homogenates under the microscope. Only single cells were subcultured into the fresh medium for 16 days, shaken at 100 rpm. The incubation was carried out at room temperature in dark conditions.

### Elicitation of Cell suspension

Pure and technical grades yeast were inoculated into PGY liquid medium, then incubated at room temperature without shaking. After incubation, yeast cells were autoclaved for 15 min at 121 $^0$C with 1 atm. The yeast cells were centrifuged and the resulting pellets were rinsed and subjected to be used as an elicitor. Elicitation was carried out by adding technical grade (5 ppm, 10 ppm) and *pure grade* (10 ppm) yeast into 50 ml of kaffir lime cell suspension culture. Kaffir lime cell suspension was treated with yeast for 4 days with 3 replicates.

After harvested, cell lines were filtered using a 100 µm nylon filter and washed using MS liquid medium to ensure that there was no yeast contamination inside cell suspension. The filtered cells were subcultured into 40 ml of MS containing 2 ppm 2,4-D. The growth of cell lines and the control group were measured using a Neubauer hemacytometer for 27 days.

## RESULTS AND DISCUSSION

### The growth of yeast

In this study, we optimized technical grade yeast as an elicitor. Commercial baker's yeast is a technical grade yeast that is very easy to obtain, widely traded, and has an affordable price (one tube pure culture's price of *S. cerevisiae* is Rp 500.000,00 while one sachet of commercial baker / technical grade yeast is Rp 5.000,00). Therefore, in this study, we compared these two types of yeasts to see their ability as an elicitor.

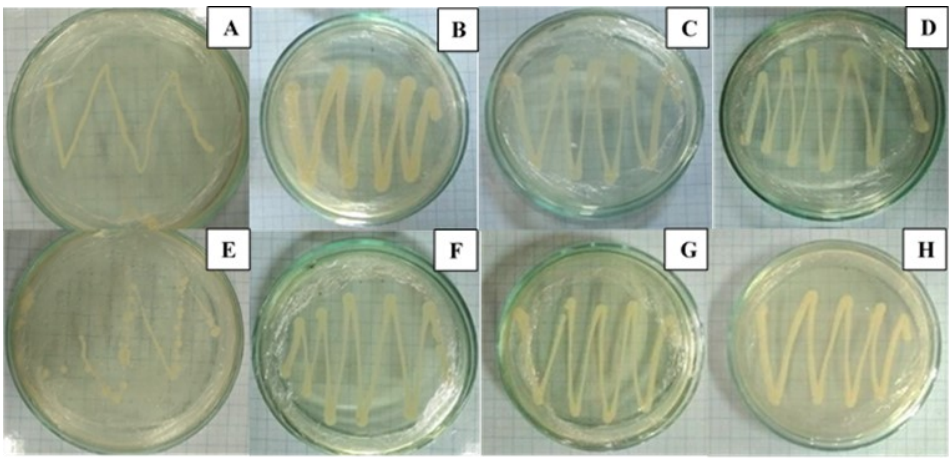


**Figure 1.** The growth of yeast on PGY solid media.
Pure grade G0(A), G1(B), G2 (C),G4 (D); technical grade G0( E ), G1 (F), G2 (G) -G4 (H). (G0: before subculture; G1: 1$^{th}$ subculture; G2: 2$^{nd}$ subculture; G4: 4$^{th}$ subculture).

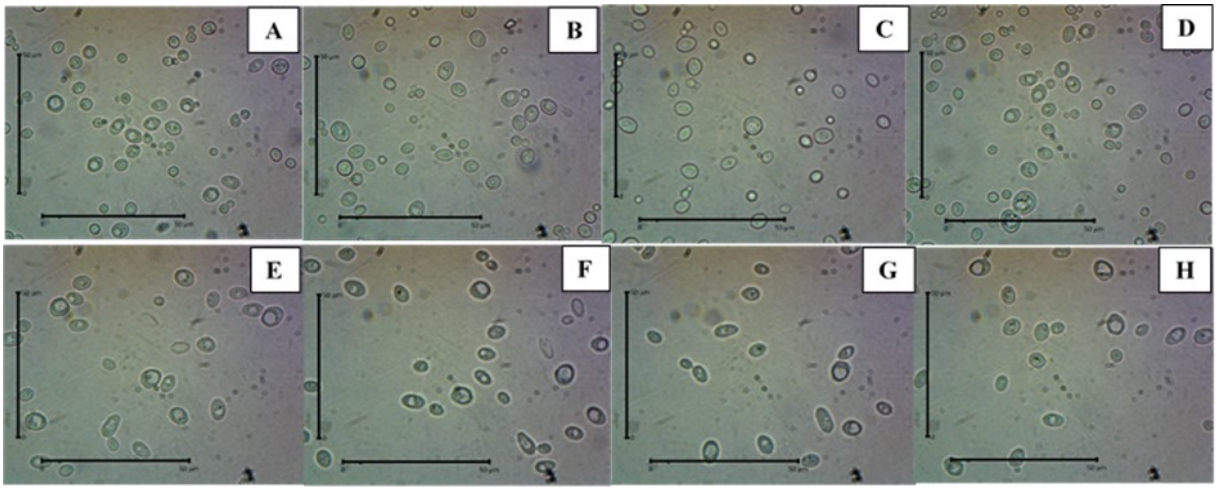


**Figure 2.** Morphology cell of pure and technical grade yeast after subculture every 7 days.Pure grade at G0 (A), G1 (B), G2 (C)-G4 (D); technical grade yeast at G0 (E), G1 (F), G2 (G)-G4 (H). (G0: before subculture; G1: 1st subculture; G2: 2nd subculture; G4: 4th subculture) At 100× magnification.

Figure 1 showed that technical grade yeast is stable during subculture until G4. This study is the first scientific using technical grade yeast as an elicitor in tissue culture. We subcultured the yeast until the 4th generation because we wanted to ensure its viability and growth stability. Technical grade yeast contains *S. Cerevisiae* and an emulsifier (sorbitan monostearate E491), affecting yeast growth. If the growth of yeast could be stabilized until the 4th subculture, we assumed that technical yeast grade is able to grow well. Thus it can be used as an elicitor. The result of morphological observations of two types of yeast (pure and technical gradeculture ) can be seen in Figure 2.

The cells of *S. cerevisiae* pure culture and technical grade yeast cultures exhibited similar shapes and size (Figure 2 and Table 1). They have spherical, oval, and elongated shapes. The size of pure and technical grade before culture and after 4th sub culture was similar. The results are consistent with the description of *S. cerevisiae* by Montes de Oca (2016). This data showed that technical grade yeast is referred to as an elicitor candidate for kaffir lime cell suspension culture. However, the morphological characters cannot be used to identify and compare the yeast because the Saccharomycetaceae family consists of members with similar cell morphology. Therefore, further research with more accurate methods such as Biochemical and DNA sequencing methods is needed.

**Table 1.** Size of pure and technical grade yeast cell before subculture and after 4th subculture.

| Grade of yeast | Subculture phase | Diameter of cells (μm) |
|---|---|---|
| Pure culture | Before subculture | 6,36 ± 0,12 |
| Pure culture | after 4th sub-culture | 6,01 ± 0,21 |
| Technical grade | Before subculture | 6,55 ± 0,47 |
| Technical grade | after 4th sub-culture | 6,21 ± 0,46 |

Furthermore, we measured the yeast growth curve. This curve was to determine the best time to apply yeast on the kaffir lime cell suspension as elicitation treatment begin. The results can be seen in Figure 3.

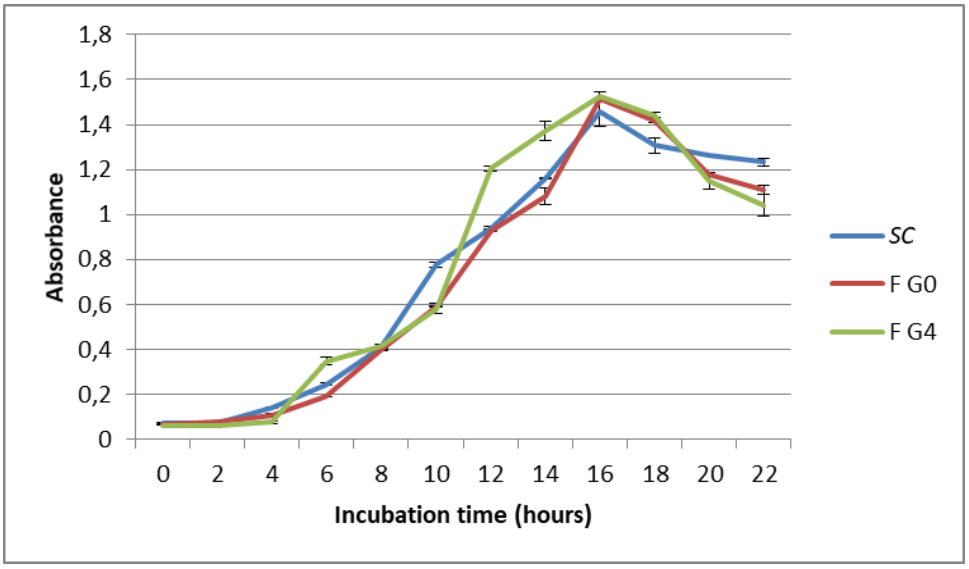


**Figure 3.** Growth curves of pure and technical grade yeasts.
Note: SC = Pure culture of *S. cerevisiae*; F = Technical grade yeast; G0 = before culture; G4 after 4th subcultur.

Sigmoid curves were achieved when both yeasts reached their maximum growth (Figure 3). The peak period of both pure and technical grade of yeast was at the 16th hour. After peak growth, cells reached their stationary phase. This peak period is the best time to harvest the yeast cells for the elicitation process. According to Klis et al. (2002), In this phase, yeast cells are at the highest growth, and the yeast cell wall, the elicitor component of the yeast is well-formed and thicker and considerably has higher turgor pressure compared to exponentially growing cells. Furthermore, at the stationary phase, the rate of yeast cells divisions is declining thus extra energy is allocated to form a compact cell wall structure (Aleu et al. 1999). According to Chen and Chen (2000), the response of plant cells to elicitors is directly related to the composition of the yeast cell wall, especially glucan, which can be recognized by plant cells as stress, so that plant cells will respond by producing secondary metabolites.

## The Comparison Growth of Cell Line After Yeast Elicitation

A Friable callus is needed as a raw material for cell suspension. The friable texture of callus facilitates the separation between cells into a single cell in cell suspension culture (Damayanti et al. 2020). The addition of 2,4-D to the culture medium caused the middle lamellae of the plant cell wall to break, thereby promoting the bonding between cells to break off and form a crumbly callus (Leksonowati et al. 2017). Therefore, 2 ppm of 2,4-D was added into the medium for callus induction and cell suspension. Callus was suspended in a sterile liquid medium containing various nutrients and growth

factor compounds were needed by cells for their growth (Damayanti et al. 2020).

Figure 4 showed the kaffir lime seeds that were used as explants in this study. Kaffir lime seeds that were used for explants were part of the endosperm. Endosperm as a food reserve for embryos contained nutrients in the form of carbohydrates and proteins. These nutrients are needed at the beginning of callus growth (Sukmara et al. 2014). Friable endosperm callus can be used as a raw material for cell suspension culture. According to Pasitvilaiturm and Pankasemsuk (2012), cell suspension cultures of *Jatropha curcas* L. Are able to grow and produce oil (Figure 5). Callus on the 25th day has a crumb texture with a moderate-yellow color (Figure 5A). G1 callus has a crumb texture with a moderate-yellow color (Figure 5B). The friable callus is needed as a raw material for making cell suspension (Damayanti et al. 2020). Therefore, G1 callus is a raw material that was subcultured into a liquid medium to form a single cell aggregate (Figure 5C).

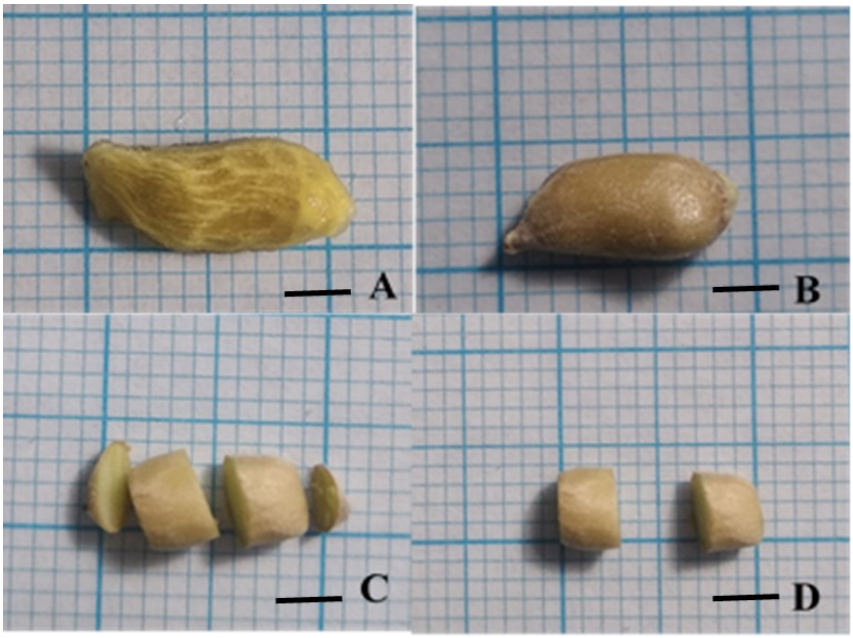


**Figure 4.** Kaffir lime seeds. The seed coat (A); the seed coat is removed (B) The seed is sliced (C); The seed is ready to culture (D). Bars: 0,3 cm.

G1Callus aged 25-30 days (early stationary phase) was transferred into the liquid medium and placed in a shaker for 21 days. In the liquid medium

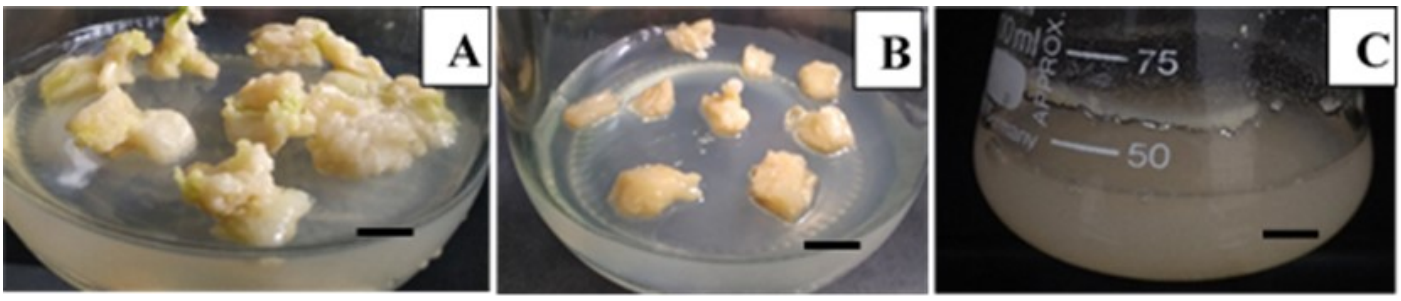


**Figure 5.** Kaffir lime callus and cell suspension. Callus on 25th day (G0) (A); Callus on 25th day (G1) (B); Suspension culture in the flask (C). Bars: 1 cm.

will be found a single cell population, explant debris, and dead cells. The suspensions aged 21 days were subcultured into the new medium for 16 days. On the 16th day, the new cell growth entered the stationary phase. Hence, this is the best time for elicitation (Damayanti et al. 2020).

Cell suspension culture consists of cells population with fast growth rate and good culture conditions make the reproduction of cells suspension culture suitable to increase the production of secondary metabolites (Moscatiello et al. 2013). It was found in mangosteen (*Garcinia mangostana* L.) culture that the production of secondary metabolites in callus treated with 100 µM methyl jasmonate or 0.5 g/l casein hydrolysate as an elicitor for 5 days and 7 days respectively was lower (21 metabolites) than in cell suspension (34 metabolites). These differences in secondary metabolites production may be due to agitation (Jamil et al. 2018). During incubation, the suspension culture was shaken using a shaker so the single cells could divide and increase the aeration. Agitation or shaking in *Jatropa Curcas* Cell suspension cultures can increase the aeration to maintain cell viability during the incubation period (Dwimahyani 2007).

Damayanti et al. (2020) showed that the beginning of a stationary phase in kaffir lime cell suspension was on the 16$^{th}$ day. Cell lines are the cells that are able to survive biotic stress and are expected to be able to produce high levels of metabolites. Cell line suspension subculture is one of the methods that could stabilize the growth of the cell line.

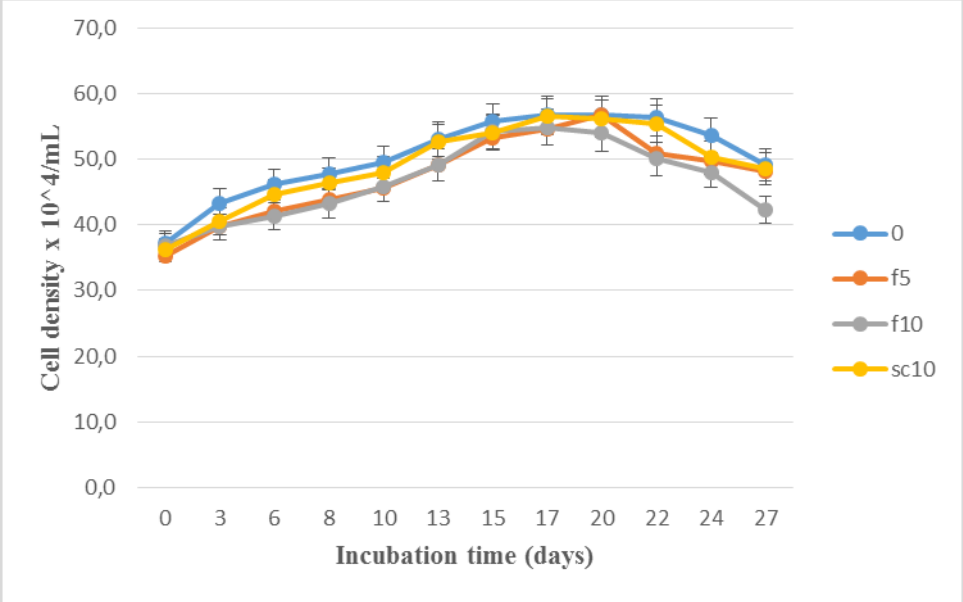


**Figure 6.** Growth curve of kaffir lime cell line after elicitation. F5 and F10 are cell lines that were elicited by technical grade yeast with 5 and 10 ppm respectively; Sc10 is cell line was elicited by pure grade yeast with 10 ppm.

Figure 6 showed the measurement of the cell growth after addition with pure and technical grade yeast. The cell density of the control group was higher than the elicitated group. This is because of screen cells' elicitation and cell lines' formation. High doses of elicitors can induce a hypersensitive

response that causes the cell to death (Namdeo 2007), so we assume that cells that can survive are cells that are resistant to biotic stress and probably be able to produce high levels of metabolites.

The growth phases of kaffir lime cell line after yeast elicitation consists of the lag phase, exponential phase, stationary phase, and death phase (Figure 6). The lag phase occurs on the 0 to 6th day. This phase occurs when the cell line adjusts to the new environment after previously being exposed to elicitors of pure and technical grade yeast. After experiencing the adjustment phase, the cell line starts to actively divide, called the exponential phase. The exponential phase occurs from the 8th day to the 17th day. In that day range, the maximum cellular division occurred. After carrying maximum cellular division, nutrients became limited in the culture medium and cell viability gradually decrease to reach the stationary phase. The graph above shows that the stationary phase starts on the 17th day until the 20th day. After the stationary phase, the nutrient content in the medium became exhausted and the toxic substances will be produced by the cells as a defense mechanism from stress (Bhojwani & Razdan 1983 in Khanpour-Ardestani et al. 2015). After that, decreasing amount of the cell density can be seen from the 24th day until the 27th day. This phase is called the death phase.

