ISSN 0215-6334 (printed)
ISSN 1907-770X (electronic)

BIOTROPIA

The Southeast Asian Journal of Tropical Biology

Vol. 30 No. 3 December 2023

Published by
SEAMEO BIOTROP
Southeast Asian Regional Centre for Tropical Biology
Bogor, Indonesia
www.biotrop.org

BIOTROPIA
Volume 30, Number 3, December 2023
ISSN 0215-6334 (printed)
ISSN 1907-770X (electronic)

EDITORIAL BOARD

BIOTROPIA, The Southeast Asian Journal Tropical Biology, is a scientific publication of SEAMEO BIOTROP published three times a year. It contains scientific research articles in any subjects of the areas of tropical biology. Contribution in the form of a review article and short communication is also considered for publication. A guide for author is found on the inside back cover.

BIOTROPIA Editorial Office
SEAMEO BIOTROP, Jalan Raya Tajur Km. 6, Bogor 16134, Indonesia
E-mail: biotropia@biotrop.org; home page: http://journal.biotrop.org/index.php/biotropia

Cover Photo: *Green bean of arabica coffee from Sembalun Area, Lombok*. The photo was taken by Zainuri.

POST-SIEGE GENOTOXIC HAZARDS IN LAKE LANAO, PHILIPPINES BY MICRONUCLEUS ASSAY

FEMA ABAMO[1,2*], ANNABELLA VILLARINO[2] AND ED LUCILLE MAE FERRANCO[2]

[1] *Mamitua Saber Research Center, Mindanao State University (MSU), Marawi City, 9700 Philippines*
[2] *Biology Dept, College of Natural Sciences and Mathematics, MSU, Marawi City, 9700 Philippines*

Received 3 September 2022 / Revised 27 July 2023 / Accepted 7 August 2023

ABSTRACT

Massive war pollutants brought by Marawi Siege in the Philippines have contaminated the city environs and the surrounding ancient Lake Lanao. Munition residues including heavy metals are reportedly genotoxic hence this study was conducted to determine the post-siege genotoxic hazards posed by the munition pollutants through micronucleus (MN) assay on the slow-moving edible gastropod snails *Vivipara angularis* Muller (locally known as "suso") thriving in the lake. MN, a biomarker of genotoxicity was examined and counted in the hemocytes of exposed juvenile and adult snails. Results revealed that MN frequencies (no. of MN/1000 hemocytes) were significantly higher in snails sampled in Lake Lanao lakeshores than in the reference site Lake Dapao. Among adult samples, there was a decreasing trend of MN frequencies with increasing distance from lakeshore fronting warzone 'ground zero' where the battle was heaviest (site A) to sampling sites away from it: sites B, C, and D (Lake Dapao) which are 8.15 km, 24.41 km, and 34.45 km, respectively. Moreover, varied patterns of micronucleation were observed between age groups and sites, i.e., in all sites except C, adults had greater MN counts than juveniles which were significant in site A only. It is a generally recognized observation that MN frequency increases with age; unexpectedly juveniles displayed significantly higher MN counts than adults in site C. The elevated MN frequencies in the snail hemocytes exposed to war pollution could be attributed to genotoxic munition residues eroded and washed into the lake water. Moreover, heavy metals which are common components of weaponries were also detected in the snail muscles, although at concentrations within safe levels but continued consumption may be cautioned to avoid biomagnification. Other genotoxins must be present in site C other than munition residues predisposing the juvenile snails. The results are baseline data on the MN frequencies in *V. angularis* exposed to war pollutants in Lake Lanao which need further investigation.

Keywords: genotoxicity, lake lanao, marawi siege, micronucleus assay

INTRODUCTION

The devastating Marawi siege, an armed conflict that lasted for five months in 2017 was the longest urban warfare in the Philippines, polluting the air, soil, and water environment and destroying biodiversity. Adding to the insurmountable damage are the numerous ill-health effects that could be suffered by the inhabitants, especially humans. High-tech ammunitions utilized both by the terrorist and the government troops included bombs, mortars, grenades such as rocket-propelled grenades (RPG), hand-held, anti-tank grenade launchers, and the likes aside from the conventional rifles, improvised explosive devices (IEDs), and carpet, and aerial bombs (Kapoor, 2017, Pareño, 2017, & Miller, 2017). These are depots of toxic pollutants such as high-melting explosives (HMX), hexahydro-1,3,5-trinitro-1,3,5- triazine (RDX), trinitrotoluene (TNT), and heavy metals with potential genotoxic threats such as lead (Pb), arsenic (As), nickel (Ni), copper (Cu) and zinc (Zn), strontium (Sr), magnesium (Mg), and barium (Ba), mercury (Hg), cadmium (Cd) antimony (Sb), iron (Fe) and chromium (Cr) (IARC 2021, Broomandi *et al.* 2020, Skalny *et al.* 2021, Chatterjee *et al.* 2017). Known components of weaponries listed in IARC (2021) are established mutagens and carcinogens causing cancers affecting the respiratory tract, liver, bone, blood, and other parts of the human body. The magnitude of weaponries used during the siege

*Corresponding author, email: fema.abamo@msumain.edu.ph

heavily contaminated the city environs and its surrounding lake with genotoxic pollutants posing health hazards to the local inhabitants as well as to the organisms thriving in the second largest lake in the country and one of the ancient lakes in the world, that is, Lake Lanao, in the province of Lanao del Sur (LDS).

Genotoxicity is the ability of chemicals to damage the genetic information of the cells thereby resulting in mutations and eventual carcinogenesis (Seukep *et al*., 2014). One reliable method to assess the genotoxic potential of some substances (such as heavy metals, pesticides including gamma radiation), is the micronucleus (MN) assay and it is used in various test organisms including man, mice, land and aquatic snails, mussels, and fishes (Silva *et al.* 2011, da Silva 2013, Bolognesi & Fenech 2012, Gentile *et al.* 2012, Luzhna & Kovalchuk, 2013, Beedanagari *et al.* 2014, da Rocha *et al.* 2016, Hayashi 2016, El Safty *et al.* 2018, Nikdehghan *et al.* 2018, and de Vasconcelos Lima 2019, Fenech *et al.* 2020). The present study made use of the freshwater gastropods *Vivipara angularis* Muller (locally known as "suso") in the MN assays instead of *B. glabrata* because they are abundant in Lake Lanao and utilized as food by the Meranaw (people of the lake). The strategic use of the snails in MN assays is anchored on their sedentary lifestyle crawling on soil and rock substratum. They use their gills as filter feeding apparatus and respiratory organs and are the first barrier to potential contaminants and the ideal target for genotoxic studies. They are tolerant to heavy metals and bio-accumulate them (Baroudi *et al*., 2020). Importantly, snails are less mobile compared to other organisms like fishes, thus they cannot easily escape from toxic substances present in their habitat. This study therefore primarily aimed to estimate the post-siege genotoxic hazards posed by munition residues in the lake through micronucleus assay using the *V. angularis* Muller thriving in the lake. It also examined the micronucleation pattern among exposed snails sampled in Lake Lanao and non-exposed snails in the reference site, Lake Dapao, LDS. Factors like the distance of the sampling sites from the war zone and the age groups of the sampled snails were considered in the MN assays.

MATERIALS AND METHODS

Study Design and Sampling Sites

Descriptive cross-sectional study design and the purposive sampling collection (accessibility of the sites and age groups of snails) were conducted in assessing the genotoxicity of the war residues by MN assay in the hemocytes of the exposed freshwater gastropods *V. angularis* of Lake Lanao. Figure 1 shows the map of the Philippines and the sampling sites, location coordinates, and the distance between sites. The first site was the lakeshore fronting Marawi City marked as ground zero (Site A) where the battle was fiercest and where residues of war were substantially deposited; the second (B) and third (C) sites were 8.15 km and 24.41 km away from site A. These three sites represent munition residues exposed sites. Site D is the reference lake (non-exposed site), Lake Dapao, LDS, 34.45 km from site A. The coordinates were determined using Garmin(R), GPSMAP 64x, and the actual sites were generated by plotting the coordinates through Google Earth. The distance of the sites from site A was calculated through Movable Type Scripts (https://www.movable-type.co.uk/scripts/latlong.html).

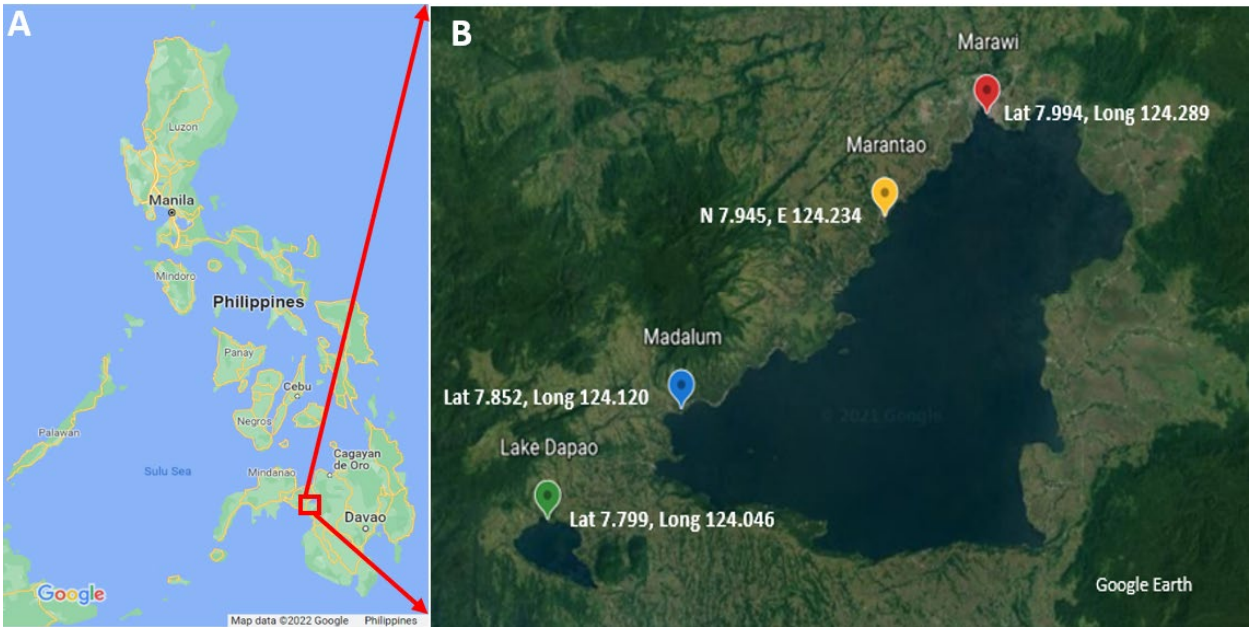

Figure 1A) Map of the Philippines showing the location of Marawi City and Lake Lanao in the red inset. B) Sampling sites (balloons) in Lake Lanao shores and their corresponding coordinates: red, site A, ground zero in Marawi city; yellow, site B; blue, site C; and green, reference site (Lake Dapao)

Test Organisms, Collection, and Identification

The test organisms were *V. angularis* Muller (class Gastropoda, phylum Mollusca, Fig. 2) Color, description, and identification of the snails were based on the work of Pagulayan & Cepillo (1991), Moneva *et al.* (2012), and Torres *et al.* (2014). Sub-adult and adult *V. angularis* have 4 whorls with shell colors ranging from dark greenish brown or greyish yellow. It is striated but not with a hammer pattern. Immature or juvenile *Vivipara* is usually up to 14-16 mm while adult length ranges from 18-31 mm. The height of the shell is 25–35 mm. The width of the shell is 20–26 mm. The shell has 5.5-6 weakly convex whorls. The last whorl is relatively large compared to that of other *Vivipara* species. The umbilicus is narrow.

Vivipara snails were randomly collected within 100 m along the lakeshores and 5-50 m perpendicular to the shores. At least 20 juveniles and 20 adult snails were purposively handpicked, systematically washed to remove mud, placed separately in containers with lake water, and transported to Research Laboratory, Biology Department, Mindanao State University, Marawi Campus. They were temporarily kept in the aerated aquaria with lettuce leaves. Micronucleus assay proceeded immediately as soon as they were brought to the laboratory.

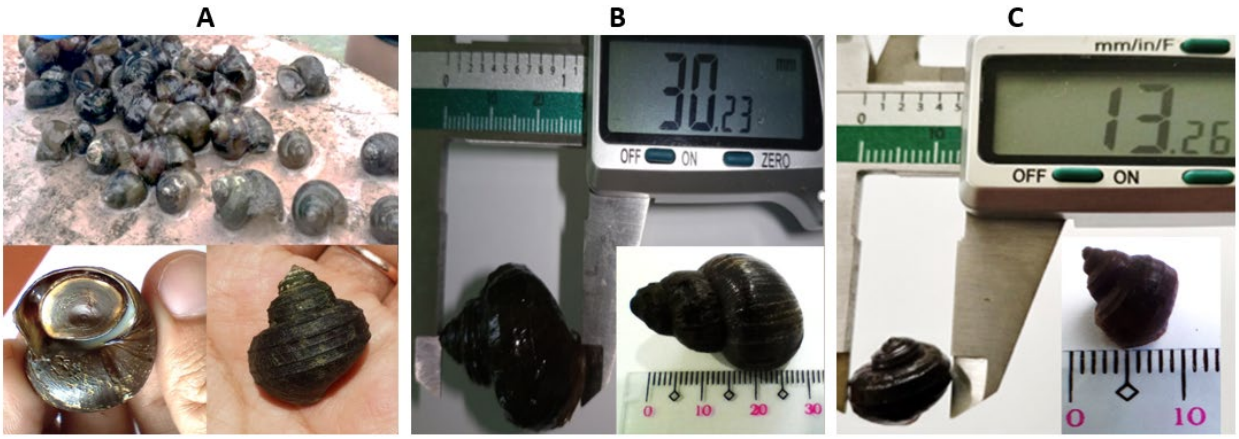

Figure 2 *Vivipara angularis* Muller snails collected from Lake Lanao (A); adults (B); and juvenile (C) samples

Micronucleus Assay

Twenty (n=20) *V. angularis, i.e.,* ten (n=10) juvenile and ten (n=10) mature gastropods from each site, including the reference site, were randomly selected, and processed for MN assay following the work of da Silva *et al.*, 2013, de Vasconcelos Lima, 2019. To collect 0.1 ml of hemolymph, the operculum of the snail was gently tapped by a micropipette tip triggering the snails to retract and release hemolymph. The hemolymph was smeared into clean slides and air-dried for 10 min, followed by adding 0.1 ml of Ringer's solution, and 0.1 ml of the 10 mM EDTA solution. The smears were placed in a moist chamber for 30 min before fixing with glutaraldehyde for 5 min, rinsed with Ringer's solution, stained with Giemsa solution (Giemsa Set, Medic) for 7 min then finally washed with distilled water and air-dried. Each slide was viewed for blind scoring of MN under a phase contrast microscope (Olympus, CKX53). Blind scoring was employed. With the help of a hand-held counter, 1000 hemocytes (cells) were examined per individual gastropod, such that, (10 adults + 10 juveniles) x 1000 cells = 20,000 cells were examined per site. Only hemocytes with an intact nucleus and cellular distinction were considered and the MN was identified and counted when its diameter was smaller than one-third the size of the nucleus, its color and texture should be similar to that of the nucleus and not in direct contact with the main nucleus. A digital camera (XCAM1080PHB, Toup Tek, China) was used to aid in counting the MN in the monitor and in taking photomicrographs.

Detection of Heavy Metals in the Snail Muscles

Muscles from adults and juvenile snails (150 g x 3 replicates) from each sampling site were harvested and immediately analyzed for heavy metals utilizing atomic absorption spectrophotometry (AAS) for Cd, Cr, and Pb, silver diethyldithiocarbamate-colorimetric (modified) for As and cold vapor AAS for Hg.

Statistical Analysis

The resulting MN frequencies between exposed and non-exposed sites and between juvenile and adult samples were analyzed statistically using Statistical Package for the Social Sciences (SPSS) for Windows. Using the Shapiro-Wilks Test, the means of the MN frequencies for sites and age groups were found not normally distributed, hence Kruskal-Wallis and the Mann-Whitney U tests were used to analyze the differences of the MN frequencies in various sites and age groups.

RESULTS AND DISCUSSION

Micronucleation by Sampling Sites

Micronucleation (Figure 3) was observed in the hemocytes of *V. angularis* in all sampling sites. Table 1 shows the mean frequencies of the MN per site and the variation was highly significant by the Kruskal Wallis test, [P=.000, H(3, N=80)=28.0326]. Indeed, MN frequencies were significantly higher in Lake Lanao sites compared to the reference site, Lake Dapao. The highest mean was recorded in site A, the ground zero where the encounter was heftiest, and the lowest in site D, the reference lake. A pairwise comparison of the mean ranks by the Mann-Whitney U test illustrated that site A was significantly higher than other sites ($P<0.5$) except for site C ($P=0.37346$). Conversely, the reference site was significantly lower than the rest of the sites ($P<.05$).