The morphology of kaffir lime cell line can be seen in Figure 7. There were three cell shapes were observed, namely spherical, comma, and elongated shapes. During the incubation period, cells change in shape due to

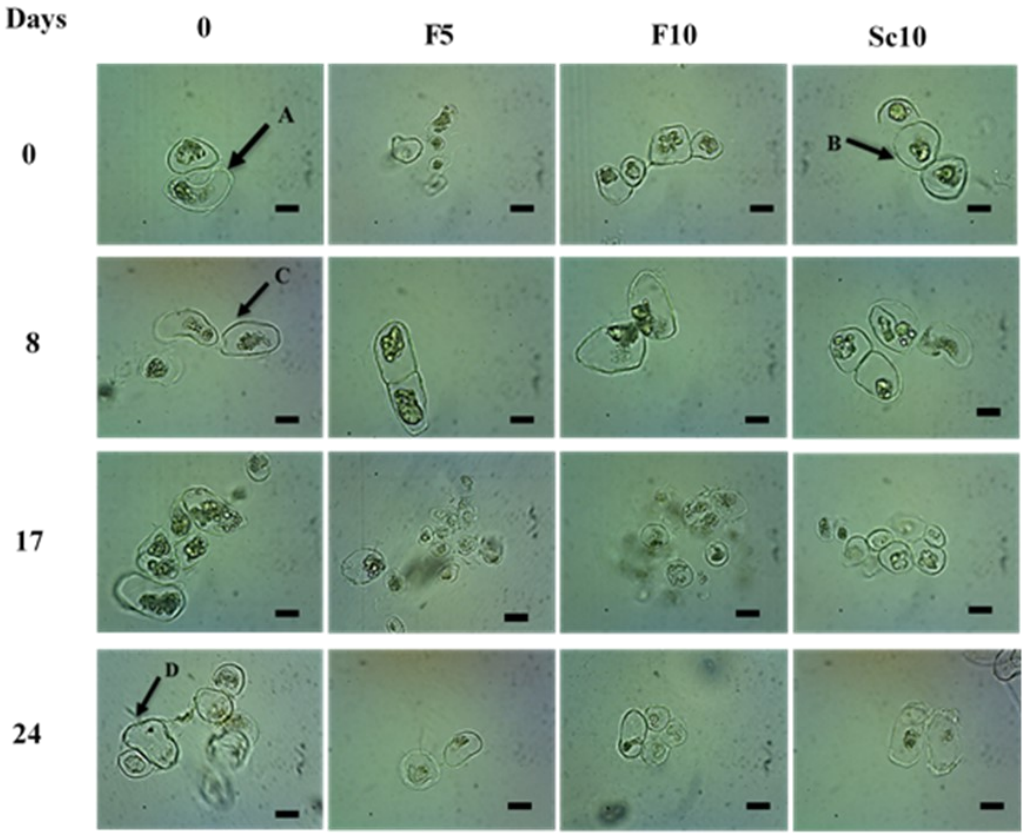


**Figure 7.** Morphology of kaffir lime cell line after elicitation from Day-0 to Day-24. (A) comma or sickle shape, (B) spherical shape, (C) elongated shape, (D) unviable cell, scale bars 100 μm. F5 and F10 are cell lines that were elicited by technical grade yeast with 5 and 10 ppm respectively; Sc10 is cell line was elicited by pure grade yeast with 10 ppm.

their response to the environment and nutrients. The spherical shape indicates that the cell is the result of previous cell division in *Stelechocarpus burahol* (BI.) Hook. F. And it is embryogenic cells with activated division in *Saccharum officinarum* L. Cells with a spherical shape will differentiate into elongated cells. In *S. burahol*, elongated cell indicated the non-viable condition and non-embryogenic cell showed in *S. officinarum* (Habibah et al. 2017; Thorat et al. 2017). According to Ogita et al. (1997) in dos Santos et. Al (2010), long binucleated cells undergo continuous cell division resulting in the development of adventitious somatic proembryos in *Larix leptolepis*. However, any species after passing through the exponential phase, the shape of the cell changes back to a spherical shape. The spherical shape of the cell persists until the cell death phase. This also occurs in *C. arizonica* cells which are spherical at the end of their growth (Sparapano & Bruno 2004).

In this study, we found that the best time for subculturing kaffir lime cell line suspension is on the 17th day, which is the final exponential phase (the initial stationary phase). The final exponential phase is better to be subcultured because when it enters the end of exponential and start the beginning of the stationary phase, the nutrient in the medium will decrease and the cells begin to produce bioactive compounds, especially secondary metabolite as a defense mechanism because of the limitation of nutrient in the medium. According to a study conducted by Khanpour-Ardestani et al. (2015), the content of acetonide compounds in *S. striata* is in the highest amount during the exponential phase and gradually decreased in the stationary phase. Subculturing the cell line with a certain period is one method to maintain cell viability to produce bioactive compounds. Sierra et al. (1992) succeeded in stabilizing alkaloid compounds in two cell line cultures of the *Tabernaemontana divaricata*. The highest production of alkaloids in the 4th day after the change of the medium and the growth remains stable during 30 subculture with a subculture interval every 9 days. The other study was found in the production of betaxanthins in callus culture of *Beta vulgaris* L. var 'Dark Detroit'. Production of betaxanthins in callus cells line of the *B. vulgaris* increased 1.8-fold after 48 subcultures with subculture intervals every 14 days (Trejo-Tapia et al. 2008). As well as the stability of synthesized verbascoside in cell line culture suspension of *Buddleja cordata* Kunth after being subcultured for 5 continuous years (Arano-Varela et al. 2020). This study provided an efficient way for further regulation of biosynthesis and production of bioactive compounds on scale-up in kaffir lime cell line culture. Analyzing post-subculture bioactive compounds and how appropriate the subculture cycle maximizes the production of secondary metabolites needs further investigation.

## CONCLUSION

Technical grade yeast is an elicitor candidate for kaffir lime cell suspension culture based on morphology and growth pattern observation as pure grade yeast. Hence we could use either pure or technical grade (commercial baker)

yeast in kaffir lime cell elicitation. The elicited kaffir lime cell line has the same growth pattern as the control cell. The initial stationary phase was on the 17th day. Subculturing kaffir lime cell line at this phase is needed to maintain the viability of kaffir lime cell line.

## AUTHORS CONTRIBUTION

D.Y.R. collected and analyzed the data and wrote the manuscript and revised it, F.D. collected and analyzed the data and wrote the manuscript, G.P.C wrote the manuscript, A.J.N. wrote the manuscript, A.B.S. analyzed the data, E.S. analyzed the data, W.A.S.T. design the research and supervised all process.

## ACKNOWLEDGMENTS

This study was funded by PDUPT (*Penelitian Dasar Unggulan Perguruan Tinggi*) from the Ministry of Research, Technology and Higher Education of Indonesia (to WAST).

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb69389
DOI: 10.22146/jtbb.69389

Research Article

# Single-dose Acute Oral Toxicity Study of Chloroform Extract of Snake Plant (*Sansevieria trifasciata* Prain.) Leaf in Wistar Rats (*Rattus norvegicus* Berkenhout, 1769)

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**Keywords:**
acute oral toxicity
chloroform extract
medicinal plants
OECD 420
*Sansevieria trifasciata*
**Submitted:**
26 September 2021
**Accepted:**
09 January 2022
**Published:**
23 March 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT

*Sansevieria trifasciata* is one of popular ornamental plants which also believed possessing therapeutic effects due to their phytochemical constituents. Secondary metabolites of plants can be toxic to other organisms; therefore, toxicity studies must be carried out to investigate adverse effects prior to further exploration as potent candidates of medicinal plants. This research aimed to evaluate toxicity and safety of consuming chloroform extract of *S. trifasciata* leaf (CESTL) in acute phase using female Wistar rats as model animal. Procedure referred to OECD Guidelines for the Testing of Chemicals, Test No. 420: Acute Oral Toxicity - Fixed Dose Procedure with single-dose administration of 2000 mg/kg bw. Results demonstrated that during 14 days of the experiment, neither mortality and sublethal effects as signs of toxicity were detected. There were no significant differences during the experiment between treatment groups and control in body weight, core temperature, individual and social behavior, food and water intake, as well as hematological profile, clinical biochemistry parameters, and relative organ weight (visceral organs indices). Almost all values were maintained within normal range (baseline) with fluctuation as normal physiological dynamics appeared relatively similar in all groups. Therefore, it can be concluded that no-observed-adverse-effect-level (NOAEL) for single-dose oral administration of CESTL with the dose 2000 mg/kg bw and can be classified in the hazard of Category 5 based on Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Based on this finding, we will continue to conduct further study to assess the repeated-dose acute oral toxicity.



## INTRODUCTION

Secondary metabolites (SMs) are by-products of plants which are not needed in their life processes. These compounds rather play a role in plant defense mechanisms against other organisms and dealing with stress from their surrounding environment. Many SMs can be used for medicinal purposes and health supplements for human and animals. The bioactive ingredients were isolated by extraction using various solvents, then the potential therapeutic properties are assessed through pharmacological research (Pagare et al. 2015).

*Sansevieria trifasciata* is one of popular ornamental plants as well as bio-remediation agent. A little is known about its efficacy as medicine and health nourishment. So far, the practice of using *S. trifasciata* for medication is only empirical, based on folk medicine and ethnobotany studies. The use of *S. trifasciata* as traditional medicine is due to its phytochemical constituents which are believed able to cure various diseases as well as to maintain body health (Dewatisari et al. 2021). Aqueous extract of *S. trifasciata* leaves has anti-diabetic effect (Qomariyah et al. 2012), ethanolic extract of *S. trifasciata* leaves possess antiallergic and antianaphylactic properties (Andhare et al. 2012). Chloroform extract of *S. trifasciata* leaves (CESTL) is potential to be developed as pharmaceutical agent due to the high content of triterpenoids, steroids, phenols, flavonoids, and alkaloids (Dewatisari 2020). However, there is still no scientific evidence provided.

As SMs are potential to be toxic to other organisms, while inappropriate dose of medicinal substances may lead to various adverse effects; therefore, series of toxicity and safety studies need to be conducted. *In vivo* experiment or preclinical trial is one of three methods commonly used for testing compounds that is essential in drug development process. The result will provide information for further test, the clinical phase trial (Derelanko & Hollinger 2002; Parasuraman 2011).

One common parameter on toxicity test is the lethal dose (LD50). So far, we cannot find any publication on the toxicity studies of CESTL. Therefore, we did not get any information regarding its LD50. However, we collected some data of LD50 of acute oral administration of *S. trifasciata* but with different solvents: LD50 of the ethanolic extract of *S. trifasciata* in Wistar rats at the dose 18000 mg/kg bw (Ighodaro et al. 2017) and LD50 of the methanolic extract of *S. trifasciata* in Wistar rats at the dose 500 mg/kg bw (Dey et al. 2014). Anbu et al. (2009) conducted acute oral toxicity test of *S. trifasciata* in Swiss mice which LD50 of the ethanolic extract at the dose 1513.5 ± 21.5 mg/kg bw, whereas the aqueous extract at the dose 1426 ± 43.6 mg/kg bw.

Toxicity test should not be limited to determining the LD50 value, as is the case with conventional procedure. As concern for animal welfare and ethics, the classical LD50 test protocol has been revised and evaluated to use fewer animals, to reduce the level of suffering, and to adopt internationally accepted methods (United Nations 2011). One of recommended methodology on toxicity tests is developed by The Organization for Economic Co-operation and Development (OECD), of which Indonesia is one of the key partners. Toxicity studies consist of acute, subchronic, and chronic periods. The acute oral toxicity tests consisted of two parts: First, the single-dose, which the substance is administered only once and the observation takes 14 days since the administration (OECD 2002). Second, the repeated-dose, which the substance is administered daily during 28 days. The dose used in the repeated-dose test is considered from the result from single-dose test (OECD 2008).

Based on the dose, Globally Harmonized System of Classification and Labelling of Chemicals (GHS) defines the level of acute oral toxicity into five categories, from Category 1 (the highest hazard) to Category 5 (the lowest hazard) as follows: 5, 50, 300, 2000, and 5000 mg/kg bw. To protect animal welfare, testing at a dose of 5000 mg/kg bw is discouraged and should not be carried out unless there is a strong reason that has a direct relevance to health and can be justified (United Nations 2011). This research aimed to study single-dose acute oral toxicity and safety levels of CESTL which follows OECD Test Guideline No 420: Acute Oral Toxicity - Fixed Dose Procedure with the dose 2000 mg/kg bw using female Wistar rats as model animal.

## MATERIALS AND METHODS

### Ethical Clearance

All procedures in this study which related to the care and use of animal for experimental model in preclinical trial have complied with animal welfare principles and did not violate the animal ethics. This is supported by the approval and issuance of Ethical Clearance by the Research Ethics Commission of Faculty of Veterinary Medicine, Universitas Gadjah Mada with the Number: 00034/EC-FKH/Eks./2021 dated on April 12th, 2021.

### Plant Material and Extraction Method

Species identification has been carried out and the result refers to *Sansevieria trifasciata* Prain. which has been approved with the issuance of Certificate of Identification by the Head of Laboratory of Plant Systematics, Faculty of Biology UGM No. 014526/S.Tb./II/2019 dated on February 25, 2019. We also grew the plant for collection (Figure 1).


**Figure 1.** Morphology of *Sansevieria trifasciata* Prain. we used in this study.

*S. trifasciata* leaves were collected by Mrs. Whika Febria Dewatisari, S.Si., M.Sc., a doctoral student of Faculty of Biology, UGM. The preparation of CESTL based on graded maceration method as follows: leaves were rinsed, finely chopped, and dried in the oven (50 ℃). The dried material *(simplicia)* then were ground into powder and soaked in chloroform with a ratio of powder : chloroform = 1 : 3 (w/v) for three days, shaking regularly to optimize the extraction. The solution was filtered and evaporated using an electric fan until completely dried. Stock of CESTL was stored in air-tight glass containers or wrapped with aluminum foil in the refrigerator (4 ℃), and taken as needed to be processed as a working solution (Dewatisari et al. 2021).

## Experimental Animals

Sample animals were nine female nulliparous Wistar rats *(Rattus norvegicus* Berkenhout, 1769) aged eight weeks old with body weight range at 135-173 (157.25±11.11) grams. Rodents, especially rats, has been used extensively in descriptive toxicity studies and drug safety tests as their systems represent human physiology (Greaves 2012).

Experiment took place at Animal House, the animal facility of Faculty of Biology UGM, the same place where they were originated. The procedure of animal care and maintaining followed standard procedures for rearing laboratory rats (NRC 2011). Rats were housed in communal cage designed for laboratory rats, made of transparent polypropylene with the size 38 x 25 x 23.5 $cm^3$, equipped with metal wire mesh for the lid, wood shaving for bedding, feeder, and rodent drinking bottle (Figure 2).


**Figure 2.** Housing for female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL

Environmental parameters are as follows: room temperature 27-29 °C, relative humidity 68-81%, standard photoperiod with artificial lighting 12 hours light : 12 hours dark. Cages were cleaned up twice a week with detergent and disinfectant.

Rats were fed with standard chow diet (Ratbio®, P.T. Citra Ina Feedmill, Jakarta) and mineral water (P.T. Berkah Tirta Jaya, Yogyakarta). Initial feed weight and water volume were determined for calculating daily food intake and water consumption.

## Study Design

Rats were assigned into three groups: the first group received CESTL, the second group received 5% Tween80 (v/v) as chloroform extract emulsifier (TWEEN), and the third group received distilled water as control-placebo (CTRL). The procedure of experiment referred to OECD Test Guideline No. 420 (OECD 2002) with a dose of 2000 mg/kg bw (Sighting study). CESTL, Tween80, and distilled water were administered orally 1 mL/individual once at the beginning of the experiment (Day 0).

## Parameters

Quantitative parameters consisted of body weight, rectal temperature, food intake, water consumption, hematological profile based on complete blood count (CBC), liver function tests based on alanine-aminotransferase (ALT) and aspartate-aminotransferase (AST) activities, renal function tests based on creatinine (CRE) and blood urea nitrogen (BUN) levels, as well as levels of fasting blood glucose, total cholesterol, and triglycerides. All parameters were observed on Day 0, 7, and 14.

Qualitative parameters as signs of toxicity consisted of mortality, sublethal effects, and clinical manifestations that lead to illness, including: morphological or physical examination, individual and social behaviors and activities, as well as stool conditions. These parameters were monitored soon after administration, intensively for four hours post-administration, and continued every day until Day 14.

## Anesthesia and Euthanasia

Before performing blood collection, rats were anesthetized by intramuscular injection of Ketamine (Kepro®, Holland) and Xylazine (Interchemie®, Holland) cocktail 0.1 mL/100 g bw (K=50 mg/kg bw, X=5 mg/kg bw). On the last day of the experiment (Day 14), rats were sacrificed by similar anesthesia procedure followed with phlebotomy for exsanguination (sera were preserved for further analysis) and perfusion using physiological saline (0.9 % NaCl w/v, Otsuka®, Indonesia).

## Collection of Visceral Organs and Calculation of Relative Organ Weights

Soon after rats being euthanized, necropsy was performed, visceral organs consisted of liver, kidney, spleen, lungs, heart, brain, and internal genital organs (ovaries and uterus) were removed, rinsed in saline, and weighed. Relative organ weight was calculated by dividing the absolute organ weight by

final body weight, then multiplied by 100 (Cattley & Cullen 2013). The formula is as follows:

$$Relative\ organ\ weight = \frac{Absolute\ organ\ weight}{Final\ body\ weight} \times 100$$

Organs were preserved in 10% neutral buffered formalin (NBF) fixative. Organs were processed for histopathological observation when the results of their functions tests indicated significant functional impairment.

### Blood Analysis

Blood samples for hematological and clinical biochemical profiles were withdrawn from orbital sinus. As much as 1 mL blood were collected in 1.5 mL ethylene-diamine-tetra-acetic acid (EDTA)-coated microtube as anticoagulant. Hematological profiles were analyzed using Sysmex®XP-100. Glucose and total cholesterol levels were measured directly using EasyTouch® rapid test strips.

Plasma for measurement of ALT, AST, creatinine, BUN, and triglycerides were separated from whole blood using centrifuge (Eppendorf®5418R) and then analyzed using Microlab®300.

### Data Analysis

Qualitative data were shown as table to compare each group. Quantitative data was tabulated in Microsoft®Excel® v.2019 and statistical analysis were performed using IBM®SPSS® v.23. based on Repeated-measures ANOVA Test to compare means over time from related group ($a = 0.05$). If significant difference is detected ($p < 0.05$), the test is continued with Bonferroni Post Hoc Test to discover which specific means differed. One-way ANOVA Test was employed to compare means between groups ($a = 0.05$). If significant difference is detected ($p < 0.05$), the test is continued with Tukey's Honestly Significant Difference (HSD) Post Hoc Test to discover which specific means differed. Reference interval for normal values were constructed based on the lowest and highest values of each variable from the animal population in this study at Day 0 or "The Baseline" (Poitout-Belissent & McCartney 2010). Results are displayed in comparison table as descriptive statistic values (mean ± standard deviation).

## RESULTS AND DISCUSSION

There is a new approach in the methodology for assessing acute toxicity study, in which it should not only focus on determining $LD_{50}$ or $LC_{50}$ since not all chemicals cause death of animals. Animals may be still alive or survive during the experiment but suffering due to physiological disturbances (sublethal effect). Therefore, signs of toxicity in animals must be clearly observed (OECD 2002).

## Signs of Toxicity

We used transparent rats cage thus we can see their natural behavior to monitor signs of toxicity. The old-style rat cages with opaque walls did not allow researcher to observe animals from the sides. Watching from the top of the cage will only interfere their activities and is stressful to the animals (ARRP 2008). In addition, the wall of the cage should be high enough to enable rats standing upright (rearing and stretching) as shown in Figure 2, because this is one of their normal behaviors and a sign of healthy rat (ARRP 2008; NC3RS 2017).

During 14-day experiment, no death or ill rats were found. Observations on general morphological and physical condition of animals, as well as individual and social behavior and their activities showed similar results as control (Table 1). It means that neither CESTL nor Tween80 which used as emulsifier of chloroform extract in aqueous media generated toxic effects that harmed the health.

The health status and psychological conditions (distress) of laboratory rats can be observed based on their morphology, behavior, and physiology. Unhealthy or stressed rats are passive, reduced activity, decreased appetite and drinking, licked their body frequently, stiffed body and guarded limbs, self-mutilate, aggressive, a lot of vocalizations as response to handling, reluctant to interact with their conspecific. Rats with health problem have messy appearance as they do not do grooming properly, fur are coarse and stiff or piloerection, hunched posture, red eyes discharge like bloody tears due to porphyrin secretion, partially closed eyelids, dilated pupils, nasal discharge or

**Table 1.** Signs of toxicity in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| PARAMETER | CESTL (n=3) | TWEEN (n=3) | CTRL (n=3) |
|---|---|---|---|
| **Mortality** | 0 | 0 | 0 |
| **Body weight (g)** | Increased by 34.67±5.37[a] $R^2$= 0.999 | Increased by 25.00±4.01[a] $R^2$= 0.990 | Increased by 18.33±7.13[a] $R^2$= 0.985 |
| **Rectal temperature (°C)** | Fluctuated, mean= 33.98±0.82[a] | Fluctuated, mean= 34.24±0.75[a] | Fluctuated, mean= 33.73±0.51[a] |
| **General morphological/ physical condition** | Normal/healthy, face, body, and tail are clean | Normal/healthy, face, body, and tail are clean | Normal/healthy, face, body, and tail are clean |
| **Individual behavior and activities** | Active, normal | Active, normal | Active, normal |
| **Social behavior and activities** | Active, normal, positive interaction | Active, normal, positive interaction | Active, normal, positive interaction |
| **Food intake (g/ individual/day)** | Fluctuated, mean= 16.68±3.59[a] | Fluctuated, mean= 15.68±3.89[a] | Fluctuated, mean= 14.05±3.41[a] |
| **Water consumption (mL/ individual/day)** | Fluctuated, mean= 25.03±3.69[a] | Fluctuated, mean= 24.43±3.19[a] | Fluctuated, mean= 22.33±3.85[a] |
| **Stool condition** | Structure, color, and smell are normal, no diarrhea detected | Structure, color, and smell are normal, no diarrhea detected | Structure, color, and smell are normal, no diarrhea detected |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water.

Values are expressed as the mean±SD. Values ended with same superscript letters in a row indicate no significant difference between groups based on one-way ANOVA test ($p > 0.05$).

runny nose, and abnormal resting position. Physiologically, ill rats have sleep disruption, they sleep most of the time or easily awakening, hypothermia, rapid and shallow breathing, abnormal heartbeat, expiration with grunting sound (Carstens & Moberg 2000; Wang et al. 2019). We did not find these signs in all animals we used in this experiment (Table 1).

Ekeanyanwu and Njoku (2014) used dimethyl sulfoxide (DMSO) as emulsifier as well as vehicle for oral administration of chloroform extract in rats. Unfortunately, we failed to dissolve CESTL in DMSO; therefore, we used Tween80 as alternative agent to emulsify chloroform extract which is nonpolar or hydrophobic, so that it can be dissolved in aqueous medium thoroughly (Parker et al. 2021). Tween80 is polysorbates, one of the food additives commonly used as emulsifier. Single-dose acute oral toxicity test resulted in very low toxicity level: dose 22 g/kg bw did not exhibit toxicity symptoms in rats (NOAEL). Acceptable daily intake of Tween80 is 0-25 mg/kg bw (FSCJ 2007).

Santos-Lopez et al. (2010) used 1% Tween80 to dissolve chloroform extract of aerial parts of *Phytolacca icosandra*, Christian et al. (2014) used 3% Tween80 to dissolve *Persea americana* leaf extract, Zakaria et al. (2015) used 8% Tween80 to dissolve chloroform extracts of *Muntingia calabura* and *Melastoma malabathricum* leaves. We used 5% Tween80 because with that concentration CESTL was completely dissolved in distilled water.

Oral administration of 5-10% Tween80 can cause diarrhea in female rats (FSCJ 2007). However, according to The Joint FAO/WHO Expert Committee on Food Additives (JECFA), oral administration of 5% Tween80 (equivalent to 2500 mg/kg bw) is considered safe (NOAEL). In this experiment, rats administered with Tween80 or CESTL did not experience diarrhea as indicated by normal stool condition (Table 1). Diarrhea is physiological response against toxic substances which enter the body via digestive tract, therefore it is one of basic parameters in acute oral toxicity tests (OECD 2002; Wang et al. 2019).

In addition to cause gastrointestinal disorders, toxic compounds can reduce or lead to loss of appetite, which can be observed by calculating daily food intake and water consumption. A decrease in food consumption followed by reduction of water consumption result in weight loss and suppress the immunity. This is a secondary effect of toxic substance on physiological condition (Morita et al. 2017). Results pointed out that oral administration of CESTL did not cause diarrhea and digestive problems, in fact, there was an increase in food intake, water consumption, and body weight compared to control (Table 1). Further toxicity studies are needed to investigate the effects of CESTL on the digestive system over a longer period of time, as well as its potential to stimulate appetite, improve digestion, and promote weight gain for underweight individuals.

Besides having impacts on the digestive physiology and growth, toxic substances may also suppress the immune system which is characterized by alteration in leukocyte count and susceptibility to disease, increased risk of

anemia, liver and kidney dysfunctions, impaired normal energy metabolism, and even mental disorders, such as stress and depression (Morita et al. 2017; Wang et al. 2019). Hematological analysis, measurement of relative organ weights, evaluation of liver and renal functions, as well as examination of metabolic profiles consisted of blood glucose, total cholesterol, and triglycerides levels can provide a comprehensive data on the toxicity and safety studies of potential therapeutic agents on physiological conditions of experimental animals in preclinical research, which will later be translated to humans dose in clinical trial phase (Etame et al. 2017; Sutrisni et al. 2019; Sukandar & Sheba 2019).

## Effect of CESTL on Hematological Profile

Evaluation of or erythrocyte profile (Table 2) demonstrated that values of red blood cell count (RBC), hematocrit (HCT), and hemoglobin (HGB) in all groups fell below the baseline on Day 7 but gradually increased on Day 14 (significant in group received Tween80). Based on this, it is vital to investigate further to see the indication of anemia or the values will recover to normal (baseline range). This condition, however, is not due to CESTL toxicity since it occurred in all groups.

The decrease of RBC, HCT, and HGB resulted in elevation of mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC). Although this is a natural regulation of the body to anticipate anemia, but it must be considered because the alteration of erythrocyte indices may change the size of the cell. It is interesting because this alteration is not significant in group received CESTL, which means the extract does not disrupt the structure and function of the erythrocyte.

We used 5% Tween80 as emulsifier to dissolve in distilled water. According to Mantskava et al. (2018), Tween80 can alter erythrocyte profile but only temporarily; therefore the use of Tween80 as emulsifier is relatively safe. The decline of MCH and MCHC correlate with hypochromia which means the anemia is caused by less hemoglobin concentration. In opposite, the elevation of MCH and MCHC do not refer to hyperchromia. The increase can be caused by erythrocyte morphology or spherocytic (Vilchez 2020).

The value of MCV in groups received Tween80 and control exceeded the baseline but it was not significant. Significant increment indicates macrocytic anemia, liver disease, and vitamin B12 deficiency (Maner & Moosavi 2021). Alteration of erythrocyte profile in control may occur with age and natural processes. Observing that erythrocyte profile in group received CESTL is more stable than the other groups, we hypothesize that this substance can be used as supplement to improve the health. Based on this finding, we are preparing to continue exploring the therapeutic effect of CESTL with lowering the concentration of Tween80 to anticipate its adverse effect on erythrocyte profile.

**Table 2**. Erythrocyte profile in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| RBC (´10⁶/mL) | 0 | 7.88±0.38[a] | 7.75±0.16[a] | 8.49±0.35[a] | 7.38 – 8.95 |
| | 7 | 7.27±0.16[a] | 6.64±0.11[b] | 7.41±0.18[a] | |
| | 14 | 7.21±0.09[a] | 6.71±0.25[ab] | 7.14±0.29[a] | |
| HCT (%) | 0 | 42.77±2.75[a] | 42.37±0.87[a] | 46.67±2.30[a] | 39.20 – 49.90 |
| | 7 | 41.10±0.60[a] | 37.10±0.40[b] | 42.00±0.86[a] | |
| | 14 | 40.07±1.10[a] | 37.57±1.27[ab] | 40.87±1.42[a] | |
| HGB (g/dL) | 0 | 14.80±0.43[a] | 14.23±0.34[a] | 15.50±0.93[a] | 13.90 – 16.80 |
| | 7 | 14.55±0.45[a] | 13.45±0.15[a] | 14.37±0.05[a] | |
| | 14 | 14.13±0.25[a] | 13.80±0.28[a] | 14.23±0.39[a] | |
| MCV (fL) | 0 | 54.20±0.90[a] | 54.67±0.42[a] | 55.00±0.75[a] | 53.10 – 55.80 |
| | 7 | 55.23±1.96[a] | 54.80±1.57[a] | 56.70±0.51[a] | |
| | 14 | 55.57±0.97[a] | 55.97±0.41[a] | 57.30±1.10[a] | |
| MCH (pg) | 0 | 18.83±1.26[a] | 18.40±0.08[a] | 18.70±0.56[a] | 17.50 – 20.60 |
| | 7 | 20.00±0.20[a] | 20.25±0.12[b] | 19.40±0.43[b] | |
| | 14 | 19.60±0.45[a] | 20.30±0.51[ab] | 19.93±0.29[ab] | |
| MCHC (g/dL) | 0 | 34.80±2.83[a] | 33.57±0.19[a] | 33.23±0.59[a] | 32.40 – 38.80 |
| | 7 | 35.40±0.60[a] | 36.25±0.05[b] | 34.23±0.61[a] | |
| | 14 | 35.27±0.53[a] | 36.23±0.66[ab] | 34.83±0.52[a] | |
| RDW-SD (fL) | 0 | 28.63±0.90[a] | 27.33±0.39[a] | 27.23±0.61[a] | 26.80 – 29.80 |
| | 7 | 29.57±1.19[a] | 28.13±0.50[a] | 28.63±0.82[a] | |
| | 14 | 28.60±0.70[a] | 28.80±0.29[a] | 28.83±0.82[a] | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water, RBC= red blood cell count, HCT= hematocrit, HGB= hemoglobin, MCV= mean corpuscular volume, MCH= mean corpuscular hemoglobin, MCHC= mean corpuscular hemoglobin concentration, RDW-SD= red blood cell distribution width- standard deviation.

Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test ($p>0.05$).

Values of leukocyte profile were fluctuating in all groups, however most of them were within baseline. Monocytes, eosinophils, and basophils were not shown as they were not detected by our machine, possibly because their numbers were very low (Table 3).

The total number of leukocytes (WBC) in group received Tween80 exceeded baseline due to the significant increase of neutrophil count. According to Thamir et al. (2013), Tween80 can increase and activate neutrophils, in the other hand, it decreases lymphocyte count. The number of neutrophils in group received CESTL fell down the baseline on Day 7 but returned to baseline on Day 14 (not significant). This result is similar to the work by Ayalogu et al. (2011) using aqueous extract of *S. senegambica*.

Elevation of neutrophil count resulted in significant increase of neutrophil lymphocyte ratio (N/L); however, the value was still within baseline. N/L is a biomarker that describes two aspects of immune system, acute and chronic inflammation (neutrophil count) and adaptive immunity (lymphocyte count). It is also one of predictive factors to identify the presence of critical illness (Liu et al. 2020; Song et al. 2021). As N/L value in group received CESTL was relatively stable during the experiment; therefore, we conclude that the extract is safe to consume as it did not trigger immune response.

**Table 3**. Leukocyte profile in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| WBC (´10³/mL) | 0 | 10.20±2.78[a] | 11.20±1.00[a] | 9.73±0.41[a] | 6.90 – 13.70 |
| | 7 | 10.70±1.39[a] | 14.75±0.12[a] | 10.13±0.45[a] | |
| | 14 | 11.10±1.84[a] | 15.37±3.10[a] | 12.07±0.99[a] | |
| NEU (´10³/mL) | 0 | 1.70±0.08[a] | 2.40±0.36[a] | 2.97±0.05[a] | 1.60 – 3.00 |
| | 7 | 1.40±0.75[a] | 2.37±1.36[a] | 2.73±0.26[a] | |
| | 14 | 2.03±0.62[a] | 4.60±0.80[b] | 2.90±1.02[a] | |
| LYM (´10³/mL) | 0 | 8.50±2.82[a] | 8.80 ±1.14[a] | 6.77±0.37[a] | 5.10 – 12.00 |
| | 7 | 8.80±1.14[a] | 11.45±0.45[a] | 7.40±0.28[a] | |
| | 14 | 9.07±1.23[a] | 10.77±2.36[a] | 9.17±0.59[a] | |
| NEU (%) | 0 | 17.93±5.61[a] | 21.93±4.16[a] | 30.17±0.85[a] | 12.10 – 31.00 |
| | 7 | 18.60±1.28[a] | 27.33±7.34[a] | 27.00±1.85[a] | |
| | 14 | 17.73±2.79[a] | 30.10±1.84[a] | 23.57±6.74[a] | |
| LYM (%) | 0 | 82.07±5.61[a] | 78.07±4.16[a] | 69.83±0.85[a] | 69.00 – 87.90 |
| | 7 | 81.40±1.28[a] | 72.67±7.34[a] | 73.00±1.85[a] | |
| | 14 | 82.27±2.79[a] | 69.90±1.84[a] | 76.43±6.74[a] | |
| N/L | 0 | 0.15±0.01[a] | 0.21±0.01[a] | 0.45±0.00[a] | 0.14 – 0.45 |
| | 7 | 0.15±0.00[a] | 0.23±0.02[ab] | 0.43±0.02[a] | |
| | 14 | 0.15±0.00[a] | 0.24±0.00[b] | 0.41±0.04[a] | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water, WBC= white blood cell count, NEU= neutrophil, LYM= lymphocyte, N/L= neutrophil lymphocyte ratio.

Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test ($p>0.05$).

Platelet count (PLT) is strongly influenced by technical factors particularly in collecting blood and waiting time at which blood samples are handled. Problems during blood collection resulted in platelet aggregation and blood clotting, so that platelet count decreased and, consequently, this affected on other variables (Tien 1995). Results showed that most of the values of thrombocyte profile were within the baseline with fluctuations as normal physiological dynamics (Table 4). Some values exceed the baseline but are not significant. Mean platelet volume (MPV) and platelet distribution width (PDW) values at group received CESTL declined significantly but returned to the baseline. This result indicated that CESTL did no harm on the normal hemostatic process.

## Effect of CESTL on Clinical Biochemistry Profile

### Evaluation of Liver and Renal Functions

ALT and AST are main parameters to evaluate liver functions. Hepatocyte injury causes the leak of those enzymes from cells into blood circulation, so that the elevation of both enzymes are detected in plasma or serum (Debelo et al. 2016). Results showed that ALT and AST activities fluctuated in all groups, some values were maintained in the baseline, whereas some others exceed the baseline but not significant (Table 5).

This result brought to conclusion that CESTL is relatively safe, did not induce hepatotoxicity. Extract of *S. senegambica* also did not show hepatotoxi-

**Table 4**. Thrombocyte profile in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| PLT (´10³/mL) | 0 | 1052.67±134.70[a] | 870.67±447.88[a] | 1092.67±66.55[a] | 239 – 1227 |
| | 7 | 869.67±470.71[a] | 1247.00±17.96[a] | 917.67±573.76[a] | |
| | 14 | 1234.67±95.83[a] | 819.00±497.39[a] | 1213.00±86.46[a] | |
| PCT (%) | 0 | 0.70±0.05[a] | 0.56±0.28[a] | 0.71±0.05[a] | 0.16 – 0.79 |
| | 7 | 0.79±0.05[a] | 0.79±0.02[a] | 0.79±0.08[a] | |
| | 14 | 0.81±0.05[a] | 0.75±0.04[a] | 0.77±0.07[a] | |
| MPV (fL) | 0 | 6.70±0.37[a] | 6.53±0.19[a] | 6.50±0.14[a] | 6.30 – 7.20 |
| | 7 | 6.43±0.34[b] | 6.23±0.09[a] | 6.37±0.05[a] | |
| | 14 | 6.53±0.26[ab] | 6.40±0.14[a] | 6.37±0.12[a] | |
| PDW (fL) | 0 | 7.60±0.57[a] | 7.47±0.31[a] | 7.17±0.17[a] | 7.00 – 8.40 |
| | 7 | 7.17±0.59[b] | 6.63±0.39[a] | 6.93±0.25[a] | |
| | 14 | 7.47±0.39[ab] | 7.03±0.05[a] | 6.90±0.22[a] | |
| P-LCR (%) | 0 | 4.87±1.59[a] | 4.53±0.90[a] | 4.20±1.02[a] | 3.20 – 7.00 |
| | 7 | 4.97±1.39[a] | 4.20±0.73[a] | 3.90±0.37[a] | |
| | 14 | 4.43±1.18[a] | 4.07±0.95[a] | 4.00±0.42[a] | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water, PLT= platelet count, PCT= plateletcrit, MPV= mean platelet volume, PDW= platelet distribution width, P-LCR= platelet large cell ratio.

Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test ($p>0.05$).

city (Ayalogu et al. 2011). *S. liberica* even has hepatoprotective activity (Ikewuchi 2012a). Hepatoprotective activity of chloroform extract is due to the flavonoids as powerful antioxidant (Khan et al. 2012). Phytochemical analysis exhibited that CESTL is rich of flavonoids (Dewatisari 2020); therefore, it is potential to be developed as new candidate of hepatoprotective agent.

The high values of ALT and AST in group received Tween80 possibly may because this substance can impair liver function through hemolysis and cholestasis (Ellis et al. 1996). However, this effect of Tween80 did not appear in group that administered with CESTL. We assume that CESTL may nourish liver structure and functions, thereby eliminate the adverse effect of Tween80. We will follow up this finding by reducing the concentration of Tween80 in the next toxicity study to anticipate the bias caused by this substance.

Creatinine (CRE) and BUN are the main parameters for evaluating renal function. These metabolic wastes are consistently excreted through the kidneys, dissolved in urine. Therefore, the increased levels of them in plasma or serum can be used as indicator of impaired renal functions (Fitria et al. 2019). Results showed that CRE and BUN levels in all groups fluctuated. Some values were outside of the baseline and significant (Table 6).

Increasing values of both compounds did not necessarily denote impaired renal function if the levels were within reference interval (baseline) or the alterations were not significant (Mulyati et al. 2019). This indicated that CESTL did not induce nephrotoxicity and safe for kidney health. Extract of

**Table 5**. Evaluation of liver functions in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| ALT (mg/dL) | 0 | 52.93±10.49$^{ab}$ | 54.90±13.98$^{a}$ | 39.27±3.23$^{a}$ | 35.40 – 74.50 |
| | 7 | 92.80±0.00$^{a}$ | 83.05±9.68$^{a}$ | 78.43±6.78$^{b}$ | |
| | 14 | 47.87±2.39$^{b}$ | 90.23±43.86$^{a}$ | 55.90±4.38$^{ab}$ | |
| AST (mg/dL) | 0 | 57.83±32.96$^{a}$ | 90.73±12.80$^{a}$ | 75.37±11.90$^{a}$ | 12.60 – 108.40 |
| | 7 | 89.00±8.16$^{a}$ | 50.50±14.29$^{a}$ | 77.33±9.39$^{a}$ | |
| | 14 | 91.03±11.80$^{a}$ | 149.27±36.67$^{a}$ | 141.63±45.25$^{a}$ | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water, ALT= alanine aminotransferase, AST= aspartate aminotransferase.
Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test (p>0.05).

**Table 6**. Evaluation of renal functions in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| CRE (mg/dL) | 0 | 0.35±0.05$^{a}$ | 0.40±0.02$^{a}$ | 0.37±0.04$^{a}$ | 0.30 – 0.40 |
| | 7 | 0.27±0.06$^{ab}$ | 0.28±0.05$^{b}$ | 0.30±0.05$^{a}$ | |
| | 14 | 0.39±0.05$^{b}$ | 0.33±0.01$^{c}$ | 0.45±0.00$^{b}$ | |
| BUN (mg/dL) | 0 | 19.08±2.42$^{a}$ | 17.09±1.53$^{a}$ | 14.68±2.54$^{a}$ | 12.0 – 22.50 |
| | 7 | 16.22±3.47$^{a}$ | 19.52±1.60$^{b}$ | 14.91±1.89$^{a}$ | |
| | 14 | 18.14±3.40$^{a}$ | 18.60±3.19$^{ab}$ | 18.73±1.43$^{a}$ | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water, CRE= creatinine, BUN= blood urea nitrogen.
Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test (p>0.05).