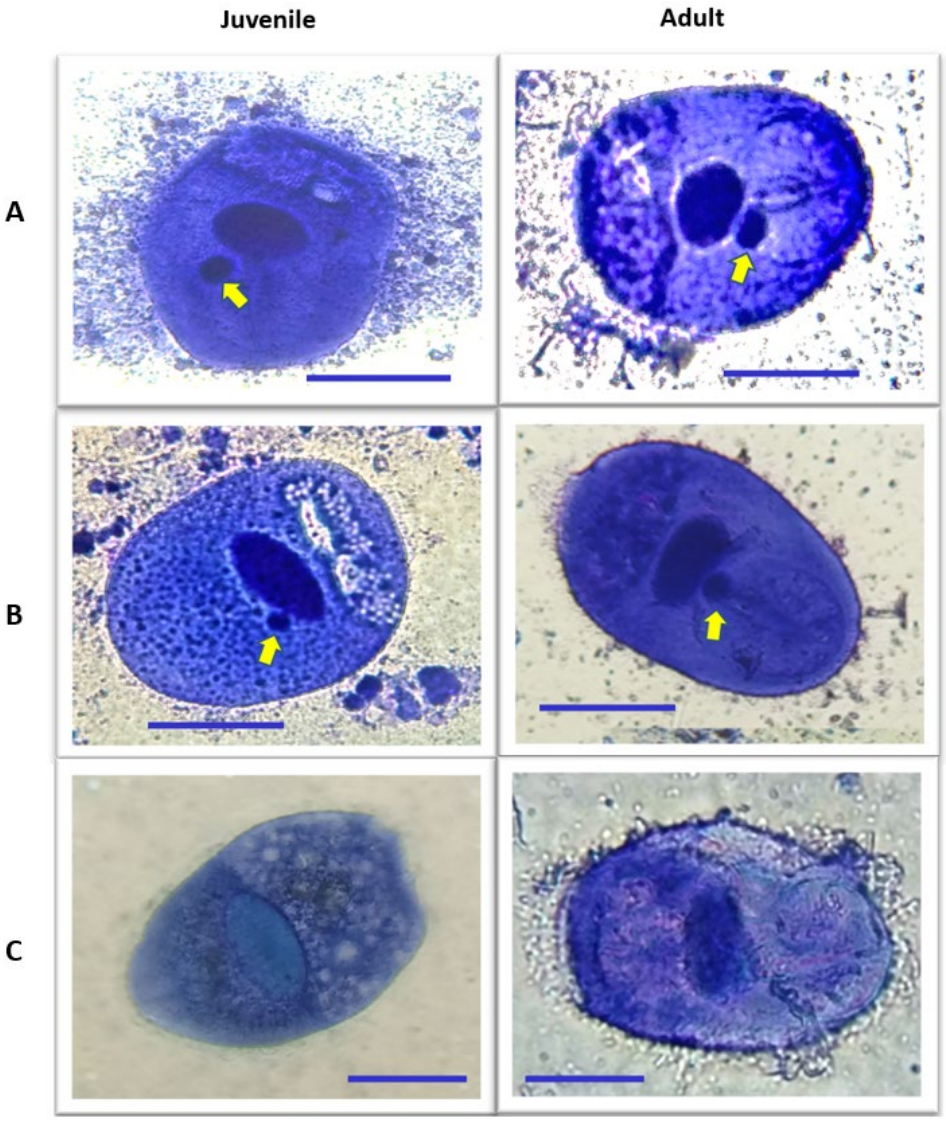

Figure 3A-B Photomicrographs of *V. angularis* hemocytes showing micronuclei (yellow arrows) in adult and juvenile samples. C, hemocytes without micronuclei are shown in C. Scale bars, 5µm

Table 1 MN frequencies in the hemocytes of adult and juvenile *V. angularis* sampled from the different sites in Lake Lanao and in Lake, Dapao, Philippines

Sampling Sites	MN frequency* Mean			SD		
	Juvenile	Adult	Total	Juvenile	Adult	Total
Site A	3.6	10	6.8	1.776	6.6	5.736
Site B	2.4	2.4	2.4	2.319	1.506	1.903
Site C	6.8	3.1	4.95	3.938	2.885	3.859
Site D	1.5	0.8	1.15	1.65	0.422	1.226
Total	3.58	4.08	3.82	3.21	5.025	4.197

*Frequency values (MN observed per 1000 hemocytes)

Spontaneous micronuclei formation can happen in any eukaryotic cell as it divides. It is often brought about by a broken chromosome fragment or rarely an entire chromosome that lags and remains outside the nucleus after cell division (Beedanagari *et al.* 2014). These are extra-nuclear bodies that contain damaged chromosome fragments or whole chromosomes that are not incorporated into the nucleus after cell division (Luzhna & Kovalchuk 2013). It can be induced by defects in the cell repair machinery and accumulation of DNA damages and chromosomal aberrations. This explains why MN was also observed in the reference lake although the count was significantly lower than the exposed sites.

As mentioned earlier, MN frequencies were shown to increase in cells of various organisms that were exposed to carcinogens and genotoxins, thus, MN assay is one of the most widely used genotoxicity biomarkers, providing an efficient measure of chromosomal DNA damage. The elevated MN counts in site A could be attributed to genotoxic chemicals most probably from war pollutants washed into the lake water where the snail samples were thriving. It must be noted that site A is fronting "ground zero" in Marawi where the encounter was densest. IARC (2021) provided a list of eroded components of munitions that are carcinogenic including heavy metals, such as As, Hg, Cd, Cr, and Pb. These heavy metals were detected in the muscle tissues of *V. angularis* from sampling sites (Table 6), and Jalova *et al.* (2021) also reported heavy metals in commonly consumed fishes sampled from Lake Lanao, though the levels detected were mostly lower than the standard limits (WHO/FAO 2010, 2011, Factor *et al.* 2012, Perelonia *et al.* 2017, and Gbogbo *et al.* 2017).

A closer look at Table 2 revealed that a higher concentration of Ar, (0.27 mg/L) from snail muscles was detected in site A, which was higher than the national acceptable limit in drinking water set at 0.010 mg/L, although the safe limit for mollusk as food is set at 1mg/kg. Nevertheless, this detection may call for some concerns due to the continued consumption of the snails by the locals because this trace metal, aside from being highly toxic and carcinogenic (Apostol *et al.* 2022), can biomagnify along with other toxic pollutants, that is, to increase in concentration in the living organisms successively at higher levels in the food chain (Hepp 2017).

The study by Sanderson *et al.*, (2017), reported that HMX and trace levels of RDX explosives compounds were found in the fiddler crabs from the live impact area (LIA) in the island of Vieques in Puerto Rico which also recorded higher levels of some metals such as arsenic. Moreover, the study by Dong *et al.* (2019) also published that arsenic-exposed human populations through drinking contaminated water had increased micronucleus formation from the buccal cells, lymphocytes, and urothelial cell samples. Moreover, Gbogbo *et al.* (2017) detected levels of arsenic in freshwater fish and shellfish from 0.2 to 2.2 mg/L in Ghana rivers and recommended in their study that the maximum quantities of the organisms considered safe for consumption ranged from 375 to 5250 g per week.

Table 2 Mean concentration of As, Cd, Cr, Pb, Hg (mg/L) detected in muscle tissues of *V. angularis* collected from the different sampling sites

Sampling Sites	Heavy Metals (mg/L)				
	Arsenic (As)	Mercury (Hg)	Cadmium (Cd)	Chromium (Cr)	Lead (Pb)
Site A	0.27	≤ 0.01*	≤ 0.025*	≤ 0.025*	0.350
Site B	≤ 0.025*	≤ 0.01*	≤ 0.025*	0.893	0.647
Site C	≤ 0.025*	≤ 0.01*	≤ 0.025*	0.92	0.507
Site D	≤ 0.025*	≤ 0.01*	≤ 0.025*	≤ 0.025*	0.54

*Machine reporting limit

Micronucleation by age groups and increasing distance from ground zero

Figure 1 shows the relative distance of the sampling sites from site A where the fiercest fight occurred. Looking at Figure 4, adult snails obviously displayed a decreasing trend of MN frequencies with increasing distance from site A but not among the juvenile samples. Comparing the MN counts between age groups, it is clearly seen that the exposed adults in site A marked significantly high MN frequencies compared to their juvenile counterparts (Table 3). These results implied that the genotoxic contaminants in these sites affected the adult snails more than the juvenile samples. It makes sense knowing that the average life expectancy of freshwater snails is 3 to 5 years (https://foliargarden.com/ life-expectancy-of-a-freshwater-snail/), and some of the adult samples must have already been at the site during the siege and thus were the most exposed to war pollutants at the highest magnitude which may have resulted in DNA damage as indicated by higher MN frequency compared to the juveniles.