*S. senegambica* also showed the same result (Ayalogu et al. 2011). Nephroprotective effect is even found in *S. roxburghiana* (Aclan et al. 2020). The nephroprotective activity of chloroform extract is due to the content of phenolics, flavonoids, and amino acids as antioxidants (Jain & Singhai 2010). Work of Dewatisari (2020) revealed that CESTL has a high content of phenols and flavonoids, which means it is potential as nephroprotective agent.

### Effect of CESTL on Glucose Level and Lipid Profile

CESTL contains triterpenoids, steroids, phenols, flavonoids, and alkaloids (Dewatisari 2020). Those phytochemical compounds have antidiabetic activity (Ota & Ulrih 2017). Alkaloids, phenolics, and flavonoids also possess antihypercholesterolemic and antilipidemic activities (Asghar et al. 2018). Flavonoids, alkaloids, and terpenoids attenuate atherosclerosis (Liu et al. 2019). Phytosterols have antidiabetic, antihypercholesterolemic, and antiatherosclerotic activities (Salehi et al. 2021). Phytochemical screening (Dey et al. 2014) and *in vitro* assay (Yumna et al. 2018) served that *S. trifasciata* has antidiabetic activity. This property is also found in *S. liberica* (Ifebi et al. 2021). The potential of CESTL as blood lipid lowering agent has not been studied. Research

by Sanad (2020) directed that the chloroform extract of *Vangueria infausta* leaf has ability to regulate blood cholesterol level. Research on *S. liberica* (Johnkennedy et al. 2014) and *S. senegambica* (Ikewuchi 2012b) demonstrated that both species possess hypocholesterolemic effect. *S. senegambica* is also able to control triglyceride level (Ikewuchi et al. 2011).

Prior to explore the potential of CESTL efficacy as herbal product to overcome health problems, toxicity studies must be carried out to determine its toxicity and safety on glucose, cholesterol, and triglyceride metabolism on normoglycemic and normolipidemic models, as we did in this study. Results showed that glucose level was maintained within baseline in all groups. Total cholesterol level increased above baseline, as well as the other groups, but then recovered. Triglyceride level exhibited similar dynamics with cholesterol level, but according to the statistical analysis the result is different. The fluctuation of cholesterol level in group received CESTL is not significant, whereas the fluctuation of triglyceride level is significant (Table 7). However, by looking the physiological dynamics (the initial value, the fluctuation, and the final result), CESTL is promising to lower cholesterol and triglyceride levels.

Based on this finding, we hypothesize that neither CESTL nor Tween80 as emulsifier disrupt the normal metabolism of glucose, cholesterol, and triglycerides, confirmed by reasonable weight gain due to normal growth of the animal. However, these results are provisional since CESTL was administered only single-dose. Therefore, we are preparing to conduct a further study with repeated-dose administration to study the effect of CESTL when consumed routinely during the acute period.

**Table 7**. Glucose level, lipid profile, and growth in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| VARIABLE | DAY | GROUPS | | | BASELINE |
|---|---|---|---|---|---|
| | | CESTL | TWEEN | CTRL | |
| Fasting glucose (mg/dL) | 0 | 94.33±14.27$^{a}$ | 145.67±13.52$^{a}$ | 177.67±41.25$^{a}$ | 75 – 233 |
| | 7 | 129.00±10.80$^{a}$ | 180.33±47.30$^{a}$ | 200.33±25.32$^{a}$ | |
| | 14 | 120.33±16.05$^{a}$ | 165.33±49.78$^{a}$ | 106.00±14.45$^{a}$ | |
| Total cholesterol (mg/dL) | 0 | 138.33±23.46$^{a}$ | 138.67±10.87$^{a}$ | 106.00±5.35$^{a}$ | 100 – 171 |
| | 7 | 276.00±93.34$^{a}$ | 247.33±68.05$^{a}$ | 240.33±16.36$^{ab}$ | |
| | 14 | 122.00±18.71$^{a}$ | 165.00±84.92$^{a}$ | 231.00±21.26$^{b}$ | |
| Triglycerides (mg/dL) | 0 | 47.77±8.35$^{a}$ | 48.93±2.65$^{a}$ | 56.90±6.19$^{a}$ | 38 – 66 |
| | 7 | 64.90±14.86$^{b}$ | 59.05±7.80$^{a}$ | 67.43±14.99$^{a}$ | |
| | 14 | 34.083±5.39$^{ab}$ | 41.63±5.20$^{a}$ | 42.40±5.74$^{a}$ | |
| Body weight (g) | Initial | 145.33±7.84$^{a}$ | 162.00±4.97$^{a}$ | 157.33±7.71$^{a}$ | 135 – 173 |
| | 0 | 156.33±1.88$^{a}$ | 169.67±5.91$^{b}$ | 161.33±6.54$^{a}$ | |
| | 7 | 167.33±3.85$^{a}$ | 176.33±3.77$^{ab}$ | 168.67±7.32$^{a}$ | |
| | 14 | 180.00±7.87$^{a}$ | 187.00±1.41$^{ab}$ | 175.67±6.94$^{a}$ | |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water.

Values are expressed as the mean±SD. Values ended with same superscript letters in a column for each variable indicate no significant difference between days based on repeated-measures ANOVA test ($p>0.05$).

### Effect of CESTL on Relative Organ Weight

Organ weight, or to be precise is relative organ weight, is endpoint parameter in toxicity study. In adults, ratio of organ weight to body weight is constant; therefore, changes in its value indicates body response to a treatment (Cattley & Cullen 2013), one of which is exposure to toxic compounds in both acute and chronic phases (Greaves 2012; Cattley & Cullen 2013). Results showed that values of almost all organs in treatment groups did not experience significant changes, except the liver (Table 8).

Liver is critical target organ in toxicity studies because it functions as the center for regulation of nutrient metabolism, producing various functional proteins, providing energy for homeostasis, as well as drug metabolism and detoxification. Liver weight in rats occupies 2-3 % of body weight (Rogers & Dintzis 2018). Decreasing of liver weight is common with age. Conversely, increasing of liver weight can occur due to the accumulation of lipids, glycogen, and other compounds due to cell damage, congestion, hypertrophy, and hyperplasia of liver cells (Greaves 2012).

Toxic effect generally elevates liver weight. In this study, treatment groups had lower liver weight than the control. Liver weight reduction is uncommon, it can be caused by hepatocyte atrophy or death due to injury or apoptosis (Cattley & Cullen 2013). Liver weight is normally influenced by physiological factors, especially blood circulation, so it is difficult to evaluate based on histopathological examination. Hepatic enzymes can increase liver weight, but this is not always the case (Greaves 2012). ALT and AST values **(**Table 5**)** indicate that the activities of both liver enzymes fluctuated during the experiment, some exhibited significant increase or decrease, however, they are not salient to cause alteration in liver weight. In this study, CESTL was administered only once (single-dose), hence, we will continue with repeated-dose administration to get more information regarding effect of CESTL on liver weight before conducting histopathological assessment.

**Table 8**. Relative organ weight in female Wistar rats as experimental animals in single-dose acute oral toxicity study of CESTL.

| ORGAN | RELATIVE WEIGHT (g) | | |
|---|---|---|---|
| | CESTL | TWEEN | CTRL |
| Brain | 1.037±0.009[a] | 1.029±0.031[a] | 0.989±0.003[a] |
| Gastrointestinal tract | 9.135±1.706[a] | 9.357±0.588[a] | 9.585±0.694[a] |
| Heart | 0.356±0.034[a] | 0.369±0.034[a] | 0.356±0.023[a] |
| Internal genital organ | 0.428±0.020[a] | 0.767±0.373[a] | 0.407±0.106[a] |
| Kidney, left | 0.380±0.035[a] | 0.435±0.028[a] | 0.423±0.075[a] |
| Kidney, right | 0.469±0.029[a] | 0.460±0.039[a] | 0.387±0.019[a] |
| Liver | 3.861±0.106[a] | 3.998±0.170[a] | 4.567±0.160[b] |
| Lungs | 0.867±0.043[a] | 0.750±0.025[a] | 0.743±0.056[a] |
| Spleen | 0.309±0.033[a] | 0.357±0.066[a] | 0.283±0.030[a] |

Abbreviations: CESTL= chloroform extract of *Sansevieria trifasciata* leaf, TWEEN= 50 % Tween80, CTRL= control/ placebo/distilled water.

Values are expressed as the mean±SD. Values ended with same superscript letters in a row for each variable indicate no significant difference between groups based on one-way ANOVA test ($p>0.05$).

## CONCLUSION

No mortality and any sublethal effects as signs of toxicity were detected on female Wistar rats used as model animal during the study. Based on all parameter values and statistical analysis results, it can be concluded that acute oral administration of chloroform extract of *Sansevieria trifasciata* leaf (CESTL) at the dose 2000 mg/kg bw (single-dose) generated no-observed-adverse-effect-level (NOAEL). Therefore, CESTL can be classified in the hazard of Category 5 based on Globally Harmonized System of Classification and Labelling of Chemicals (GHS). Build upon this finding, we are preparing to conduct further study to assess the repeated-dose acute oral toxicity of CESTL to provide more information prior to the exploration of its potential therapeutic effects.

## AUTHORS CONTRIBUTION

LF designed the research and supervised all the process; ICPG and WBTS conducted the experiment and responsible for data collection; ICPG, WBTS, and MIM analysed the data and constructed interpretations of the results for discussions. LF wrote the manuscript as compilation of concepts from all authors.

## ACKNOWLEDGMENTS

This work is supported financially by the "Hibah Kolaborasi Dosen dan Mahasiswa" (KDM) of the Faculty of Biology Gadjah Mada University of the year 2021 (Grant Number: 1009/UN1/FBI/KSA/PT.01.03/2021, dated on March 25, 2021). We are very grateful to Mrs. Whika Febria Dewatisari, S.Si., M.Sc. for the idea, and for providing the valuable CESTL, as well as the time for sharing her knowledge with us.

## CONFLICT OF INTEREST

The authors have no conflicts of interest to declare, and there is no financial interest to report.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb67412
DOI: 10.22146/jtbb.67412

Research Article

# The Influence of Sex and Weather on the Activity Budget of Javan Slow Lorises (*Nycticebus javanicus*) in Garut Regency, West Java

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**Keywords:**
daily activity
Javan slow loris
nocturnal
radio telemetry
**Submitted:**
29 June 2021
**Accepted:**
18 December 2021
**Published:**
28 March 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT

The Javan slow loris (*Nycticebus javanicus*) is a nocturnal primate endemic to Java. Previous studies on slow loris activity are limited to general daily activity, and there is a lack of research on the potential sex differences in slow loris activity. This study aims to analyze differences in the daily activity of the Javan slow loris based on sex. From August to December 2018, the daily activity of six wild Javan slow lorises was recorded using behavioral observations with instantaneous point sampling at 5-minute intervals. Differences in male and female slow loris activity were analyzed using the Generalized Linear Mixed Model (GLMM). We set sex and weather as fixed factors and individuals as random effects. The results of this study showed that females spent more time feeding and less time resting than males. In addition, the Javan slow loris behavior was affected by temperature and humidity like other slow loris species.



## INTRODUCTION

Daily activity budgets are key behavioral indicators of the survival strategies that species adopt to maximize individual fitness and longevity, and may also reveal the core requirements for a species' existence within a habitat (Repi et al. 2019). Daily activity budgets vary widely among primate species and are dictated by various factors, including social structure, nutritional requirements and ecological factors such as habitat type, food resource availability, and season (Korstjens et al. 2010; McFarland et al. 2014; Strier 2017).

Daily activity budgets are further influenced by morphological and reproductive differences between the sexes; leading to differences in nutritional requirements (Key & Ross 1999; Lodberg-Holm et al. 2021). It is generally agreed that the females of a species bear the greatest direct costs of reproduction through the energy consumption required for gestation and lactation, while males bear indirect costs of reproduction through consuming energy by

maintaining territories and guarding mates against rivals (Key & Ross 1999; Sussman et al. 2005; Thompson & Georgiev 2014). Differences in the strategies employed to efficiently exploit resources to meet varying nutritional needs between the sexes may lead to variations in activity budgets (Anirudh et al. 2020).

The Javan slow loris (*Nycticebus javanicus*) is a small nocturnal primate endemic to Java (Lehtinen 2013). The species is under threat due to habitat loss and wildlife trafficking in Southeast Asia and is classified as Critically Endangered by the IUCN Red List (Nekaris et al. 2015) and listed as Appendix 1 on CITES (Nekaris et al. 2008). Javan slow lorises are socially monogamous primates that live in family units consisting of a mated pair and several offspring (Barrett et al. 2021). Unlike many other socially monogamous primates, recent research has shown that adult slow loris males are actively involved in juvenile development and act as "social fathers" to offspring within their family unit; engaging in play behavior with juveniles to strengthen social bonds and to provide motor training for young individuals (Fernandez-Duque et al. 2009; Barrett et al. 2021).

The Javan slow loris has a geographic distribution in West, Central, and East Java (Nekaris et al. 2014; Voskamp et al. 2014; Wirdateti et al. 2019). Their habitat in West Java includes primary forest, secondary forest, and bamboo forest (Pambudi 2008). Javan slow lorises are also found outside of protected areas in traditional plantation gardens and forest gardens in Sumedang, Ciamis, and Tasikmalaya, West Java (Winarti 2003; Winarti 2011)

Previous studies on the daily activity budget on Javan slow lorises in plantation areas have found that the Javan slow loris spends 10-26% of its active time foraging (Reinhardt et al. 2016; Romdhoni 2021), with 5-15% of the total activity budget attributed to feeding (Reinhardt et al. 2016; Romdhoni 2021). Resting, including sleeping, constitutes 4-16% of their activity budget, while 14-39% of the daily activity budget is attributed to traveling (Rode-Margono et al. 2014; Reinhardt et al. 2016; Romdhoni 2021). Ecological factors, including habitat connectivity, food resource availability, rainfall, temperature, and humidity have also been shown to significantly affect slow loris behavior (Rode-Margono et al. 2014; Reinhardt et al. 2016; Cabana et al. 2017; Barrett et al. 2021).

Previous studies on the daily activity of slow lorises have been limited to general daily activity and studies into the sex-based differences in the daily activity of Javan slow lorises are lacking. A detailed understanding of the activity budget of threatened species such as the Javan slow loris is critical to their conservation efforts. Knowledge of how the activity budget of the Javan slow loris differs between the sexes may allow for more specialized management plans both in-situ and ex-situ. Therefore, this study aims to identify differences in adult Javan slow loris activity budgets based on sex while also considering the effects of temperature and humidity.

## MATERIALS AND METHODS

### Study Area

This research was conducted at the Little Fireface Project (LFP) field site from August to December 2018. The research was conducted in traditional plantation gardens, referred to as *talun*, in the village of Cipaganti, Garut, West Java, Indonesia (Figure 1).

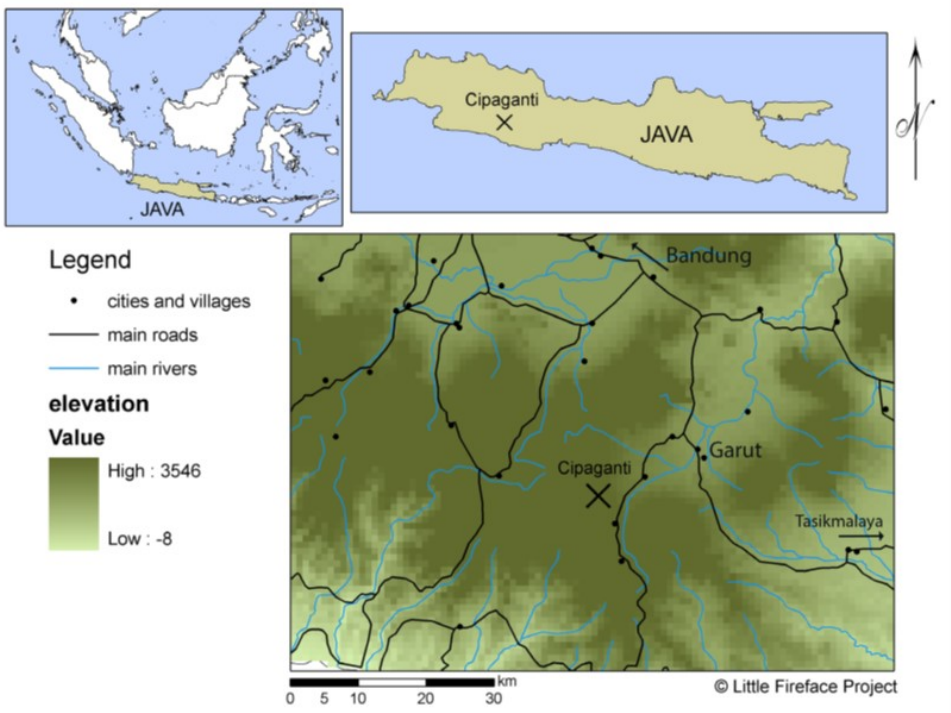


**Figure 1.** Location of the field site (Rode-Margono et al. 2014).

### Behavioral Observations

We collected data on six adults Javan slow lorises (three males and three females) fitted with VHF radio-collars (BioTrack, UK). We followed one individual per night, with a total duration of 12 hours per observation. We did not follow multiple lorises simultaneously because each individual has their own home range. Due to limited human resources we focused on one individual per night. The behavior of slow lorises was collected using an instant point sampling technique with 5-minute intervals (Altmann 1974). If the Javan slow loris performed a rarely observed behavior between the 5-minute intervals, we collected data on an ad libitum basis (Altmann 1974; Rode-Margono et al. 2014). We recorded the behavior data using a detailed behavioral ethogram adapted from Rode-Margono et al. (2014) (Appendix 1). We collected the weather data (temperature and humidity) via a HOBO weather station.

### Data Analysis

We used a Generalized Linear Mixed Model (GLMM) to see differences in activity budget. We set weather (temperature and humidity) and sex as fixed factors and individuals as random effects. We used IBM SPSS Statistic v 26. We considered a p-value of 0.05 as the threshold for significance.

## RESULTS AND DISCUSSION

### Results

#### Sex based differences in activity budget

The research was conducted on six individuals for a total of 378.33 hours of observation over 35 days. The total instances of active observation were 1107 times and the total time of active observation was 92.67 hours.

We found a significant difference between male and female slow lorises in feeding ($p < 0.001$) and resting ($p = 0.021$) behavior (Table 1). Females spent more time feeding (8.32%) than males (2.93%) and less time resting (3.25%) than males (7.17%). The percentage of other behaviors did not change between male and female slow lorises (Figure 2).

#### Environmental factors affecting activity budget

With an increase in temperature, slow lorises spent significantly more time feeding ($p < 0.001$) and sleeping ($p < 0.001$) and significantly less time doing the following behaviors: alert ($p < 0.001$), forage ($p = 0.008$), social ($p < 0.001$), and travel ($p < 0.001$) (Table 1). Humidity also had a significant influence on the time spent alert, feeding, foraging, resting, sleeping, and socializing (Table 1).