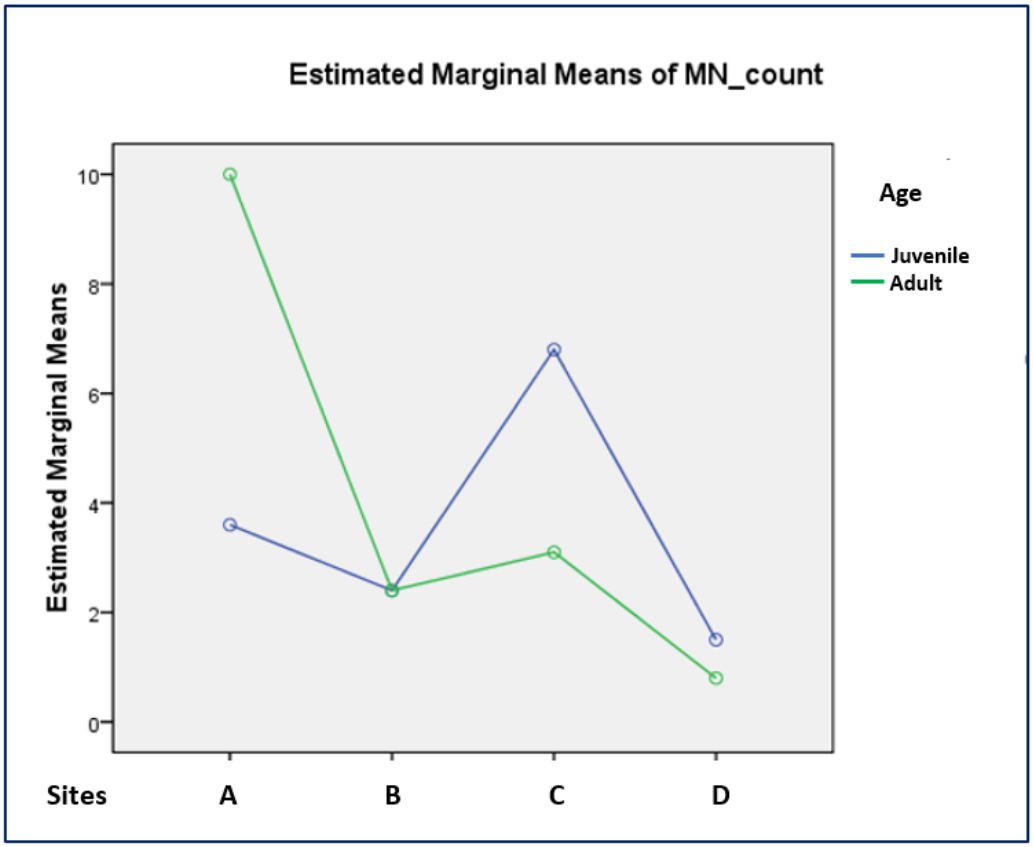

Figure 4 Profile plots of the estimated marginal means of MN frequencies in different sites and age groups showing a downward trend among adults from sites A, and B to D, except in site C which showed an unexpected increase

Table 3 Pairwise comparison of MN frequencies between age groups at different sites by Mann-Whitney U

Sites	Comparison of MN frequencies between juvenile and adult	
	Sig. level	Remarks
Site A	0.011	Significantly different (adult has higher count)
Site B	0.853	Not significantly different
Site C	0.023	Significantly different (adult has lower count)
Site D	0.579	Not significantly different

On the other hand, in site C, which is 24.41 km away from site A, a different MN pattern was observed wherein juveniles displayed higher MN counts than adults. These results were unexpectedly alarming and need further studies. This must be attributed to some genotoxic substances other than the munition residues due to war which particularly predisposed the young snails. One possible explanation is the nearby brassware industry which utilizes brass, a metal alloy primarily composed of Cu and Zn. Copper alloy with a higher amount of Cu is known to cause 'contact killing', possibly via redox cycling and production of reactive oxygen species (ROS) groups that can cause lethal damage to cell membranes and oxidative damage to DNA (Grass *et al.* 2011 and Bille *et al.* 2013). DNA has a higher affinity to Cu2+ which may affect the integrity of DNA and the normal process of DNA replication and transcription (Govindarajua *et al.* 2013) and consequently micronucleus formation (Nikdehghan *et al.* 2018)Substantial evidence has shown the possible genotoxicity of Cu and Zn in aquatic organisms. Moreover, looking back at Table 2, Cr was detected highest in site C although it was closely followed by site B. The study by El Safty *et al.* (2018) demonstrated that Cr- and Ni-exposed workers of the electroplating industry had increased MN signifying cytogenetic damage caused by these heavy metals.

The observed MN frequencies in this study are comparable to the works of Silva *et al.* (2011) and de Vasconcelos Lima (2019) who tested the genotoxicity of gamma radiation and heavy metals and domestic sewage sludge, respectively against freshwater snails *B. glabrata* using the MN assay. The assay was also utilized by da Silva *et al.* (2013) when they tested tobacco leaves in the land snails *Helix aspersa.* Their results considered MN assays as a reliable parameter to measure genotoxic hazards in the environment using snails. Although the MN frequencies in the present study are baseline data to approximate genotoxicity in the munition-laden Lake Lanao using *V. Angularis*. Further studies must be conducted to verify the genotoxic chemicals in crucial sites.

CONCLUSION

Post-siege genotoxic hazard in Lake Lanao, LDS, Philippines brought by Marawi Siege in 2017 was assessed through MN assay using V. angularis snails sampled from different sites in Lake Lanao and in the reference site Lake Dapao. Results demonstrated a higher MN frequency in snails sampled from munition-exposed sites in Lake Lanao lakeshores than in the reference site, Lake Dapao. Among the adult samples, there was a decreasing trend of MN frequencies with increasing distance from lake shore fronting ground zero at Marawi City where the siege was heaviest. A remarkable difference was also found in the MN frequency patterns between the age groups. In all sites, except site C, adults had higher MN frequencies than juveniles and the difference was significant in site A. Moreover, it is generally accepted that MN frequency increases with age, but unexpectedly, MN frequencies were significantly higher among the juvenile samples than adult samples in site C. The post-siege genotoxic hazards possibly posed by the war pollutants in Lake Lanao near ground zero cannot be overlooked. Local leaders must be informed of the results for possible interventions to protect the health and well-being of the populace around Marawi and Lake Lanao.

ACKNOWLEDGEMENT

The authors are truly grateful to the Philippine Council for Health Research and Development - Department of Science and Technology (PCHRD-DOST) Grants-in-Aid program for the generous financial support for this project.

REFERENCES

Alborz A. 2013. Environmental characteristics and prevalence of birth defects among children in post-war Iraq: implications for policies on rebuilding the Iraqi education system. Med Confl Surviv 29(1):26-44. https://doi.org/0.1080/13623699.2013.765197

Apostol GLC, Valenzuela S, Seposo X. 2022. Arsenic in Groundwater Sources from Selected Communities Surrounding Taal Volcano, Philippines: An Exploratory Study. Earth 3: 448-459. https://doi.org/10.3390/earth3010027

Baroudi, F., Al-Alam, J., Fajloun, Z., & Millet, M. (2020). Snail as sentinel organism for monitoring the environmental pollution; a review. *Ecological Indicators*, *113*, 106240. https://doi.org/10.1016/j.ecolind.2020.106240

Beedanagari S, Vulimiri S, Bhatia S, Mahadevan B. 2014. Chapter 43 – Genotoxicity biomarkers: Molecular basis of genetic variability and susceptibility. In: Gupta R, editors. Biomarkers in Toxicology, Academic Press. p 729-742. https://doi.org/10.1016/B978-0-12-404630-6.00043-9.

Bille T, Grimes M, Podini D. (2013). Copper Induces DNA damage on unfired brass cartridge casings. https://www.promega.com/-/media/files/resources/conference-proceedings/ishi-24/poster-abstracts/21-daniele-podini.pdf . Retrieved May 5, 2021.

Bolognesi C, Fenech M. 2012. Mussel micronucleus cytome assay. Nat Protoc 7: 1125-1137. https://doi.org/10.1038/nprot.2012.043.

Broomandi P, Guney M, Kim JR, Karaca F. 2020. Soil contamination in areas impacted by military activities: A critical review. Sustainability 12(21): 9002.

Camama CG, Torres MA, Manting MM, Gorospe JG, Demayo CG. 2014. Compartmentalization in the shells of four lake populations of *Vivipara angularis*. Ann Biol Res 2014, 5(3): 16-21.

Chatterjee S, Deb U, Datta S, Walther C, Gupta DK. 2017. Common explosives (TNT, RDX, HMX) and their fate in the environment: Emphasizing bioremediation. Chemosphere 184:438-451. https://doi.org/10.1016/j.chemosphere.2017.06.008

da Rocha SM, da Rocha CAM. 2016. Micronucleus test in bivalve mollusks as an important tool for xenobiotic exposure risk assessment. Acta Fish Aquat Res 4(1):70-79. https://doi.org/10.2312/ActaFish.2016.4.1.70-79

da Silva FR, Erdtmann B, Dalpiaz T, Nunes E, Ferraz A, Martins, TL, Dias J F, da Rosa DP, Porawskie M, Bona S, da Silva J. 2013. Genotoxicity of Nicotiana tabacum leaves on Helix aspersa. Genet Mol Biol 36(2):269-275. https://doi.org/10.1590/S1415-47572013005000020

de Vasconcelos Lima M, de Siqueira WN, Silva HAMF, de Melo Lima Filho J, de França EJ, de Albuquerque Melo AMM. 2019. Cytotoxic and genotoxic effect of oxyfluorfen on hemocytes of *Biomphalaria glabrata*. Environ Sci Pollut Res Int (4):3350-3356. https://doi.org/10.1007/s11356-018-3848-3

Dong J, Wang JQ, Qian Q, Li GC, Yang DQ, Jiang C. 2019. Micronucleus assay for monitoring the genotoxic effects of arsenic in human populations: A systematic review of the literature and meta-analysis. Mutat Res Rev Mutat Res 780: 1-10.

Earth 2022, 3, 448–459 Arsenic in Groundwater Sources from Selected Communities Surrounding Taal Volcano, Philippines: An Exploratory Study. https://doi.org/10.3390/earth3010027

El Safty AMK, Samir AM, Mekkawy MK, Fouad MM. 2018. Genotoxic Effects Due to Exposure to Chromium and Nickel Among Electroplating Workers. Int J Toxicol 37(3): 234-240. https://doi.org/10.1177/1091581818764084

Factor CJB, de Chavez ERC. 2012. Toxicity of Arsenic, Aluminum, Chromium and Nickel to the Embryos of the Freshwater Snail, Radix quadrasi von Möellendorf 1898. Philipp J. Sci 141 (2): 207-216.