### Discussion

#### Javan slow loris daily activity based on sex

Our study found that across eight main behaviors observed in Javan slow lorises, two key behaviors, feeding and resting, differed significantly in proportion of total activity budget between the sexes. Our data indicates that female Javan slow lorises attributed significantly more of their active time to feeding than males. In contrast, males rested significantly more than females within their daily activity budget (Figure 2). These findings are in direct contrast to studies on other monomorphic primate species, including other *Nycticebus* species. Research on the feeding time of captive greater slow lorises (*N. coucang*) and eastern lesser bamboo lemurs (*Hapalemur griseus*) revealed no significant difference between the sexes (Duncan 1982; Grassi 2002). How-

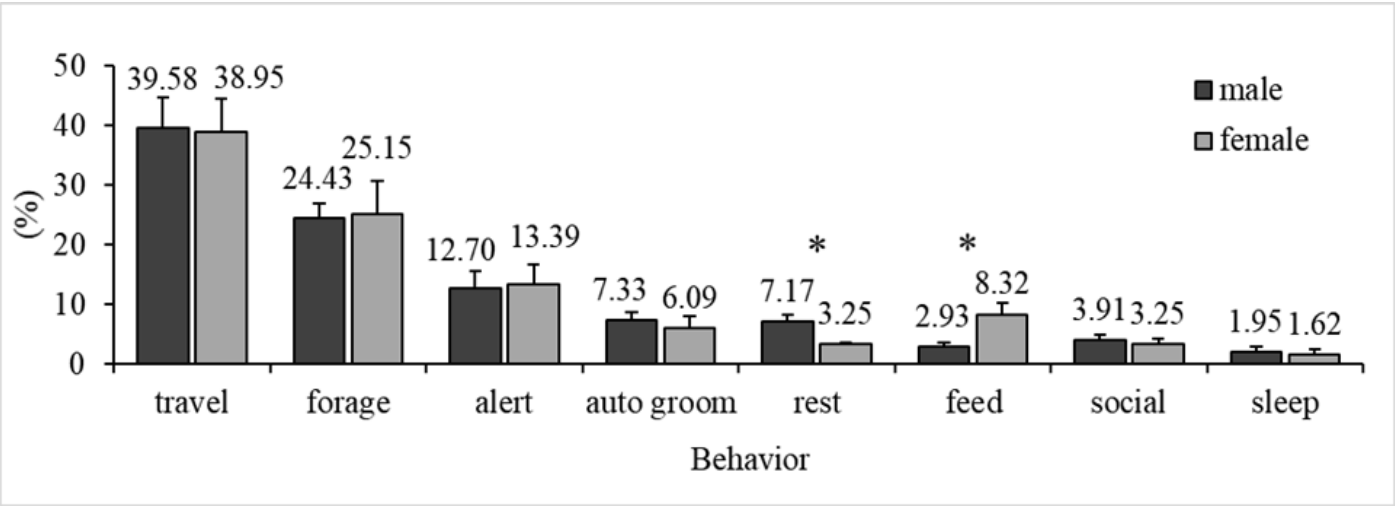


**Figure 2**. Javan slow loris behavior percentage based on sex (3 females, 3 males). *Significantly different ($p < 0.05$).

**Table 1.** Results of the generalized linear mixed model explaining the effect of sex, temperature, and air humidity on the behavior of six Javan slow lorises in Cipaganti, West Java.

| Behavior | Factor | Coefficient | Std. Error | t-value | Sig. |
|---|---|---|---|---|---|
| **Alert** | Sex | -0.039 | 0.506 | -0.078 | 0.938 |
| | Temperature | -0.131 | 0.009 | -15.435 | 0.000* |
| | Humidity | -0.008 | 0.002 | -3.574 | 0.001* |
| **Feed** | Sex | 1.432 | 0.412 | 3.473 | 0.001* |
| | Temperature | 0.125 | 0.010 | 12.327 | 0.000* |
| | Humidity | 0.100 | 0.003 | 32.872 | 0.000* |
| **Forage** | Sex | 0.040 | 0.294 | 0.136 | 0.892 |
| | Temperature | -0.017 | 0.006 | -2.768 | 0.008* |
| | Humidity | 0.020 | 0.002 | 12.473 | 0.000* |
| **Groom** | Sex | -0.414 | 0.438 | -0.945 | 0.350 |
| | Temperature | -0.015 | 0.011 | -1.400 | 0.169 |
| | Humidity | 0.002 | 0.003 | 0.790 | 0.434 |
| **Rest** | Sex | -1.114 | 0.464 | -2.401 | 0.021* |
| | Temperature | 0.016 | 0.012 | 1.361 | 0.181 |
| | Humidity | -0.029 | 0.003 | -9.436 | 0.000* |
| **Sleep** | Sex | 0.093 | 3.011 | 0.031 | 0.975 |
| | Temperature | 1.048 | 0.037 | 28.558 | 0.000* |
| | Humidity | 0.053 | 0.009 | 5.976 | 0.000* |
| **Social** | Sex | -0.839 | 1.274 | -0.659 | 0.514 |
| | Temperature | -0.290 | 0.022 | -13.327 | 0.000* |
| | Humidity | 0.015 | 0.007 | 2.179 | 0.035* |
| **Travel** | Sex | -0.217 | 0.313 | -0.694 | 0.491 |
| | Temperature | -0.108 | 0.005 | -21.448 | 0.000* |
| | Humidity | -0.002 | 0.001 | -1.339 | 0.188 |

*Significantly different ($p < 0.05$)

ever, direct comparisons between the activity budgets of captive individuals and wild individuals are complicated by the fact that individuals in captivity do not face the same challenges as wild individuals in terms of food availability and predation risk and environments vary widely (Melfi & Feistner 2002). Regarding research on wild individuals, Wiens (2002) found no significant differences in the daily time budget of resting, feeding or social behavior between male and female greater slow lorises; however the inclusion of sexually immature individuals in the data analysis of this study may obscure potential differences in reproduction-related changes to the activity budget between adult male and female slow lorises.

As a monogamous species, the Javan slow loris lacks sexual dimorphism, and males and females are roughly the same size and weight (~905g) (Barrett et al. 2021). Monomorphic species are generally expected to have similar energy expenditure outside of reproduction costs (Key & Ross 1999; Cabana et al. 2017). However, female nutritional requirements are closely linked to reproduction; during gestation and lactation, females may adjust their activity budget to compensate for the increased energy requirements of reproduction (Ganzhorn et al. 2004).

In other monomorphic primates such as ring-tailed lemurs (*Lemur catta*), females have been shown to become more selective in nutrient intake in periods of lactation (Rasamimanana & Rafidinarivo 1993; O'Mara & Hickey 2014) which may lead to changes to daily activity. Research on feeding time in male and female sifakas (*Propithecus verreauxi*) also showed a significant difference at the end of the lactation period, in addition to differences in diet (Koch et al. 2017). In contrast to our study, Anirudh et al. (2020) found significant differences in feeding and foraging behavior between male and female adult Philippine slow lorises (*N. menagensis*), as males were observed feeding significantly more while females spent significantly more time foraging. They also observed that feeding activity for male individuals was well distributed throughout the active period, whereas for females, most of the feeding behavior occurred at the beginning of the active period. These findings suggest that males and females may adopt different foraging strategies to maximize individual requirements, however as the authors note, the study was conducted on individuals released from captivity; therefore, observed behavior may differ from their wild counterparts (Anirudh et al. 2020). Female Javan slow lorises have a gestation period of 6 months, a relatively long period considering their small size (Poindexter & Nekaris 2017) and may result in significant changes to nutritional requirements during reproduction. Two of the three females observed in this study were known to have been in a period of gestation, and subsequently lactation, during the period of data collection. Their higher energy requirements during this time likely explains our feeding behavior findings.

An increase in the proportion of time spent on one activity within an activity budget causes a decrease in the proportion of time spent on one or more other activities. As the other behaviors analyzed in this study were not significantly different between the sexes, this suggests that the increased feeding behavior exhibited by the focal female individuals directly resulted in less time spent resting. Conversely, as males have lower nutritional requirements than females, they may be able to balance energy expenditure by increasing period of resting rather than increasing energy intake (Reinhardt et al. 2016)

### Javan slow loris daily activity in relation to environmental factors

In line with previous studies on the effects of environmental factors on Javan slow loris activity, this study found that temperature and humidity had significant impact on several behaviors. Temperature had a significant positive relationship with sleeping and feeding behavior, and a significant negative relationship with foraging, traveling, social, and alert behavior. Humidity had a significant positive influence on sleeping, foraging, feeding, and social behaviors and a significant negative influence on resting and alert behavior. Environmental factors may directly affect slow loris behavior through changes in energy requirements and indirectly through changes to the habitat in response to fluctuations in climatic conditions (Reinhardt et al. 2016). The slow

lorises in this study live in an agricultural area between 1100-1500m asl, covered with cultivated fields, abandoned fields, tree plantations, and bamboo patches. Each field is often bordered by trees, creating a connected canopy for the lorises (Rode-Margono et al. 2014).

Slow lorises are specialized exudativores that also feed on insects, nectar, and small vertebrates and rarely eat fruits (Wirdateti et al. 2005; Nekaris & Bearder 2007). According to Cabana et al. (2017), the Javan slow loris eats exudates (38-60%) predominantly and insects (12-27%) with flower nectar consumed seasonally and fruit consumed rarely. They consumed exudates from *Accacia deccurens* and nectar of *Calliandra calothyrsus* (Rode-Margono et al. 2014; Cabana et al. 2017; Romdhoni 2017). Male slow lorises consume more exudates and nectar, while female slow lorises consume more arthropods (Romdhoni 2017). While exudates, the staple food source of Javan slow lorises, are available year-round, it is of low nutritional value and therefore slow lorises must supplement this diet with other food sources (Cabana et al. 2017). Other key slow loris food sources such as insects, nectar, and flowers are seasonally available and this may affect slow loris feeding and foraging behaviors (Cabana et al. 2017).

Increased humidity may affect the activity of arthropods (Reinhardt et al. 2016). Insects comprise approximately 10-18% of the Javan slow loris's diet, including flying insects such as Lepidoptera and Coleoptera, and insect consumption varies between seasons (Wiens et al. 2006; Starr & Nekaris 2013; Cabana et al. 2017). In higher humidity, insects have been shown to fly lower (Shamoun-Baranes et al. 2006). Slow lorises cannot leap, so they may take advantage of lower flying insects during periods of increased humidity by selectively feeding more on insects. This study did not analyze food source selection and therefore cannot draw further conclusions on potential changes to feeding behavior because of environmental factors.

Rode-Margono & Nekaris (2014) and Reinhardt et al. (2016) did not find a correlation between temperature and Javan slow loris activity. We found, however, that Javan slow lorises decreased feeding and sleeping at lower temperature. We also found that travel and forage time increased at lower temperature, suggesting a possible need to travel farther searching for food to compensate for the lower food availability in the colder period (Campera et al. 2021). Nekaris et al. (2021), found a higher use of canopy bridges during hotter periods, suggesting a positive relationship between travelling and temperature. Our study might be temporally limited, and other patterns might emerge if a larger data collection period is considered.

## CONCLUSION

Significant differences in the activity of male and female slow lorises were found in feeding and resting behavior. Male slow lorises fed more and rested less, while female slow lorises ate more and rested less. The Javan slow loris behavior is affected by environmental factors (temperature and humidity).

## AUTHORS CONTRIBUTION

K.A.I.N. directed the long-term field project, conceptualised the project, and acquired the funding. H.R. wrote the original draft manuscript and conducted the field work. H.B. supervised field research activities. H.R. conducted the statistical analysis. D.P., E.I, K.H., M.C., and K.A.I.N. reviewed, revised, and proofread the manuscript.

## ACKNOWLEDGMENTS

Our gratitude goes to the Little Fireface Project staff and volunteers, for helping us in the field, D Ahmad, Y Nazmi, A Nunur, R Hidayat, D Rustandi, E Brown, A Ardiansyah, MH Rizky, and A Sokonegoro for their assistance in collecting data. We thank the Nocturnal Primate Research Group of Oxford Brookes University, the Garut District Perhutani, the Garut Natural Resources Conservation Center (BKSDA), and Ministry of Research and Technology (KEMENRISTEK). Funding for all aspects of the fieldwork in Cipaganti came from Amersfoort Zoo, Augsburg Zoo, Brevard Zoo, Cleveland Zoo and Zoo Society, Columbus Zoo, Conservation International Primate Action Fund and Margot Marsh Biodiversity Fund, Cotswold Wildlife Park, Disney Worldwide Conservation Fund, Ernest Kleinwort Charitable Trust, European Human Behaviour and Evolution Society, Henry Doorly Zoo, International Primate Protection League, Lee Richardson Zoo, Leverhulme Trust (RPG-083), Longleat Safari & Adventure Park, Marjorie Coote Foundation, Mazuri Zoo Feeds, Memphis Zoo, Mohamed bin al Zayed Species Conservation Fund (152511813), Moody Gardens, Nacey Maggioncalda Foundation, National Geographic (GEFNE101-13), Naturzoo Rhein, Paradise Wildlife Park, People's Trust for Endangered Species, Phoenix Zoo, Primate Society of Great Britain, Plumplori, the Royal Geographic Society with IBG, San Francisco Zoo, Shaldon Wildlife Trust, Sophie Danforth Conservation Biology Fund, University's Federation for Animal Welfare, Whitley Wildlife Conservation Trust, and ZGAP.

## CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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## APPENDIX

**Table A**. Ethogram of Javan slow loris adapted from Rode-Margono et al. (2014).

| No | Behavior | Description |
|---|---|---|
| 1 | Alert | Remain stationary like in "rest" but active observation of environment or observer. |
| 2 | Feed | Actual consumption of a food item. |
| 3 | Forage | Stationary or movement associated with looking for food (often including visual and olfactory searching). |
| 4 | Auto groom | Lick or use tooth comb on own fur. |
| 5 | Rest | Remain stationary, often with body hunched and eyes are open. |
| 6 | Sleep | Remain stationary in huddled position with head between the knees, or eyes are visible but closed. |
| 7 | Social | All interactions with conspecifics, including aggression, allogrooming, play, and other social behaviors. |
| 8 | Travel | Continuous, directed movement from one location to another. |

Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb67360
DOI: 10.22146/jtbb.67360

Research Article

# Effect of Cryoprotectans and Cryopreservation on Physiological and Some Biochemical Changes of *Hopea odorata* Roxb. Seed

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**Keywords:**
*Hopea odorata*
cryoprotectant
cryopreservation
recalcitrant seed
seed viability
**Submitted:**
04 July 2021
**Accepted:**
20 December 2021
**Published:**
04 April 2022
**Editor:**
Miftahul Ilmi

## ABSTRACT

*Hopea odorata* Roxb. is a forest plant from Dipterocarpaceae family that has important economic and ecological functions in the ecosystem. Generative propagation of *H. odorata* is limited because of its recalcitrant seed that cannot be stored for long periods at room temperature or even at low temperature. Cryopreservation is a seed storage technique that has the potential to prolong the shelf life of recalcitrant seeds. The aim of this study was to evaluate the effect of cryoprotectant and cryopreservation treatment on seed viability and biochemical change (electrolyte leakage, total malondialdehyde, total phenol) of *H. odorata* seeds. Fresh seeds of *H. odorata* were treated with two types of cryoprotectans namely PVS1 as non penetrating cryoprotectant and PVS2 as penetrating cryoprotectant, each type of cryoprotectant with four different concentrations (25, 50, 75 or 100%(w/v)) and four different immersion times (30, 60, 90 or 120 mins). Seeds were then stored in two different temperatures, at room temperature (28±2ºC) or in liquid nitrogen (-196±2ºC) for 24 hours to evaluate the cryoprotectant toxicity. The results showed that *H. odorata* seeds stored at room temperature and immersed either in 100%, 75% or 50% of PVS1 possess a higher viability as well as germination percentage, germination rate, vigour index and maximum growth potensial. In addition, they have lower value of electrolyte leakage, total malondialdehyde and total phenol compared to those seeds treated with PVS2. Meanwhile, both type of cryoprotectants and cryopreservation treatment in this study have not been able yet to increase seed viability of *H. odorata*. Cryopreservation treatments caused an increase in the total of malondialdehyde and electrolyte leakageas and these leads the inability of *H. odorata* seeds to germinate. PVS1 cryoprotectant seems to cause less toxic effects on *H. odorata* seeds but it can not prevent the negative impact of cryopreservation treatment.



## INTRODUCTION

*Hopea odorata* Roxb. is a native forest plant in India, Bangladesh, Myanmar,Vietnam and several Indochina countries (Ly et al. 2017). *H. odorata* seedlings have a fast growth rate, reaching 30-40 cm at the age of 6-9 months

and are ready to be transplanted into the field. The diameter of its stem can reach 53 cm at the age of 25 years with a survival rate of almost 100% (Joker & Salazar 2000; Junaedi & Frianto 2012). Therefore, *H. odorata* is one of the species recommended for establishing Dipterocarpaceae forest (Weinland 1998), such as implemented in several countries like Malaysia, Sri Lanka, Vietnam and Cambodia (Chua et al. 2010; Ashton et al. 2011). On its economical value, *H. odorata* is widely used as a source of timber, gum or resin, and for construcion purposes (Orwa et al. 2009; Junaedi & Frianto 2012).

The high demand of wood and resin of *H. odorata* were not accompanied by the restoration process yet. Thirty to fifty percent of *H. odorata* population from three generations (± 300 years) continues to decline due to exploitation and habitat conversion for agriculture purposes. Declining population problem cause *H. odorata* to be categorized as plant species that are vulnerable to ecological extinction according to International Union for Conservation of Nature and Natural Resources (IUCN) (Ly et al. 2017). Propagation of *H. Odorata* currently is still limited to vegetative technique because of the seed storage problem. *H. odorata* flowers bear fruit only every one or two years, fruit ripening occurs for three months from anthesis and produces recalcitrant seeds (Sasaki 2008; Orwa et al. 2009).

Recalcitrant seeds are sensitive to desiccation and it differ to orthodox seeds which have desiccation tolerance. Recalcitrant seed normally cannot be stored for a long time under conditions that cause low moisture content of the seeds, such as high temperature, low humidity, tightness material container and direct light exposure. It is because they have active metabolism and high respiration rate under those conditions (Lodong et al. 2015). It has been reported that some recalcitrant seeds from family Dipterocarpaceae showed a decrease in their germination percentages if stored at room temperature (28±2C$^{o}$), for example *Vatica chinensis* decreased the germination percentage to 69.95% when stored at mositure content (MC) of 52.24% , *Hopea ponga* has germination percentage of 40% when stored at MC of 29%, and *Shorea seminis* has germination percentage of 30.11% when stored at MC of 49 % to 47%) (Sukesh & Chandrashekar 2011; Sukesh & Chandrashekar 2013; Zanzibar et al. 2019).

Cryopreservation is an alternative storage process for any biological constructs such as cell, tissue or organelles at very low temperatures (Jang et al. 2017). Cryopreservation procedure involves the utilization of liquid nitrogen to quickly deep freeze the cell, allowing suppresion of enzymatic activity, and metabolic activity will turn to stand still conditions trough out the storage periods until thawed (Kartha 1985; Radha et al. 2012). The most critical factor for the effectiveness of cryopreservation technique is the right choice of protective compounds that can prevent cells from chilling injury. Cryoprotectant is nonelectrolyte chemical used to reduce the amount of intracellular water and protect the cell for extracellular ice formation caused by very low temperatures (Joshi 2016). There are several cryoprotectants that

can be categorized according to the way of penetrating the cells, namely (a) small substances that are able to penetrate the cells through the cell wall and plasma membrane, (b) substances that are able to penetrate only through the cell wall and (c) substances that do not penetrate through the cell walls or the plasma membrane. Plant Vitrification Solution (PVS) and its modification for obtaining successfulness in cryopreservation has been reviewed by Zamecnik et al. (2021). Jitsopakul et al. (2012) reported that application of PVS2 solution as common cryoprotectan with 15 min loading treatment increase the germination percentage of cryopreserved seeds *Vanda tricolor*. Meanwhile, the effectiveness of cryopreservation techniques to maintain cell viability was also found in seed of *Percea americana* cv. Velvick. An exposure of this seeds into PVS2 and vitrification solution L (VSL) for 20 min caused a highest regrowth level after seeds were immersed into liquid nitrogen (O'Brien et al. 2021). Based on these findings, cryopreservation technique looks promising to be applied to recalcitrant *H. odorata* seeds, so that the seeds can be stored for a longer period without losing its viability. In addition, this kind of research has never been reported previously. The purpose of this study was to evaluate the effect of cryoprotectant treatment on seed viability and some biochemical change within the seed including electrolyte leakage, total malondialdehyde, total phenol of *H. odorata* seeds following storage at room temperature (28±2$^{o}$C) or liquid nitrogen (-196±2$^{o}$C).

## MATERIALS AND METHODS

### Materials

The experiment was held at Research Center for Plant Conservation and Botanical Gardens, Bogor and Plant Physiology Laboratory, Faculty of Biology, Universitas Gadjah Mada, Yogyakarta. The mature seeds of *H. odorata* were collected from Bogor Botanical Garden in December 2020. The seeds were classified as mature when 2/3 of the wings turned the color to dark brown and the pericarp or the fruit coat has changed as well from green to brown (Joker & Salazar 2000). The wings were removed for ease of the experiment.