Fathi RA, Matti LY, Al-Salih HS, Godbold D. 2013. Environmental pollution by depleted uranium in Iraq with special reference to Mosul and possible effects on cancer and birth defect rates. Med Confl Surviv 29(1): 7-25. https://doi.org/10.1080/13623699.2013.765173

Fenech M, Knasmueller S, Bolognesi C, Holland N, Bonassi S, Kirsch-Volders M. 2020. Micronuclei as biomarkers of DNA damage, aneuploidy, inducers of chromosomal hypermutation and as sources of pro-inflammatory DNA in humans. Mutation Research/Reviews in Mutation Research 786: 108342. https://doi.org/10.1016/j.mrrev.2020.108342

Gbogbo F, Otoo SD, Asomaning O, Huago RQ. 2017. Contamination status of arsenic in fish and shellfish from three river basins in Ghana. Environ Monit Assess 189(8): 400. https://doi.org/10.1007/s10661-017-6118-9.

Gentile N, Mañas F, Bosch B, Peralta L, Gorla N, Aiassa D. 2012. Micronucleus assay as a biomarker of genotoxicity in the occupational exposure to agrochemicals in rural workers. Bull Environ Contam Toxicol. 88(6): 816-22. doi: 10.1007/s00128-012-0589-8

Govindaraju M, Shekar HS, Sateesha SB, Vasudeva Raju P, Sambasiva Rao KR, Rao KSJ, Rajamma AJ. 2013. Copper interactions with DNA of chromatin and its role in neurodegenerative disorders. J Pharm Anal. 3(5): 354-359. doi: 10.1016/ j.jpha.2013.03.003.

Grass G, Rensing C, Solioz M. 2011. Metallic copper as an antimicrobial surface. Appl Environ Microbiol 77(5): 1541-1547. https://doi.org/10.1128/AEM.02766-10.

Hans Sanderson, Patrik Fauser, Ricky Steven Stauber, Jesper Christensen, Per Løfstrøm & Thomas Becker (2017) Civilian exposure to munitions-specific carcinogens and resulting cancer risks for civilians on the Puerto Rican island of Vieques following military exercises from 1947 to 1998, Global Security: Health, Science and Policy, 2:1, 40-61, DOI: 10.1080/23779497.2017.1369358

Hayashi M. 2016. The micronucleus test—most widely used in vivo genotoxicity test—. Genes and Environ 38(1): 1-6. https://doi.org/10.1186/s41021-016-0044-x

Hepp LU, Pratas JA, Graça MA. Arsenic in stream waters is bioaccumulated but neither biomagnified through food webs nor biodispersed to land. Ecotoxicol Environ Saf 139: 132-138. https://doi.org/10.1016/j.ecoenv.2017.01.035

International Agency for Research on Cancer (IARC) 2021. IARC monographs on the identification of carcinogenic hazards to humans [Internet]. Int. Agency Res. Cancer. URL https://monographs.iarc.who.int/list-of-classifications (accessed 3.23.21).

Jalova MC, Lomantong AD, Calibo LG, Camarin MM. 2021. Assessment of Heavy Metals Found in Commonly Consumed Fishes from Lake Lanao, Philippines. Isr J Aquac Bamidgeh 73: 1-9. https://doi.org/10.46989/001c.24939.

Kapoor K. 2017. Snipers, bombs, mortars-Philippines troops battle against Islamists. World News. [accessed 2017 Dec 29]. Available at: https://sg.news.yahoo.com/snipers-bombs-mortars-philippine-troops-battle-against-islamists-125808644.html

Luzhna L, Kathiria P, Kovalchuk O. 2013. Micronuclei in genotoxicity assessment: from genetics to epigenetics and beyond. Front Genet 4:131. https://doi.org/10.3389/fgene.2013.00131

Moneva CS, Torres MA, Demayo CG. 2012. Applications of geometric morphometric analysis in describing sexual dimorphism in shell shapes in Vivipara angularis Muller (Family Viviparidae). Anim Biol Anim Husb 4(1): 14-19.

Nikdehghan N, Kashiri H, Hedayati A. 2018. CuO nanoparticles-induced micronuclei and DNA damage in Cyprinus carpio. Aquac Aquar Conserv Legis (AACL) Bioflux 11(3): 925-936.

Pagulayan IF, Cepillo JC. 1991. The Reproductive Biology and Laboratory Maintenance of Vivipara angularis Muller (Prosobranchia: Viviparidae). Transactions NAST PHL 13: 535-568.

Pareño R. 2017. US delivers weapons for PAF amid Marawi siege. Philippines: philstar.com; [accessed 2017 July]. Available from:https://www.philstar.com/headlines/2017/07/29/1722681/us-delivers-weapons-paf-amid-marawi-siege

Perelonia KB, Abendanio, CC, Raña, JA, Opinion AG, Villeza JT, and Cambia, FD. 2017. Heavy Metal Contamination in Water and Fishery Resources in Manila Bay Aquaculture Farms. The Philipp J Fish 24(2): 74-97. https://doi.org/10.31398/tpjf/24.2.2016A0014

Savabieasfahani M, Ali SS, Bacho R, Savabi O, Alsabbak M. 2016. Prenatal metal exposure in the Middle East: imprint of war in deciduous teeth of children. Environ Monit Assess 188(9): 505. https://doi.org/10.1007/s10661-016-5491-0

Seukep AJ, Noumedem JA, Djeussi DE, Kuete V. 2014. Genotoxicity and teratogenicity of African medicinal plants. In: Toxicological survey of African medicinal plants: Elsevier. p. 235-275.

Silva L, Silva B, Melo A, Silva R, Amancio F. 2011. Micronuclei as biomarkers of genotoxicity of gamma radiation in aquatic environments. INAC 2011: International nuclear atlantic conference Nuclear energy: new jobs for a better life, Brazil. [Accessed 2017 August 14]. Available at: https://inis.iaea.org/collection/NCLCollectionStore/_Public/42/107/42107631.pdf

Skalny AV, Aschner M, Bobrovnitsky IP, Chen P, Tsatsakis A, Paoliello MMB, Buha Djordevic A, Tinkov AA. 2021. Environmental and health hazards of military metal pollution. Environ Res 201:111568. https://doi.org/10.1016/ j.envres.2021.111568

World Health Organization/Food and Agriculture Organization (WHO/FAO). (2010). Joint Expert Committee on Food Additives (JECFA) Meeting (72nd: Rome, Italy), Pp. 16. https://apps.who.int/iris/handle/10665/44520

WHO/FAO (2011). JECFA Meeting (73rd: Switzerland). Evaluation of certain food additives and contaminants: Technical Report Series 960: 228. https://apps.who.int/iris/handle/10665/44515

BIOTROPIA Vol. 30 No. 3, 2023: 263 - 281 DOI: 10.11598/btb.2023.30.3.1858

ESTIMATION OF CHANGE IN ABOVEGROUND BIOMASS IN FOUR NATIONAL FORESTS IN BANGLADESH

AJOY MOJUMDER[1], MD SAHADAT HOSSAIN[2] AND TIMOTHY A. VOLK[2*]

[1]*Department of Environmental Science, Stamford University Bangladesh, Dhaka 1217, Bangladesh*
[2]*Department of Sustainable Resources Management, SUNY College of Environmental Science and Forestry, Syracuse, NY 13210, USA*

Received 27 October 2022 / Revised 28 August 2023 / Accepted 4 September 2023

ABSTRACT

Forest biomass helps mitigate climate change impacts through the sequestration of atmospheric carbon dioxide and potentially storing it for long periods. Deforestation and unsustainable timber harvesting cause the reduction of forest biomass, resulting in the reduced carbon sequestration capacity and altered natural balance of forest ecosystems. We used Landsat 8 Operational Land Imager (OLI) to compare the aboveground forest biomass (AGB) changes between 2014 and 2020 for the four national forests that represent important forest cover zones in Bangladesh. We found no considerable change in AGB in the Sundarbans mangrove forest and the Ukhiya hill forest from 2014 to 2020. In contrast, the average AGB content in Nijhum Dwip mangrove forest decreased from 44.36 $Mg.h^{-1}$ in 2014 to 37.46 $Mg.h^{-1}$ in 2020. The average AGB of the Madhupur deciduous forest also decreased from 110.01 $Mg.h^{-1}$ in 2014 to 107.22 $Mg.h^{-1}$ in 2020. The decreased biomass contents could be attributed to anthropogenic factors as indicated by the presence of human activities and this information will be helpful for forest restoration and management in Bangladesh.

Keywords: change detection, forest biomass, GIS

INTRODUCTION

To achieve the International Panel of Climate Change (IPCC) 1.5°C goal, 730 billion tonnes of CO_2 (730 Pg of CO_2 or 199 Pg of C) must be removed from the atmosphere by 2100. Currently, forests absorb about 7.6 billion tons of CO_2 per year (Harris and Gibbs 2021), and some projections indicate that they could sequester around 36.7 billion tons in 2100 (Masson-Delmotte *et al.* 2022). Global forests store about 60% (~ 862 gigatons) of total terrestrial carbon and sequester close to 80% of all terrestrial aboveground and 40% below-ground organic carbon respectively. However, forest degradation, largely due to anthropogenic activities and climate change, results in CO_2 emissions of about 12 to 20% of global greenhouse gases (Pan *et al.* 2011).

Bangladesh is one of the Asian countries with a high deforestation rate (~2,600 ha annually), largely due to human activities (Macdicken *et al.* 2015). With climate change impacts affecting the weather conditions in Bangladesh, forest degradation has been exacerbated including the alteration of forest composition, structure, and biophysical processes (Littell *et al.* 2010). The combination of climate change and human actions is contributing to deforestation (Deb *et al.* 2018).

The forests of Bangladesh are categorized into four subcategories based on their geographic conditions: Swamp Forests, Mangrove Forests, Plain Land Sal Forests, and Hill Forests (Akhter *et al.* 2013). As per the Bangladesh Forest Department (BFD 2020), forests account for 12.8% of the total land area and are classified into ten classes based on the terrain, climate, location, and management principles including Bamboo Forest, Rubber Plantation, Hill Forest, Mangrove Forest, Mangrove Plantation, Forest Plantation, Shrubs with scattered tree, Swamp Forest and Swamp Plantation. Bangladesh has 2.6 million hectares of forest cover, of which about 50% is

*Corresponding author, email: tavolk@esf.edu

in rural settlements and holds 66% of the country's aboveground forest biomass. As a result, the designated forests only total 1.6 million hectares when excluding unclassified state forests and village forests (Table 1). According to BFD (2020) estimation, total carbon (C) stock on land is 1275.6 million Mg C, of which 80.5% is stored in soils (0–30 cm), 15.3% in above-ground biomass, 3.6% in below-ground biomass, 0.5% in dead wood biomass, and 0.1% in litter biomass. A total of 21.5% of the carbon stock in the entire country is stored in forests, with hill and mangrove forests contributing 9.7% and 5.2% of the total stock, respectively.

Forest resource assessment and monitoring- such as the abovementioned estimates- is important for sustainable forest management, supporting the livelihoods of communities, maintenance of ecosystem services, and carbon sequestration (Angelsen 2009). However, at present there is no complete time-series study of forest biomass and carbon in Bangladesh and how these factors change over time, which is an essential first step in understanding both the current biomass and carbon stocks as well as monitoring future carbon sequestration or losses in different regions of the country. This shortcoming is attributed to limited resources to conduct a conventional forest inventory, which is time-consuming and expensive (Unni 1983).

Recent forest inventory techniques employ digital technology, such as remote sensing and GIS, which can provide quality, quantity, and distribution data for assessment and monitoring. These techniques have been used in spatial decision support systems for forest land classification and planning, modelling, conservation, stand density, timber harvesting, (Köhl *et al.* 2006) and for identification of endangered plant species, faunal habitat zoning, detecting tree damage, mortality, insect infestation, and change (Mukul *et al.* 2014). These methods measure both qualitative (e.g., types) and quantitative (height, basal area, woody biomass, etc.) forest characteristics with ~75% accuracy (Ali *et al.* 2015).

Table 1 Forest types and their characteristics in Bangladesh (BFD 2020; Khan and Millate-e-Mustafa 2001; Rahman 2021)

Forest Type	Description
Hill Forests	Hill forests of Bangladesh (0.67 million ha) are mostly mixed-evergreen forests situated in the southeast (Chittagong Hill Tracts) and northeast (Sylhet Hill Tracts) (Mukul *et al.* 2016). The dominant species include *Dipterocarpus turbinatu, Swintonia floribunda*, *Lophopetalum fimbriatum*, *Duabunga sonnerationides, Salmalia insignis*, *Syzygium grande* (Reza and Hasan 2019).
Plain land Sal Forests	Plain land Sal Forests, also known as Sal moist forest (0.12 million ha) is mainly located in central Bangladesh and dominated by *Shorea robusta* with *Phyllanthus emblica, Terminalia chebula, T. Belerica*, *Careya arborea*, etc. (Chowdhury and Koike 2010).
Natural Mangrove Forests	The Sundarbans mangrove forest (0.6 million ha) is the single largest productive natural mangrove forest in the world and is in the southern coastal regions. It contains dense evergreen species dominated by *Heritiera fomes*, *Avicennia officinalis*, *A. Alba*, *Hibiscus tiliaceus*, *Excoecaria agallocha*, etc. (Reza and Hasan 2019).
Coastal Plantation Forests	Afforestation along the long shoreline was started to maintain and safeguard coastal ecosystems which currently cover an area of 0.11 million hectares. Many different types of mangrove species are planted, including *Sonneratia apetala, Avicennia officinalis, A. Marina, A. Alba, Amoora cucullata, Bruguiera sexangula, Excoecaria agallocha, Xylocarpus mekongensis, Heritiera fomes, Ceriops decandra, and Nypa fruticans* (Chowdhury *et al.* 2020; Islam *et al.* 2021).
Un-classified State Forests	This is a 0.73 million hectares area in the Chittagong Hill Tract and is managed by district councils. Major species include *Bambusa balcooa, B. Burmanica, Thyrsostachys oliveri, Melocanna baccifera, Hevea brasiliensis,* etc.
Swamp Forests and Swamp Plantation	Covers 0.02 million hectares and is mostly found in the north-eastern region (Sylhet and Sunamganj district). Major species include Hijol or Indian oak (*Barringtonia acutangular*), Koroch (*Pongamia pinnata*), Pitali (*Trewia nudiflora*), and Borun (*Crataeva magna*) (Sohel *et al.* 2023).
Privately Owned Village Forests and Forest Plantation	Also known as homestead forests, span 0.27 million hectares and are dispersed throughout the county.

Bangladesh conducted its first national forest resource assessment in 2007 (Altrell *et al.* 2007), however, there is little information available on earlier data-collecting techniques and there are inconsistencies in methodology, and data-sharing arrangements (Costello *et al.* 2016). In response to the growing need for data on trees and forest resources, a second, more thorough institutionalized forest inventory was released in 2020. It was based on remote sensing and included biophysical, land-use, and socioeconomic data (Henry *et al.* 2021). In Bangladesh, forestry information is still highly valued and is included in significant national plans, strategies, and policies due to the severe burden that energy and food production places on the country's forests (BFD 2020). As a result, inventories of forest resources are important for managing Bangladesh's endangered forests sustainably.

This study's objective is to estimate the change in forest aboveground biomass between 2014 and 2020 in four forest zones (Mangrove Forest, Hill Forest, Sal Forest, and Coastal Plantation Forest) in five districts of Bangladesh (Table 2) using Landsat 8 imagery data. The main purpose is to understand the change in total tree biomass and generate maps showing changes in aboveground biomass.

MATERIALS AND METHODS

Study Location

This study focused on four designated forests in Bangladesh that provide socioeconomic and ecological benefits but are also deteriorating and losing forest cover because of human pressures and natural disasters (Figure 1).

The Sundarbans Mangrove Forest covers 927,700 hectares and is the biggest continuous stretch of mangroves in Bangladesh. They are highly productive, have a rich floral and faunal diversity, and have a significant impact on the national and regional economies and climate (Mahmood *et al.* 2021). A million people rely on the Sundarbans for their livelihoods, and the area offers numerous direct benefits like fuelwood collection, fishing, and protection from tropical cyclones and tidal surges for their lives and property (Aziz and Paul 2015). It also manages coastal and riverbank erosion, aids in the sequestration and storage of carbon, and supports soil nutrient cycling (Hale *et al.* 2019; 2015). Bangladesh has over 60% of the Sundarbans, which make up 4.13% of the country's total landmass and 38.12% of its total forest area (BFD 2017). However, the Sundarbans are under threat from both natural and man-made sources, including erosion, tropical cyclones, salinity intrusion, and sea level rise, as well as from human settlement, overfishing, industrial development, pollution, and tourism (Giri *et al.* 2011; Uddin *et al.* 2019). Consequently, from the 1990s to the 2000s, there was a net loss of 1.1% (10000 ha) of Bangladesh's Sundarbans; hence, it is critical to continuously monitor the Sundarbans' forest cover to maintain this global heritage (Giri *et al.* 2015).

Madhupur Sal Forest is a Plainland Sal Forest, one of the most abundant and ecologically appealing forest types (Islam *et al.* 2023) but it has been severely damaged by agriculture and other human activities (Rahman *et al.* 2009). Around 7,079.4 hectares of Sal Forest were destroyed between 1972 and 2013 as a result of the quick destruction and severe disruptions caused by both local and indigenous inhabitants (Al Faruq *et al.* 2016). The Madhupur Sal Forest is surrounded by densely populated areas, which puts pressure on the forest's resources and accelerates their degradation (Rahman *et al.* 2009). The main causes of forest degradation are encroachment, illegal tree merchants, shifting cultivation, ethnic people's energy and wood consumption, and the introduction of exotic species (Yasmin *et al.* 2010).