### Methods

#### Cryopreservation Procedure

*H. odorata* seeds were immersed in two different types of cryoprotectants, the first was Plant Vitrification Solution Number 1 (PVS1) which consists of mannitol and the second was Plant Vitrification Solution 2 (PVS2) which consists of DMSO 15% (w/v), ethylene glycol 15% (w/v) and glycerol 30% (w/v) in 0.4 M sucrose. For each type of cryoprotectant four different concentrations were prepared namely 25%, 50%, 75% or 100% (w/v). The seeds were immersed in each type of cryoprotectant for 30 mins, 60 mins, 90 mins or 120 mins. For control, seeds were immersed in distilled water and put in cryo-tubes. Both control and seeds treated with cryoprotectant were then stored at room temperature (28±2$^{o}$C) or subjected to liquid nitrogen (-

196±2°C) for 24 hours. Seeds that have been subjected to liquid nitrogen were thawed with warm water (40°C) for 3 minutes. Seeds were rinsed with distilled water to remove any residual cryoprotectant. Subsequently, seeds were sown in polybags (15 cm diameter, volume ± 4.420 $cm^3$) containing sterile sand (90% sand grains that passed a ± 5 mm sieve) and placed in a greenhouse with average air temperature of 30.5°C and 53% of humidity, 147x100 Lux of light intensity, and 74% of soil moisture (average condition at midday). The germination tests were carried out using three replications of 25 seeds. The other biochemical tests were performed using three replicates as well.

### Physiological and Biochemical Observation Variables

Observation variables were divided into physiological variables (moisture content, germination test) and biochemical variables (electrolyte leakage, total malondialdehyde, total phenol). Normal seedling category and germination test were carried out according to procedure of seed testing protocol of International Rules for Seed Testing (ISTA) 2015 and 2018.

Moisture content was calculated using the formula :

$$MC = \frac{M2 - M3}{M2 - M1} \times 100\%$$

Where, M1 = weight of petridish; M2 = weight of petridish and seed before drying; M3 = weight of petridish and seed after drying.

Germination percentage was calculated using the formula :

$$GP = \frac{first\ count + final\ count}{Number\ of\ germinated\ seeds} \times 100\%$$

Where, the first count was determined at 7 days after sowing (DAS) and the final count was determined at 12 DAS.

Germination rate was calculated using the formula :

$$GR = \sum_{i=1}^{12} d$$

Where, *d* is additional percentage of normal seedling/etmal (1 etmal = 24 hours).

Vigor index was calculated using the formula :

$$VI = \frac{\sum seedlings\ of\ first\ count}{total\ planted\ seed} \times 100\%$$

Maximum growth potential was calculated using the formula :

$$MGP = \frac{\sum germinated\ seed}{\sum planted\ seed} \times 100\%$$

Total Malondialdehyde was determined using 1 gr of *H. odorata* seed extract according to method explained by Zhang and Huang (2013) and calculated using the formula:

$$MDA = [6.45 \times (A_{532} - A_{600}) - 0.56 \times A_{450}] \times 5$$

Where, $A_{532}$ = absorbance of 532 nm, $A_{600}$ = absorbance of 600 nm, $A_{450}$ = absorbance of 450 nm.

Total phenol was determined using Folin-Ciocalteu method and total phenol was determined using a UV-Vis spectrophotometer at 765 nm and three times reading were performed to obtain the variations of absorbance value.

Percentage of electrolyte leakage was measured using a method explained in Stewart and Bewley (1980) and calculated using the formula :

$$C = \frac{Cl}{Ch} \times 100\%$$

Where, C = percentage of electrolyte leakage, Cl = Leachate or first conductivity, Ch = Homogenate or final conductivity.

### Data Analysis

Data analysis was performed using the Analysis of Varince (ANOVA) test an.d continued with the Duncan's Multiple Range Test (DMRT). Data were calculated using Statistical Product and Service Solutions (SPSS) software version 16.0 with $\alpha = 5\%$.

## RESULTS AND DISCUSSION

### Effect of cryoprotectant on *H. odorata* seed stored at room temperature

Results showed that different types and concentrations of cryoprotectant had a significant effect on physiological and biochemical variables ($p<0.05$) (Table 1). Meanwhile, immersion time variations did not show any significant effect on moisture content, germination percentage and germination rate. Non-significantly immersion time variation (I) on the observed variables has implications for its interaction with other treatments variation (T-C / C-I / T -C-I). This indicates that different duration of immersion in this study gave the same effect on *H.odorata* seeds stored at room temperature regardless of the type of cryoprotectants.

**Table 1**. Analysis of variance for the effect of cryoprotectans, concentration, and immersion time on the variable observations and its interactions.

| Source of variation | P-value | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | MC | GP | GR | VI | MGP | EL | MDA | PH |
| Type (T) | .000* | .000* | .000* | .000* | .000* | .000* | .000* | .000* |
| Concentration (C) | .001* | .000* | .000* | .000* | .000* | .000* | .000* | .000* |
| Immersion time (I) | .182ns | .095ns | .412ns | .002* | .000* | .000* | .000* | .000* |
| T*C | .056 ns | .000* | .000* | .000* | .000* | .000* | .000* | .000* |
| T*IT | .010* | .449ns | .183ns | .000* | .000* | .000* | .000* | .000* |
| C*IT | .997ns | .790ns | .307ns | .286ns | .000* | .000* | .000* | .000* |
| T*C*IT | 1.00ns | .652ns | .232ns | .894 ns | .000* | .000* | .010* | .000* |

Note : * = treatment gave significant effect on observed variables at $\alpha=5\%$. ns = treatment gave non-significant effect on observed variablesat $\alpha=5\%$. Type (PVS1 and PVS2). Concentration (25%, 50%, 75%, 100%). Immersion time (30, 60, 90 or 120 min). MC: Mouisture Content, GP: Germination Percentage, GR: Germination Rate, VI: Vigour Index, MGP: Maximum Growth Potensial, EL: Electrolyte Leakage, MDA: Total Malondialdehyde, PH: Total Phenol. (Rohmah 2021).

Chemical compound of DMSO (dimethyl sulfoxide, $Me_2SO$) as a component of PVS2 was reported to be toxic to cell membranes, especially when used at room temperature (Aronen et al. 1999; Bettoni et al. 2019). In the study of damar (*Agathis damara*) seeds, it has been reported that high germination rate (84,67%) was obtained from seeds that have been subjected to vitrification for one hour without any cryoprotectant. Cryoprotectant normally used to increase seed viability of recalcitrant or intermediate seeds. However, the application of cryoprotectant and vitrification for one hour lowered seed viability of damar (Djam'an et al. 2006). The effectiveness of cryoprotectant and duration required to immerse the seed into cryoprotectant seems different and it depends on the type of seed cultivar and pre-cryopreservation treatment such as the loading treatment (Wardani et al. 2019; O'Brien et al. 2021).

Regardless of immersion time of *Hopea odorata* seeds in the cryoprotectants, both PVS1 and PVS2 cryoprotectants with concentrations of 25%, 50%, 75% or 100% were generally able to reduce seeds moisture content above the critical moisture content value (lethal MC) of *H. odorata* (Figure 1). All treatment combinations significantly reduce the MC of *H. odorata* seeds compared to those of control that showed moisture content value of seeds about 36,89 - 45,31%. According to Orwa et al. (2009), the lethal MC for *H. odorata* seed is 33% at 35°C, in which seeds will die within five days under that condition due to dehydration. Immersing seeds in cryoprotectants before vitrification is important to prevent seeds from mechanical damage due to intracellular fluid crystallization. However, type of cryoprotectants, concentrations, and immersion time must be determined so that it may not cause the water content of the seeds decreased below the lethal MC, especially for recalcitrant seeds (Berjak & Pammenter 2013). The results of this study were in line with the finding that application of 35% cryoprotectant effectively reduce the moisture content of Sugi (*Crypometria japonica*) seeds and increase its germination. It was also reported that application of PVS2 for 2 hours was significantly able to maintain the critical

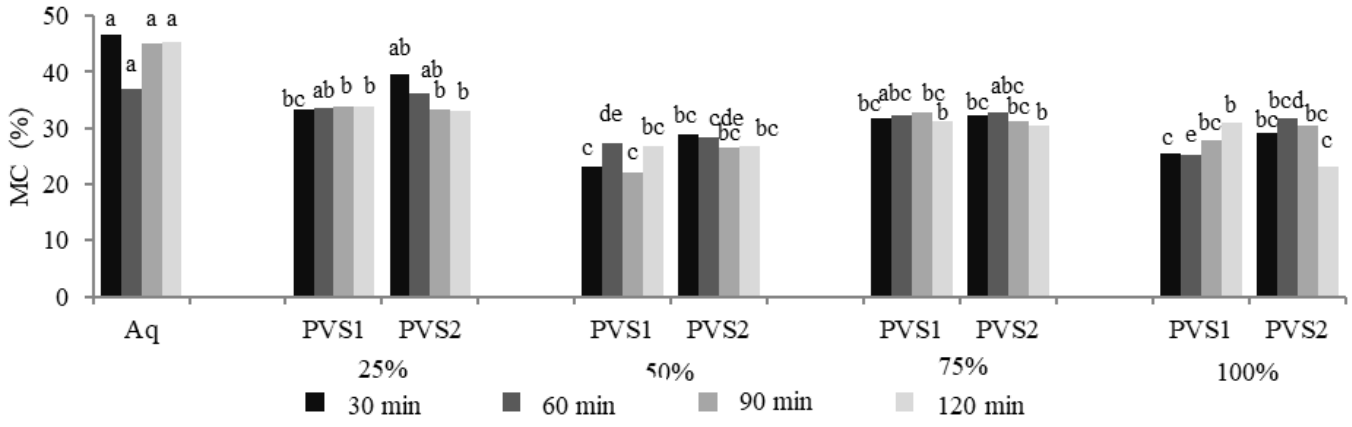


**Figure 1.**Interaction of cryoprotectant types, concentrations and immersion times on percentage of moisture content (MC) of *H.odorata* seed stored at room temperature. Note: Aq= Aquadest. The similar letter above each column indicates that the treatment gave a nonsignificant effect based on DMRT test (α=5%). (Rohmah 2021)

moisture content of *Hibiscus sabdariffa* seeds (14.28%) compared to control (10.71%) or vitrification at a temperature of -5ºC (11.53%) (Kim et al. 2009; Suhendra et al. 2014).

Figure 2 showed that PVS2 treatment for *H. odotara* seeds at all concentrations and immersion time resulted in the germination percentage (GP) less than 50% compared to those treated with cryoprotectant PVS1 or control. Decreasing value of seed GP can be caused by seed moisture content that below the critical limit. However, seed moisture content of *H. odorata* presented in Figure 1 showed that the PVS2 application was generally able to maintain the seeds moisture content that is appropriate to keep seed viability (38.58% to 26.56%). The fact that seed germination value is low suggests that there might be a toxic effects of PVS2 cryoprotectant.

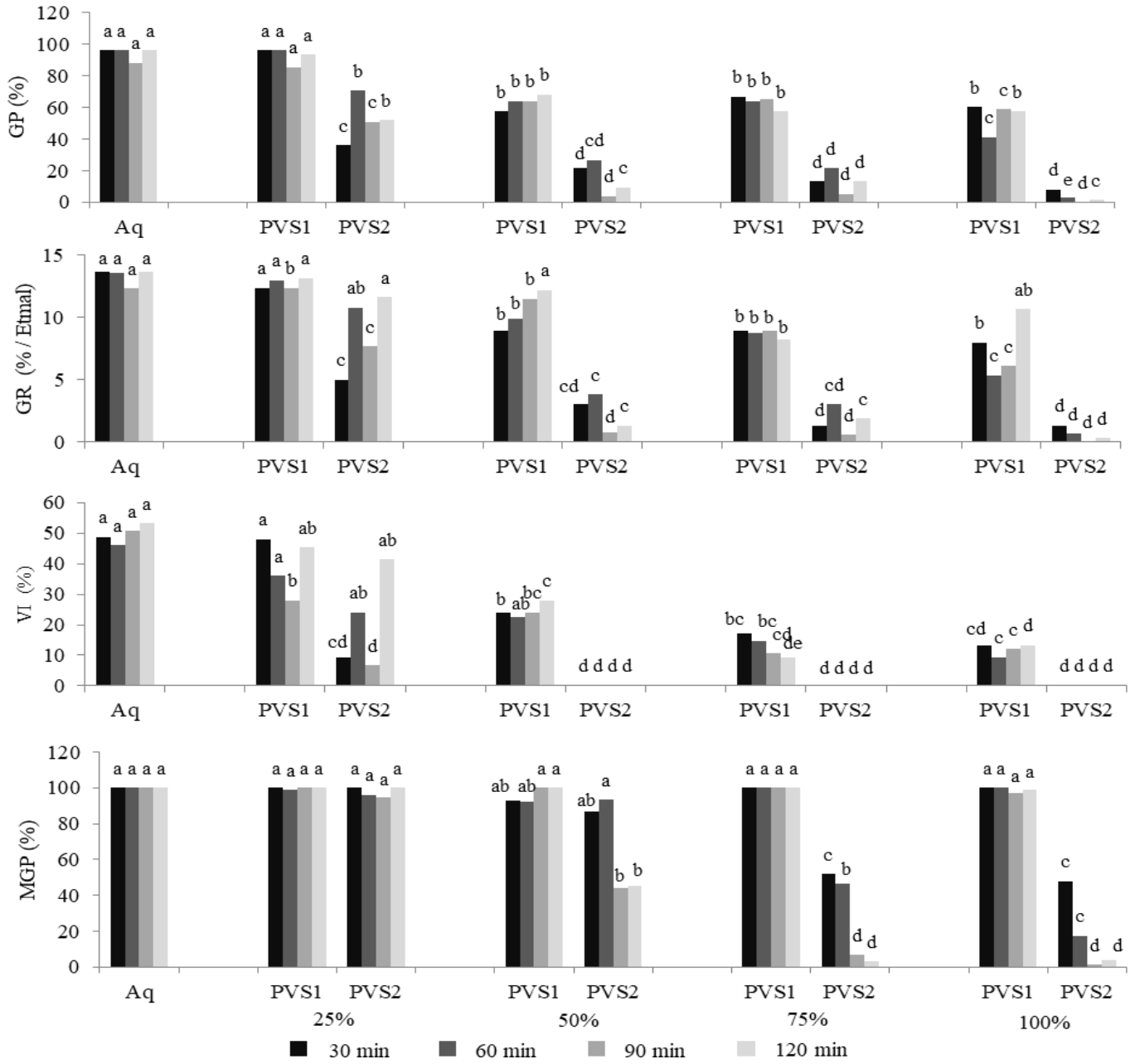


**Figure 2.** Interaction of cryoprotectant types, concentrations and immersion times on percentage of germination percentage (GP), germination rate (GR), vigor index (VI), and Maximum Growth Potential (MGP) of *H.odorata* seed at room temperature. Note: Aq= Aquadest;Etmal = 24 h. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

The results showed that PVS2 treatment decreased the average of germination rate percentage (GR), vigor index (VI), and Maximum Growth Potential (MGP) compared to those of PVS1 treatment (GR: 5.34 - 16.38%; VI: 9.33 - 48.0%; MGP: 92 - 100%) (Figure 2). *H. odorata* seeds that were immersed in PVS2 showed very small VI percentage (0% - 4%), except at concentration of 25% and immersion time of 120 mins (41.33%), but in general the value was lower than PVS1 treatment or controls. Treatment of 75% PVS2 with immersion time 90 mins (6.7%) and 120 mins (30.7%) was significantly decrease the percentage of MGP of *H. odorata* seed compared to PVS1 in the same concentration and immersion time (MGP: 100%). *H. odorata* seeds treated with PVS2 of 50% for 90 min or 120 min showed a decline in MGP, whereas the higher concentration of PVS2 (75% or 100%) start lowering the percentage of MGP at 30 min immersion. These results indicate that PVS2 treatment with a concentration of more than 50% with immersion time more than 60 mins caused inhibition for germination of *H. odorata* seeds. Meanwhile, the percentage of VI after PVS2 treatmentis were lower than PVS1,this result confirmed that *H. odorata* seeds can successfully germinate but not able to grow in the normal category of ISTA (2018). It was also reported that application of PVS2 on shoots tissue of *Pimpinella pruatjan* increased survival to 90% when applied before explants were stored in liquid nitrogen, whereas after freezing with liquid nitrogen the percentage of survival was only 40%) (Roostika et al. 2013). Best (2015), reported that PVS2 is dangerous for the structure of phospholipid bilayer, causing a decrease in the permeability function or turgidity of the cell membrane. The effectiveness of PVS2 could be increased by the addition of melatonin or vitamin C to reduce the effects of toxicity and accumulation of oxidative compounds due to low temperature stress. Burritt (2012), reported that addition of reactive oxygen species (ROS) binders in cryoprotectants will improve plant tissue adaptation mechanisms.

Results presented in Figure 3 showed that treatments of PVS1 or PVS2 at concentrations of 25%, 50%, 75% or 100% with 30 minutes immersion time did caused any significant difference on electrolyte leakage (EL) percentage compared to control, but there was a tendency for a slight increase in electrolyte leakage in those seeds treated with longer immersion time of PVS1 or PVS2. The increasing percentage of EL after seeds were immersed into croprotectants might be due to cryoprotectant concentration that was lower compared to the intracellular fluid, and it promotes hypotonic conditions for cells. Under hypotonic conditions, the solutes in the seeds will move to the extra cellular which in this experimental unit is a cryoprotectant compound with a lower concentration.

Total malondialdehyde (MDA) of *H. odorata* seeds that were immersed into 25% PVS1 for 30 mins showed a lower value (5,110 nmol $g^{-1}$ FW) than immersion into 50% PVS1 (5,211 nmol $g^{-1}$ FW), 75% PVS1 (5,379 nmol $g^{-1}$ FW) or 100% PVS1 (5.451 nmol $g^{-1}$ FW). In addition, total MDA increased concomitant with longer immersion time even when the concentration of

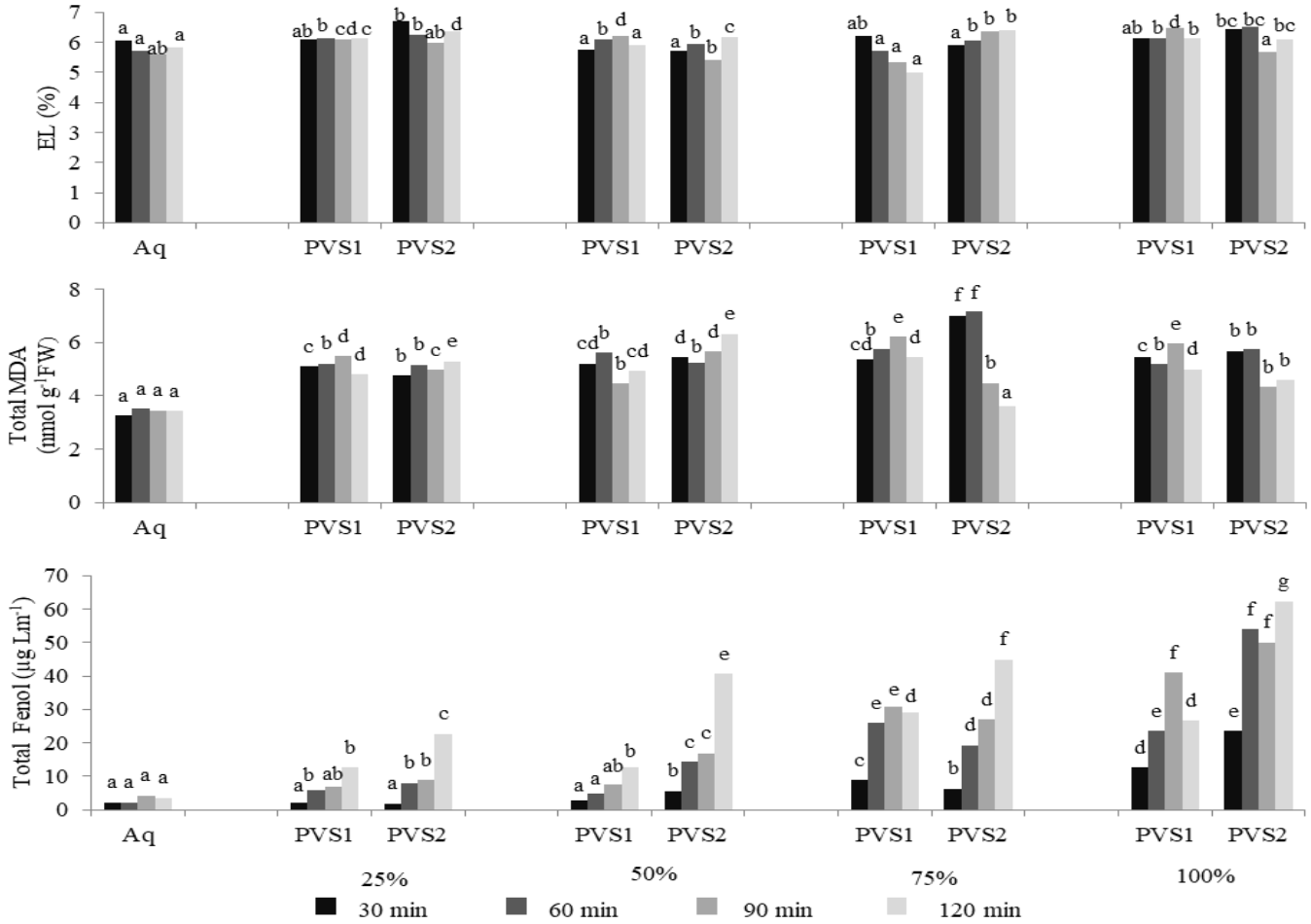


**Figure 3.** Interaction of cryoprotectant types, concentrations and immersion times on percentage of electrolyte leakage (EL), total malondialdehyde (MDA), and total phenol (PH)at room temperature. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

PVS1 used was 25%. The similar pattern of increasing total MDA was also seen in the PVS2 treatment. Overall, both PVS1 and PVS2 treatments caused an increased in total MDA compared to control. Regarding to the germination percentage, application of PVS1 (25%) had similar value with control, however greater concentration of PVS1 or PVS2 tend to reduce the germination percentage of *H. odorata*. This results indicate that PVS1 of 25% was not toxic for *H. odorata* seeds and has a prospect to be used as a cryoprotectant. Malondialdehyde is a reactive compound in cell membranes resulting from the denaturation of polyunsaturated fatty acids (PUFA) due to biotic and abiotic stresses from the extracellular environment. An excess of MDA accumulation can damage the property of cell membrane, such as its selective permeability function (Olvera-Carrillo et al. 2011).