The Ukhiya Hill Forest, Whykhing, and Teknaf forest ranges make up Cox's Bazar Forest Lands, which enclose 21,848 hectares of the Chittagong Hill Forest (Nur *et al.* 2016). Tropical evergreen and semi-evergreen vegetation, which makes up this forest, is very important for the variety of flora and fauna and for sustaining the livelihood of the local population. However, the majority of Bangladesh's deforestation took place in this forest area because of population settlement, shifting agriculture, and illicit logging (Reddy *et al.* 2016; Salam *et al.* 1999). Since 1991, over 931,447 Rohingya refugees have migrated to Bangladesh (UNHCR 2023); the bulk of them are now living in camps built by indiscriminately

removing 1,747.45 ha of natural vegetation and leveling hills in the upazilas of Teknaf and Ukhiya in the Cox's Bazar district (Hossain and Haider 2020; Sarkar *et al.* 2023). As a result, it caused changes to the environment, including topsoil loss that resulted in unproductive land, erosion, a potential risk of landslides, and a threat to the biodiversity of the local and regional areas (Mahmood *et al.* 2021). Hasan *et al.* (2021) showed that in 2016, Ukhia's forest area had fallen by 82% because of severe deforestation. Therefore, effective change detection and analysis through appropriate monitoring are essential for the conversion of this protected area and sustainable management (Akhtar *et al.* 2022; Hossain and Moniruzzaman 2021).

The Nijhum Dwip Forest is a Coastal Plantation Forest that is a distinctive mangrove ecosystem and unique wildlife habitat for the native spotted deer population (*Axis axis*), and a wintering site for a significant number of migratory water birds, including several endangered species (Nishorgo Network 2018). Natural disasters like cyclones and tidal erosion, as well as human activities like unlawful harvesting and turning forest areas into agricultural land, have caused a loss of over 42% of forest area from 1990 to 2020 (Islam *et al.* 2021). To preserve and sustainably manage this protected area, the Government of Bangladesh (GoB) designated 16,350 hectares of Nijhum Dwip as a national protected area in 2001 (Hossain *et al.* 2016).

Table 2 Study area and forest types included in this forest biomass assessment.

BFD Class (BFD 2020)	**Study area**	**Study locations**	**Area covered (ha)**
Hill Forests	Ukhiya Hill Forests	District: Cox's Bazar Upazila: Ukhia(21°16'59.88" N and 92°05'60.00" E)	26,180
Coastal Plantation Forests	Nijhum Dwip	District: Noakhali Upazila: Hatiya(22° 05' 35" N and 91° 00'13.7" E)	16,352
Plain land Sal Forests	Madhupur Sal Forest	Distract: Tangail Upazila: Madhupur(24°37'0.12" N and 90°01'30.00"E)	36,692
Natural Mangrove Forests	Sundarbans Mangrove Forest	District: Khulna Upazila: Dacope Administrative Range: Khulna Range(22°46'45" N and 89°43'49"E)	74,672
		District: Khulna Upazila: Koyra Administrative Range: Nalian Range(22°4'26.6" N and 89°24'2.6" E)	61,450
		District: Bagerhat Upazila: Sarankhola Range:(22°2'56.4" N and 89°48'10.2" E) and Management Range: Sarankhola Range Mongla(22°4'7.7" N and 89°39'17.9" E) Chandpai Range	132,495
		District: Bagerhat Upazila: Mongla Administrative Range: Chandpai Range(22°4'7.7" N and 89°39'17.9" E)	130,995

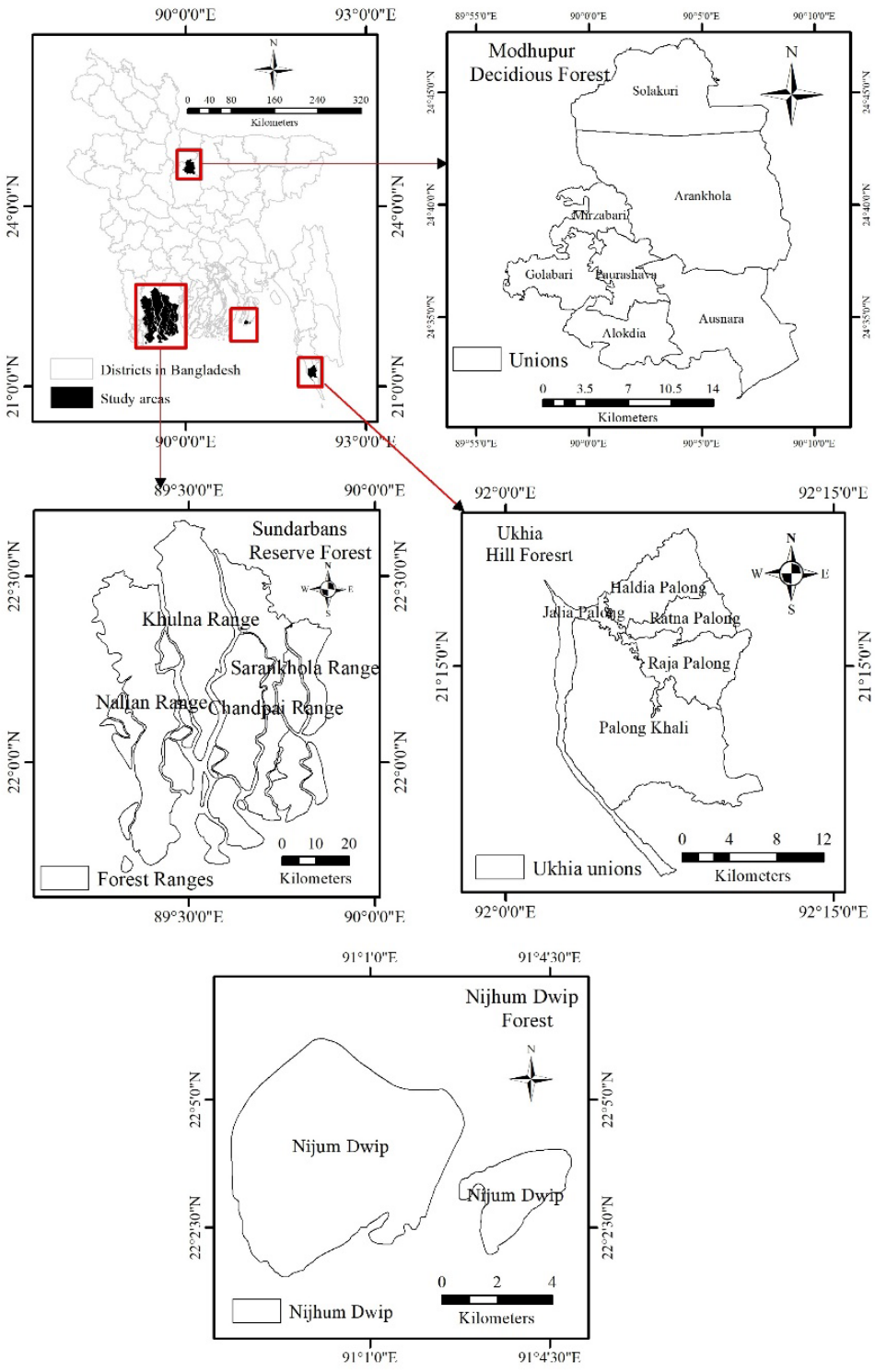

Figure 1 The country-level map shows the locations of study areas, with expanded maps for each of the regions where changes in forest biomass were assessed

Aboveground Biomass (ABG) Data Acquisition and Estimation

This study used multi-spectral Landsat-8 satellite images, with a 30 x 30 m resolution, for the years 2014 and 2020 acquired from the USGS open-source satellite database (https://earthexplorer.usgs.gov/) (Table 3). The images were processed and analyzed in ArcGIS 10.2.1.

This study followed the procedure used by Tripathi *et al.* (2010) for calculating carbon sequestration (Figure 2). The Normalized Difference Vegetation Index (NDVI) (Eq. 1) was calculated from satellite data (Tucker 1979) and used to calculate the fraction of photosynthetically active radiation (FPAR) (Hu *et al.* 2017; Sims *et al.* 2006) (Eq. 2), photosynthetically active radiation (PAR) (Sellers *et al.* 1995) (Eq. 3), and absorbed photosynthetically active radiation (APAR) (Eq. 4) (Field *et al.* 1995).

$$NDVI = \frac{(NIR-RED)}{(NIR+RED)} \; or \; \frac{APAR}{PAR} \quad (1)$$

Again,

$$FPAR = (1.24 \times NDVI) - 0.168 \quad (2)$$

PAR is restricted to just a portion of sunlight's spectrum from 400 to 700 nanometers (nm). PAR is estimated from sunrise to sunset and is given by the formula:

$$PAR = \frac{GHI}{365} \, Wm^{-1} \quad (3)$$

Where, GHI is the Global Horizontal Irradiance (Rn), retrieved from the World Bank solar irradiance project (Suri *et al.* 2020).