The effect of cryoprotectant on total phenol (FE) is presented in Figure 3. *H. odorata* seeds that were immersed into PVS1 of 25% or 50 % for 30 min, 60 min or 90 min still has relatively similar total phenol content compared to control. Whereas greater PVS1 concentration or PVS2 caused an increased in total phenol content within the seeds. According to Ma et al. (2016), excessive phenol accumulation in plant tissues may indicate oxidative stress that could be due to environmental stress. Phenol as a non-enzymatic

antioxidant group will suppress lipid peroxidation products such as malondialdehyde, proline or group of *reactive oxygen species* (ROS). In this experiment, a reduction of total MDA that concomitant with an increase in total phenol was found in *H.odorata* seeds treated with PVS2 of 75% or 100% and immersion time of 90 minutes or 120 minutes. However, germination percentage, germination index, vigor index and maximum growth potential of *H,odorata* seeds were not supported by those treatments.

## Cryoprotectant and Cryopreservation Treatment of *H. odorata* Seed (-196±2ºC)

Variations of cryoprotectants, concentrations, immersion times and their interactions did not significantly affect the moisture content (MC) of cryopreserved seeds, except for the interaction between types of cryoprotectans and immersion times (T-I). Cryoprotectant immersion times only affected the value of total MDA and total phenol (Table 2). Results of analysis of variance did not show any significant effect of the immersion time on germination variables (GP, GR, VI, MGP) of *H.odorata* seeds following cryopreservation. All seeds were become necrosis and could not germinate. Therefore, the results of Duncan's test of germination variables after cryopreservation were not presented.

**Table 2.** Analysis of variance for effect of cryoprotectans types, consentration, and immersion time on the observations variables and its interactions following cryopreservation.

| Source of variation | P-value | | | |
|---|---|---|---|---|
| | MC | EL | MDA | PH |
| Type (T) | .476$^{tn}$ | .720$^{tn}$ | .000$^{*}$ | .000$^{*}$ |
| Concentration (C) | .112$^{*}$ | .571$^{tn}$ | .000$^{*}$ | .000$^{*}$ |
| Immersion time (I) | .845$^{tn}$ | .232$^{tn}$ | .000$^{*}$ | .000$^{*}$ |
| T*C | .158$^{tn}$ | .640$^{tn}$ | .000$^{*}$ | .000$^{*}$ |
| T*IT | .608$^{tn}$ | .320$^{n}$ | .000$^{*}$ | .000$^{*}$ |
| C*IT | .943$^{tn}$ | .380$^{tn}$ | .010$^{*}$ | .001$^{*}$ |
| T*C*IT | 1.00$^{tn}$ | .616$^{tn}$ | .009$^{*}$ | .000$^{*}$ |

Note : * = treatment gave significant effect on observed variables at α=5%. $^{ns}$ = treatment gave non-significant effect on observed variablesat α=5%. Type (PVS1 and PVS2). Concentration (25%, 50%, 75%, 100%). Immersion time (30, 60, 90 and 120 min). MC: Mouisture Content, EL: Electrolyte Leakage, MDA: Total Malondialdehyde, PH: Total Phenol. (Rohmah 2021)

Figure 4 showed that variations in type of cryoprotectans, concentrations and immersion times mostly did not caused any significant difference on MC of *H. odorata* seeds following cryopreservation compared to control. A slight increase of MC was found in *H.odorata* seeds treated with 25% of PVS1 or 25% PVS2 with immersion times of 30 mins or 60 mins

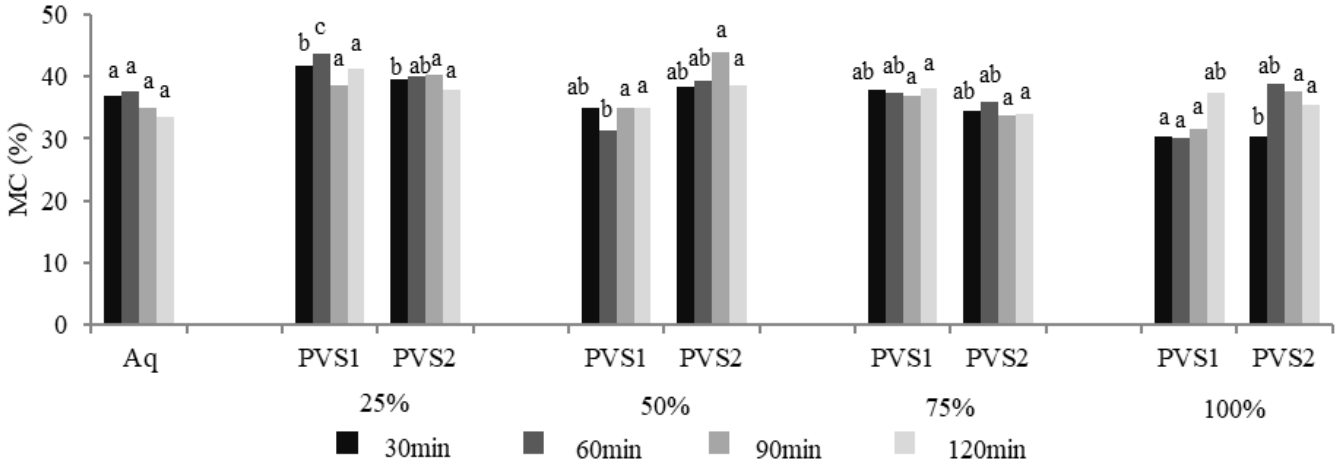


**Figure 4.**Interaction of cryoprotectant types, concentrations and immersion times on percentage of moisture content (MC) of *H.odorata* seed following croyopreservation. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

(41.58% and 39.66% respectively). According to Yan et al. (2014) an increase of MC following cryopreservation might be due to immersion treatment that was carried out previously, in which the solvent of cryoprotectant moves into the seed tissue.

All variation of cryoprotectants and immersion times of *H. odorata* seeds that followed by cryopreservation did not caused any significant difference effect on the electrolyte leakage (EL) compared to the control (Figure 5). This results indicate that both PVS1 or PVS2 did work effectively as a seed protector of cell membrane damage that may occurred following low temperature treatment . The increase of electrolyte leakage after cryopreservation is an indicator that normally used to determine condition of cell membranes. Ntuli et al. (2011) reported that the amount electrolyte leakage will increase if the cell membrane is mechanically damaged due to ice crystals or suffer from physiological damage due to accumulation of oxidative compounds in response to chilling injury. The research report of Pukacki and Juszczyk (2015) stated that cryopreservation *Acer pseudoplantanus* as recalcitrant seeds had electrolyte leakage accumulation more than 50% and indicated a breakdown of cell membrane integrity.

Results presented in Figure 5 also showed that variation of immersion times and cryopreservation tend to increase the total malondialdehyde (MDA) of *H.odorata* seeds. Total MDA in *H. odorata* seeds treated with PVS2 and followed with liquid nitrogen treatment had a higher value (5.767-12.905 nmol $g^{-1}$ FW) than those seeds subjected to liquid nitrogen but treated with PVS1 (4.927-8.898 nmol $g^{-1}$FW) regardless of its concentration and time of immersion. The total MDA in the H. odorata seeds treated with PVS2 and continued with cryopreservation was significantly higher than control (immersion into distilled water: 4,372-5,792 nmol $g^{-1}$ FW). This result indicates that PVS2 which was initially used to minimize damage of cryopreservation actually increases the risk of deterioration and seed viability of *H. odorata.*

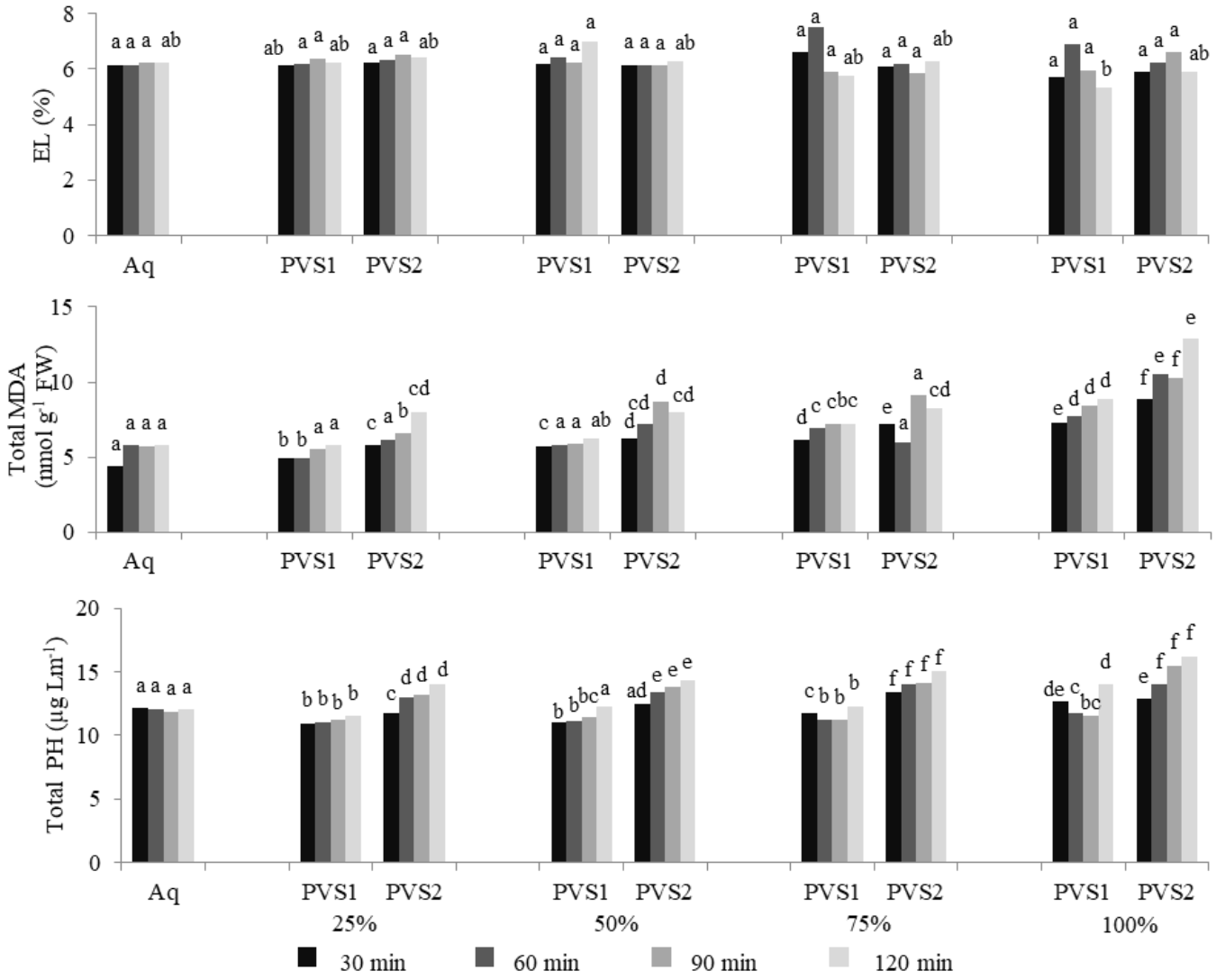


**Figure 5.** Interaction of cryoprotectant types, concentrations and immersion times on percentage of electrolyte leakage (EL), total malondialdehyde (MDA), and total phenol (PH) after cryopreservation. Note: Aq= Aquadest. The similar letter in the graphic indicates that the treatment gives a nonsignificant effect at DMRT test (α=5%). (Rohmah 2021)

Vendrame et al. (2014) stated that PVS2 at high concentrations promoted osmotic stress and cell death. O'Brien et al. (2021) reported that PVS2 at high concentrations (100%) with an immersion time of 30-40 mins caused damage to meristem cells and decreased generative capacity of avocado (*Persea americana*) shoot tip cells. Wang et al. (2014) suggested that DMSO in PVS2 could be replaced with glycerol to prevent the osmotic shock.

Total phenol of *H. odorata* seeds following cryopreservation increased in both PVS1 and PVS2 treatments regardless of the duration of immersion in those cryoprotectants. According to Galluzzi et al. (2013) an increase in the amount of total phenol as a group of non-enzymatic antioxidant compounds is a normal response of seed tissue that face oxidative stress due to the extreme temperature of liquid nitrogen. Phenol is regulated to suppress the accumulation of oxidative compounds such as $O2^{\bullet-}$ and $H_2O_2$ or products of lipid peroxidation processes such as malondialdehyde, proline and acrolein that cause damage to plant tissues (Narayanan et al. 2015).

## CONCLUSION

From the results and discussion it can be inferred that immersion of *H. odorata* seed into Plant Vitrivication Solution Number 1 (PVS1) at room temperature resulted in higher percentage of physiological variables and lower values of biochemical variables compared to those immersed into Plant Vitrivication Solution Number 2 (PVS2) at higher concentration (50% - 100%). PVS1 and PVS2 of 25% did not caused significant difference in physiological variables. Meanwhile, combination of cryoprotectant and cryopreservation treatments were not able to increase the viability of *H. odorata* seeds. Cryopreservation treatment increased the amount of total malondialdehyde and total phenol content in *H. odorata* seeds. Based on this result , it seems that PVS1 is kind of cryoprotectant that had less toxic effect on *H. odorata* seeds but still can not prevent the negative impact of cryopreservation treatment. Further research still important to be conducted to obtain appropriate cryopreservation storage techniques for H. odorata seeds, such as encapsulation in alginate capsules to form artificial seeds, which can be combined with dehydration using PVS1.

## AUTHORS CONTRIBUTION

L.A.R. collected plant samples, carried out experiments, analyzed the data, and wrote the manuscript. F.F.W. and A.H.W. help in data collection and analysis. D.L. and K.D. designed the research, supervised all the processes laboratory analysis, and edited the manuscript.

## ACKNOWLEDGMENTS

The authors would like to thank Universitas Gadjah Mada Yogyakarta that has funded this project through RTA (Recognisi Tugas Akhir) Research Grant year 2020 (Letter task No : 2488/UN1.P.III/DIT-LIT/PT/2020, date 12th May 2020), and to the Center for Plant Conservation Research, Indonesian Institute of Sciences (LIPI), Bogor for giving permission to the authors to use materials and experimental equipments in Seed Bank Laboratory.

## CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

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Journal of Tropical Biodiversity and Biotechnology
Volume 07, Issue 01 (2022): jtbb65227
DOI: 10.22146/jtbb.65227

Review Article

# The Effect of Accumulation of Leaf Litters and Allelochemicals in the Soil to the Sustainability of the Newly Introduced Crop Plants

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**Keywords:**
Cacao
Leaf litters
Soil organic carbon
Sustainability
Vanilla
**Submitted:**
12 April 2021
**Accepted:**
26 November 2021
**Published:**
04 March 2022
**Editor:**
Ardaning Nuriliani

## ABSTRACT
Indonesia is the second-largest vanilla production and the third-largest cocoa production in the world, but it sustained for a short period. The unsustainability of these crops is speculated to occur because of the change in leaf litter accumulation which affected the sustainability of soil organic carbon that plays an important role in maintaining soil health and fertility. To find out methods that could improve the sustainability of the production, a literature review was conducted. The articles, related to the sustainability of vanilla and cacao production, were collected using Google Scholar, Wiley Online Library, ResearchGate, and Google Chrome browser. Keywords were employed to find the articles including soil organic carbon, cocoa plantation, vanilla, leaf litter, and allelochemical. This current article review found that introducing crop by clearing of previously existing vegetation could severely reduce the rate of leaf litter accumulation. Consequently, in a prolonged period, the soil organic carbon and soil fertility are very low and are unable to support the healthy growth and production of the crops. To restore production, the plantation then is returned to more traditional agroforestry such as replanting shading trees and addition of mulch. However, in the higher density of canopy, the crop production is low attributed partly to the decreasing soil pH which increases the impact of allelochemical. This review concluded that the sustainability of leaf litter accumulation is crucial to maintain soil health, but mitigation is required to reduce the impact of allelochemical accumulation.



## INTRODUCTION
Indonesia is the second-largest vanilla production in the world after Madagascar (Arya & Lenka 2019), but this achievement is not sustainable as the case found in Bali (Figure 1). The production was slowly increased into the top production and then decreased very sharply. The ensuing increase in production was not found for more than 5 years after the top production (BPS Bali. a). Indonesia is also an important cacao producer, the 3rd largest in the world, but the production is also not sustainable (Schaad & Fromm 2018). In Bali, the production of cacao was showing a slightly similar trend to that of vanilla production, i.e. slow increased into top production and followed by

a sharp decrease (BPS Bali. b). The period of decreasing production occurred for ca. 4-5 years (Figure 1). Since these two crops, particularly cacao, is the source of income for millions of smallholder farmer and family in Indonesia, a majority in Sulawesi Island (Witjaksono & Asmin 2016), the sustainability of these crop production is regarded as very important to be maintained. Various factors may involve in the fluctuation of production, but this review focusing discussion on the accumulation of leaf litter and allelochemical in the plantations to find out sustainable methods. Both, the accumulation of leaf litter and allelochemical are related since the accumulation of leaf litter decreases soil pH and increases the impact of allelochemical.

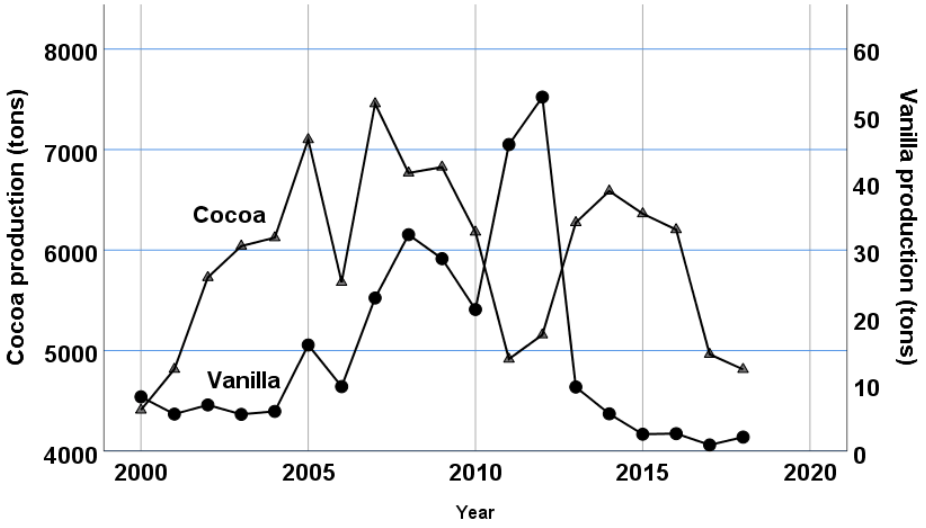


**Figure 1.** Production of vanilla and cacao in Bali from 2000-2018. Data were collected from BPS Bali (https://bali.bps.go.id/dynamictable).

This study is regarded as very important, firstly, it is because clearing up trees before the introduction of new crops will cut off organic carbon supply from the leaf litter of the trees which subsequently could deteriorate soil organic carbon. This organic carbon is responsible for soil health and fertility. Secondly, clearing up trees affects the hydrological mechanism and leads to drought during the dry season and flooding during rainy seasons. After the plantation then unproductive, the abandoned landscape is unable to support people economically and environmentally.

## MATERIALS AND METHODS

This review was written after reading and understanding literature collected using search engines, such as Wiley Online Library, Google Scholar, Research Gate, and Google Chrome browser. Free access relevant articles from a journal found in those search engines were then opened and downloaded. There were more than 100 articles found related to leaf litter, soil organic carbon, cacao production system, allelochemical, and nutrient release from decomposing plant materials. About 30 papers were selected for this review based on the publishing date and its relevance to the written topic. Data col-

lected from these papers include leaf litter accumulation and soil organic carbon level, the decomposition rate of leaf litter, and the type of allelochemical produced by plants. Each data variable was then analyzed descriptively and presented in tables.

## RESULTS AND DISCUSSION

### Leaf litter accumulation and soil organic carbon level

The trees vegetation in agroforestry could produce a substantial amount of leaf litter (Table 1) which becomes an important source of soil organic carbon and nutrients (Ampitan et al. 2021; Ledo et al. 2020; Sauvadet et al. 2020; McGrath et al. 2000; Mehta et al. 2013; Mutshekwa et al. 2020). Whilst soil organic carbon improves soil porosity and water percolation, the increased amount of available nutrient in soil improve growth and production of the crop (Shaxson & Barber 2003; Sanz et al. 2017).

**Table 1.** The accumulation of leaf litter in various types of vegetations. By using litter traps, accumulation of leaf litter was observed for 1-2 years and using carbon dating, the accumulation of carbon was observed for 256 years.

| No | Type of vegetation | Tree species | Leaf litters (kg/ha) | References |
|---|---|---|---|---|
| 1 | Dry forest | *Cordia alba* | 1134 | (Castellanos-Barliza et al. 2018) |
| 2 | Boreal forest | | 7250 | (Kyaschenko et al. 2019) |
| 3 | Agri-horti-silvi culture | Okra, Manggo, Teak | 831.25 | (Singh et al. 2019) |
| 4 | Cacao plantation | Cacao | 3130 | (Muoghalu & Odiwe 2011) |
| 5 | Agroforest tree | *Celtis australis*, *Grewia optiva*, *Bauhinia variegata* & *Ficus roxburghii* | 2190 | (Singhal et al. 2019) |

Relative to the boreal forest, the accumulation of leaf litters in the plantation is much lower (Table 1). In cacao plantations (Muoghalu & Odiwe 2011), the production of leaf litter was about 43 % of that found in the boreal forest (Kyaschenko et al. 2019). In agri-silviculture, the accumulation of leaf litter reported was 831.25 kg/ha (Singh et al. 2019), but in the boreal forest, the accumulation was 7250 kg/ha (Kyaschenko et al. 2019). The data indicated that decreasing plant diversity in the plantation relative to the diversity of plants in native forest resulted in a decrease amount of leaf litter accumulation. This decrease subsequently then reduces soil organic carbon which is very important for the soil health and nutrient available in the soil.

The contribution of leaf litter to the accumulation of soil organic carbon has widely been studied. Novara et al. (2015) reported that the addition of leaf litter to soil increased soil organic carbon by up to 13% relatively to the soil without the addition of leaf litter. This increase in soil organic carbon is mainly attributed to worm activity. More recent reports (Liebmann et al.

2020) show that the contribution of litter to mineral related organic carbon in topsoil was 1.88 g C $m^{-2}$ for 22 months which is equal to ca 18.800 kg C $Ha^{-1}$. Therefore, soil organic carbon in the plantation is lower than in the forest after the conversion of forest into cropland (Aryal et al. 2018; Kassa et al. 2017; Machado et al. 2017) have taken into account to sustain the newly introduced crop production.

Under the various conditions of the agricultural system employed and the type of crop introduced, the landscape of the crops might continuously deteriorate which eventually makes the soil unable to provide a healthy growth condition. This deterioration can be estimated by assuming that crop production is linearly correlated with the soil conditions. In cacao plantations, the deterioration is commenced ca 6 years after the initial productions which are indicated by a sharp decrease in productions (Figure 1). This sharp decrease occurred just after the crop showing the top productions. In the vanilla plantation, the deterioration is commenced ca 4 years later than that in cacao plantation. This is very likely attributed to the higher commercial yield produced by cacao, i.e. more than 7000 tons rather than vanilla, i.e. ca 55 tons during the period of the first top production. The removal of the yield from the plantation, particularly during the top production, possibly causes a substantial decrease of nutrients available in the soil. Although this nutrient loss could be replenished by increasing input, such as fertilizer, the decrease of soil organic carbon content is unable to be replenished instantly.

In the ensuing period, production of cacao can still be restored but the top production is about 870 tons lower than the first top productions. Unlike the cacao, in vanilla plantations, restoring production did not occur for more than 5 years. Indicating that the deterioration of soil growth medium in the crop landscape is relatively hard to heal possibly because the agricultural system employed does not support the sustainability of soil organic carbon which is regarded as central to soil health (Ramesh et al. 2019). Thus, the addition of mulch to a crop plantation draws more attention to the sustainable production of the crop (Shaxson & Barber 2003). For example, to heal the landscape in cacao production, Acheampong et al. (2019) have experimented by mulching cocoa plants using coffee husk. These authors concluded that simple mulching techniques could significantly improve the cropping of cacao. The other experiment was reported by Riedel et al. (2019) using agroforestry and rehabilitation pruning. These authors highlight the potential of agroforestry to reconcile ecologically sustainable land. Since both mulching and agroforestry improve soil organic carbon, those experiments suggesting that the sustainability of cacao production can be improved by maintaining soil organic carbon. For sugarcane crops, it was estimated that sustaining soil organic carbon after the removal of crop yield requires the addition of ca 3 ton/ha organic materials (Gmach et al. 2021). This kind of technique may become an important method for improving the sustainability of cacao or vanilla plantations.

## Soil fertility

Litterfall and litter decomposition are key elements of nutrient cycling in tropical forests (Cole et al. 2020; Froufe et al. 2020; Castellanos-Barliza et al. 2018; Mehta et al. 2013). Before providing crop plants with nutrients, the leaf litter produced by trees in plantations undergo a complex interaction with soil involving biotic and abiotic factors (Keller & Phillips 2019). This interaction which then leads to leaf litter decomposition is also affected by the type of agricultural system employed (Sauvadet et al. 2020). In agroforest cocoa plantation, the time required for 99% leaf litter decomposition was 2.6 years, much faster than in conventional cocoa plantation, i.e. 3.5 years (Asigbaase et al. 2021). This is indicating that the supply rate of organic carbon into the soil of agroforest plantations was faster than the supply in the conventional plantations. This enhancement, according to the authors, is attributed to the improvement of soil conditions. It has been acknowledged that under harsh environmental conditions such as low temperatures, waterlogging, anoxic, acidic sites, and drought, the decomposition rate of leaf litter is low (Aerts et al. 2012; Xie et al. 2020). The faster accumulation and subsequent decomposition of leaf litter in agroforestry, the likely make higher soil perforation enables better drainage and lengthens soil moisture. This mechanism is in agreement with reports which showed that forest soil has the highest soil perforation and moisture followed by agroforestry and pasture (Suárez et al. 2021). This is also indicating that higher plants diversity in plantations resulted in more healthy soils. Among the type of trees, the decomposition rate is varied (Table 2).

**Table 2.** The decomposition rate of leaf litter in various plant vegetations.

| No | Plant type | Decomposition rate (k) | References |
|---|---|---|---|
| 1 | *Theobroma cacao* | 1.03 | (Muoghalu & Odiwe 2011) |
| 2 | *Grewia optiva* | 2.12 | (Singhal et al. 2019) |
| 3 | *Celtis australis* | 2.30 | |
| 4 | *Bauhinia variegate* | 1.64 | |
| 5 | *Ficus roxburghii* | 1.05 | |
| 6 | Angiospermae (include Magnoliid) | 2.52 | (Liu et al. 2014) |
| 7 | Eudicot | 6.18 | |

Since the supply rate of nutrients available in the soil is the function of the rate of the leaf litter decomposition, the sustainability of crop production in a plantation then depended upon the type of shading tree in the crop plantation. The crop plant or shading tree that produces leaves with a high decomposition rate will provide nutrients faster than the plants that produce leaf with a lower decomposition rate. For example, the decomposition rate of cacao leaf was 1.03 (Muoghalu & Odiwe 2011) and the decomposition rate of Angiospermae was 2.52 (Liu et al. 2014). Assuming that cacao plantation is

developed using a monoculture system and Angiospermae trees are also grown in a monoculture, the time taken by the cacao plants to acquire recycled nutrients was twice longer than the Angiospermae plantation. Consequently, if the cacao plantation is established by clearing off previous vegetation, the supply of soil organic carbon into the soil will undergo a lag period for a relatively long period which then deteriorates the previously accumulated soil organic carbon. Resuming process by the cacao plants is very low and after reaching a certain low soil organic carbon level, the soil could not provide a condition for healthy production. This includes soil moisture and nutrient available in the soil. The production then drops sharply and the plantation is then abandoned because of its economically inefficiency. It is speculated that by establishing cacao plantation in an agroforest system that consists of crop plants and forest trees, the supply of leaf litter can be maintained which subsequently provided soil with richer amount of organic carbon and nutrients.

However, although the agroforestry system provided a healthier growth medium for the crop plants, the type of nutrient release and environmental condition generated by the shading trees might not complement the requirement or growth condition of the crop. This is particularly because the nutrient requirement of a particular crop is specific to species. Various studies have been reported which showed various organic carbon, fertility, and nutrients content in different vegetation diversity. Suárez et al. (2021) evaluated soil quality in various agroforestries in the Colombian Amazon. They found that the general indicator of soil quality is decreasing from forest, agroforestry, and pasture. Importantly, these authors found that the establishment of agroforest cacao plantations improves soil fertility by 42% relatively to degraded pasture. Matos et al. (2020) reported that litter quality supported the restoration of soil and Saputra et al. (2020) reported that complex agroforest increases soil organic carbon. These studies collectively showed that the more diverse the plant population, the higher soil organic carbon level and soil fertility.

In the native forest, N content in soil was higher than in the plantation (Machado et al. 2017), but in pasture monoculture, potassium was released at a higher level (Piza et al. 2021). The type of nutrient in leaf litter produced by non-agroforest and by different agroforest was studied by Rangel-Mendoza and Silva-Parra (2020). This study compares the concentration of N, P, Ca, Mg, K and it is found that leaf litter collected from mixed Cacao-*A. peregrina* L agroforestry contains a higher concentration of N, P, Mg and K. This is indicating that different types of vegetation provided different supplemental nutrients for the growth of crop plants and it was improved after the diversity of vegetation is increased. However, a very basic question remains unanswered whether or not the improvement of the soil health linearly correlated with the crop yield as previously been proposed.

## The accumulation of allelochemicals in plantation

The agroforestry system is not a new system for vanilla plants because of their growth characteristics. The vanilla plants are naturally grown in mixed culture with trees as tutor or shading plants. Therefore, related to a monoculture cacao plantation, the accumulation of soil organic carbon and nutrient in the vanilla plantation can be considered as healthier. However, the yield of the vanilla plants is also not sustainable, as the case found in Bali (Figure 1). So, it is speculated that other factors associated with leaf litter, i.e. allelochemical, could also become an important constraint for the growth and production of crops. The increasing amount of leaf litter accumulation in the soil makes soil not only rich in organic carbon and nutrient but also allelochemical. This is particularly because the compound is also present in the leaf (Iqbal et al. 2019). The higher amount of leaf litter accumulation, the more likely allelochemical accumulated in the soil and the impact is more severe.

Plants have long been known to produce various organic compounds to encounter adverse environmental conditions, such as drought, light, disease, and predators. The chemical compounds are known as secondary metabolites comprised of phenolic compounds, alkaloid, and terpenoid (Macías et al. 2019). A group of organic compounds that are released into the environment for inhibiting the growth of other plants is known as allelochemical. This compound can be released via volatilization from leaves, exudation from the root, and leachate or release from plants residue (Zhang et al. 2021). According to these authors, allelochemical released from plant residue has the most negative effect on the performance of recipient plants since the allelochemical consist of whether water or non-water-soluble compounds.

In plants, this toxic allelochemical may be stored in a membrane-bound vesicle or released via the root system into the apoplast by exocytosis (Bonanomi et al. 2006; Weston et al. 2012). The other allelochemical is produced and stored in leaves and then it released into the environment during leaf litter degradation. Toxicity of the allelochemical is rapidly decreased under aerobic condition but it is increasing and becoming stable under anaerobic condition (Bonanomi et al. 2006). Suggesting that crop plants developed in an anaerobic condition, such as water logging during prolonged rain seasons, will encounter an increasing number of toxic compounds when the accumulation of leaf litter increased. Therefore, since the leaf litter accumulated on the soil surface could be originated from the crop itself, both agroforestry and monoculture systems will be facing the allelochemicals problems. This is particularly because toxic compounds previously stored in a membrane-bound vesical in plants are then released freely into environments during decompositions. This toxic compound could inhibit the root system of whether the crop or the shading trees. Allelochemical released by the crop and inhibited the growth of the crop is known as autotoxicity. This mechanism could substantially reduce crop yield (Singh et al. 1999). The other toxic compound could also inhibit the growth of other plants. Zhang et al. (2015) reported that leaf litter from different plants species affected differently to the growth

of seeds. For example, extract from *Populus canadensis* inhibited the growth of rape seed, but the extract from *Prunus persica* was beneficial to the germination of the rape. Indicating that a plant species may be sensitive or tolerant to an allelochemical produced by other species.

Deheuvels et al. (2012) had previously reported that the production of cacao was lower in plantations using a high density of canopy rather than in high-density cocoa plants. These results deviate from the previous discussion which showed that agroforestry increases soil organic carbon and nutrient which should support a higher yield. The high organic carbon in the soil also increased soil porosity which makes the soil healthier, i.e. it is more aerobic and lengthens soil moisture. Thus, the negative correlation between soil health and crop yield, under the high density of canopy (Deheuvels et al. 2012), is very unlikely attributed to the increase of soil organic carbon and nutrient nor by soil porosity. One of the various possible causes is the presence of allelochemical release from leaf litter of the crop and the canopy. According to Bonanomi et al. (2006), the effect of allelochemical is increased in anaerobic soil conditions and in lower soil pH levels. So, the lower cacao production in the higher density of canopy, most likely caused by lowering soil pH level which increases the toxicity of allelochemical. This possibility is in agreement with the report by Fang et al. (2017) which showed that soil pH was lower under the condition of higher soil organic carbon and a report by Cornelissen et al. (2018) which showed that crop yield positively correlated with soil pH.

In other studies, Bhowmik & Doll (1982) showed that the addition of corn residue reduced corn production and the addition of soybean residue reduced production of soybean. It is suggested that allelochemical originated from the crop could also inhibit the root physiology of the crops themselves. This also indicates that allelochemical previously stored in a safe vesical in plants then can be dangerous for the plants after it is released into the environment during leaf litter decompositions. This possibility then can be inferred for cacao productions, since cacao plantations accumulated ca 3130 kg/ha/year leaf litters (Muoghalu & Odiwe 2011). Under the condition of anaerobic and low soil pH, the impact of allelochemical released by cacao leaf could be substantial to inhibit production. Therefore, to sustain productivity, whether, in agroforest or monoculture, soil porosity and good drainage are required to enhance aeration and to improve soil pH level. In acidic soil, the application of biochar or charcoal has been found to improve soil pH and plant growth (Cornelissen et al. 2018).

The impact of the toxic compound is reported more severe in a shallow-rooted crop than in deep-rooted crops (Chalker-Scott 2007) which highlighted the difference between cocoa and vanilla. Unlike cacao plants which have relatively deeper-rooted systems, vanilla plants that belong to the orchid family are shallow-rooted plants and rarely shed their leaves. So, the contribution of vanilla leaf litter to the landscape is much lower than that of cacao leaves litter. Accordingly, less amount of allelochemical was originated from leaf

litter of the vanilla plants. However, because vanilla is a shallowed-rooted plant, they are very sensitive to drought and mulching is regarded as crucial to inhibit water loss from the soil surface. Allelochemicals that could inhibit the growth of vanilla are most likely originated from shading trees and mulch materials. Vanilla plants are mulched with various types of plants materials, such as coconut husk, leaf litter of various trees, etc. Under the condition of anaerobic and lower pH, allelochemical from the shading trees and mulch materials could also affect the growth of vanilla. Metabolites produced by the shading trees and mulch material that potentially inhibit the growth of vanilla are shown in Table 3.

The effect of mulching on the vegetative growth of vanilla plants has been reported (Adiputra et al. 2019). This study showed that the growth of vanilla was reduced by the addition of taro clippings but increased by the addition of dry leaf and coconut husk mulch. These authors concluded that vanilla plants are sensitive to allelochemical released by taro clipping but tolerant to allelochemical released by dry leaf and coconut husk.

## CONCLUSION

Maintaining soil organic carbon level previously existed in the plantation is required to improve the sustainability of the newly introduced crop. This can be achieved by retaining the tree population and addition of mulch. However, since the increase of organic carbon could decrease soil pH which increased the negative effect of allelochemical, the addition of biochar or charcoal is regarded as crucial to increase soil pH.

## AUTHORS CONTRIBUTION

I G.K. Adiputra. read and understanding relevant articles which are searched and downloaded using search engines before written into a manuscript.

**Table 3.** Type of allelochemical released by plants species

| No | Plant Family | Species | Compounds | Reference |
|---|---|---|---|---|
| 1 | Arecaceae | *Cocos nucifera* | Phenolic compounds | (Gonçalves et al. 2019) |
| 2 | Dennstaedtiaceae | *Pteridium Gled. Ex Scop* | Selligueain A | (De Jesus Jatoba et al. 2016) |
| 2 | Rubiaceae | *Coffee arabica* | Caffeine | (Asfew & Dekebo 2019) |
| 3 | Fabaceae | *Gliricidia sepium* | Coumarine | (Takemura et al. 2013) |
| 4 | Moraceae | *Artocarpus heterophyllus* | Aqueous methanol extract | (Noguchi & Takami 2015) |
| 5 | Araceae | *Colocasia esculenta* | Benzoic acid | (Asao et al. 2003) |
| 6 | Moraceae | *Artocarpus heterophyllus* | squalene | (Biswas & Chakraborty 2013) |
| 7 | Sterculiaceae | *Theobroma cacao* | Theobromine, Polyphenol | (Hii et al. 2009) |
| 8 | Fabaceae | *Leucaena leucocephala* | Mimosine | (Sahid et al. 2017) |

## ACKNOWLEDGMENTS

I would like to thank my colleagues and all member of the staff of the Biology Department, Faculty of Information Technology and Sciences, University of Hindu Indonesia, who in various ways supported and encouraged my work.

## CONFLICT OF INTEREST

There is no competing interest regarding the manuscript.

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