APAR is the available energy in vegetation for photosynthesis that is calculated each month and the product of PAR surface irradiance and the fraction of photo-synthetically active radiation (FPAR).

$$APAR = FPAR \times PAR \quad (4)$$

According to Field (1995), biomass is the function of APAR and light use efficiency (LUE). In practice, it is impacted by environmental variables like temperature and vapor pressure deficit (VPD), which can be derived from meteorological factors (Eq. 5).

$$\varepsilon \; or \; LUE = \varepsilon^{\circ} \times T_1 \times T_2 \times W \; (g/MJ) \quad (5)$$

Where T_1 and T_2 represent the effect of high and low temperatures on LUE i.e., relate to plant growth regulation (acclimation) by temperature; W represents the effect of moisture on LUE; and ε° represents the maximum light utilization rate under ideal conditions which is globally uniform maximum 2.5 g/MJ. The T_1 denotes the depressant effect on the Net Primary Product (NPP) of high and low temperatures restricting the process of photosynthesis.

Table 3 Landsat 8 data source and resolution used to estimate aboveground biomass in four forest types in Bangladesh

Forest Type	Satellite Data	Spatial Resolution (m)	Acquisition Date
Hill Forest	Landsat-8 OLI/TIRS C2 Level 2	30×30	02.03.2014 & 02.13.2020 (Path: 136, Row: 45)
Nijhum Dwip Forest	Landsat-8 OLI/TIRS C2 Level 2	30×30	02.03.2014 & 02.04.2020 (Path: 136, Row: 45)
Madhupur Sal Forest	Landsat-8 OLI/TIRS C2 Level 2	30×30	01.25.2014 & 02.11.2020 (Path: 137, Row: 43)
Sundarbans Reserve Forest	Landsat-8 OLI/TIRS C2 Level 2	30×30	01.14.2014 & 02.13.2020 (Path: 137, Row: 45)

The plant acclimation by temperature (i.e., T_1 and T_2) and effect of moisture on LUE i.e., W which is the function of the ratio of Estimated Evapotranspiration (EET) and Potential Evapotranspiration (PET) were estimated by following (Eq. 6), (Eq. 7) and (Eq. 8) (Field *et al.* 1995; Potter *et al.* 1993).

$$T_2 = C \left\lfloor \frac{1}{1+exp^{(0.2 \times T_{OPT}-10-T_{mon})}} \right\rfloor \times \left\lfloor \frac{1}{1+exp^{(-0.3 \times T_{OPT}-10+T_{mon})}} \right\rfloor \quad (6)$$

Where:

T_{opt} = Mean temperature during the month of maximum NDVI in 2014 and 2020. Islam and Mamun (2015) found October has the maximum NDVI for Bangladesh and the mean temperature of this month was 26.4 ^{0}C in 2014 and 31.2 ^{0}C in 2020 (BMD 2020).

T_{mon} = Mean monthly air temperature in 2014 and 2020. Based on satellite image acquisition, our study used average mean air temperature for January and February as 19.41 ^{0}C for 2014 and 22.12 ^{0}C for 2020 (BMD 2020).

C = constant = 1.185

And,

$$T_1 = 0.8 + 0.02 \times T_{opt} - 0.0005 \times (T_{opt})^2 \quad (7)$$

$$W = 0.5 + \frac{EET}{PET} \quad (8)$$

PET is the function of temperature and latitude while EET is the function of precipitation (P) and net solar irradiance (Rn), which is being converted to the equivalent evaporation through specific heat of water Field *et al.* (1995). When EET exceeds PET, NPP is no longer restricted by water. This study used a coefficient value of 0.85 for water scaler (W) from a study in Bangladesh (Islam and Alam 2021). Therefore, the amount of biomass was estimated from the ratio amount of APAR and PAR (or NDVI) multiplied by LUE that helps plant biomass increment (Eq. 9).

$$Biomass = APAR \times LUE\ (g/m^2) \quad (9)$$

The amount of biomass content per m^2 was estimated based on the number of pixels of clusters of Landsat-8 OLI/TIRS C2 Level 2, 30 m sensor multiplied by the squared resolution of the image. The estimated biomass was converted to megagrams per hectare (Mg.ha^{-1}), divided into four groups, and then mapped using the "natural break" method.

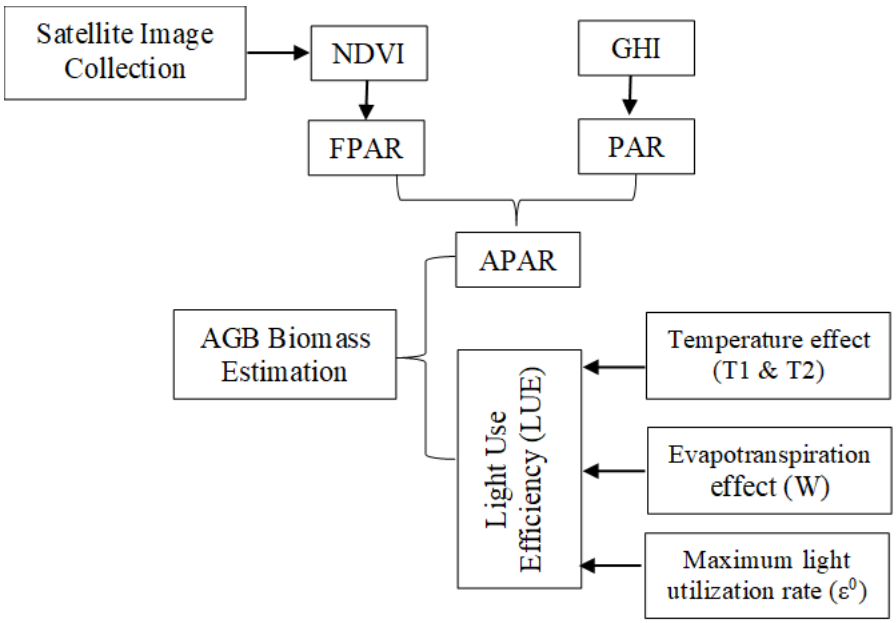

Figure 2 Simplified workflow of satellite-based aboveground forest biomass estimation

RESULTS AND DISCUSSION

The average AGB content per hectare in Sundarbans Reserve Forest slightly increased from 89.73 $Mg.ha^{-1}$ in 2014 to 90.76 $Mg.ha^{-1}$ in 2020 (Table 4), but the changes varied across the forest. The maximum AGB content increased (4%) at Sharankhola Range from 98.13 $Mg.ha^{-1}$ to 102.36 $Mg.ha^{-1}$ and decreased (2%) at Chandpai Range from 81.76 $Mg.ha^{-1}$ in 2014 to 80.0 $Mg.ha^{-1}$ in 2020 respectively (Figure 3a and Figure 3b). According to Global Forest Watch (GFW 2022), Bagerhat and Khulna lost 0.05% of tree cover during 2000-2021 which resulted in 64.9 MT CO_2 and 13.9 MT CO_2 emissions respectively. Sundarbans Reserve Forest is under pressure due to the high dependency of local poor communities on its natural resources for their survival and livelihoods and the current unsustainable management practices (Mahmood *et al.* 2021). One step to address this was conducted by the Forest Department and other partners by planting around 290,000 ha of coastal mangrove forests by involving local stakeholders via a co-management method (SER 2022).

In contrast, the Madhupur Sal Forest in Tangail district lost around 14,000 ha (9.1%) of tree cover from 2001 to 2021 which released 555 MT of CO_2 emissions to the atmosphere (GFW 2022). The average AGB content per hectare in Madhupur Sal Forest has decreased from 110.01 $Mg.ha^{-1}$ in 2014 to 107.22 $Mg.ha^{-1}$ in 2020 respectively but changes also varied across this area. The maximum AGB content increased (16%) at Golabari from 118.54 $Mg.ha^{-1}$ to 137.66 $Mg.ha^{-1}$ and decreased at Mirzabari union (14%) from 111.24 $Mg.ha^{-1}$ in 2014 to 95.35 $Mg.ha^{-1}$ in 2020 respectively (Figure 4a and Figure 4b). Illegal felling and settlement, grazing, encroachment, industrial and agricultural expansions, agroforestry (banana, pineapple, garlic, zinger, etc.), and firing range practice by the military are found as the underlying causes of deforestation in Madhupur Sal Forest (Mollick *et al.* 2018; Rahman *et al.* 2009). Cox's Bazar lost around 9,600 ha (22%) of its forest cover during the last two decades (2000-2021) resulting in 4.6 MT CO_2 emissions (GFW 2022).

Similar to the Madhupur Sal Forest, the average per hectare AGB content in the Ukhia hill forest also decreased. The maximum AGB content in Jalia Palong union increased approximately doubled from 4.08 $Mg.ha^{-1}$ to 8.17 $Mg.ha^{-1}$ but the biomass density numbers remain low in this area. The biomass in the rest of this region remained the same or increased slightly from 2014 to 2020 (Figure 5a and Figure 5b). Ullah *et al.* (2022) identified overpopulation, agricultural and industrial extensions, poverty, unemployment, and illegal logging as the proximate drivers of deforestation in the Teknaf area. Activities contributing to these changes include excessive timber and fuelwood extraction, infrastructure extension, agricultural expansion, encroachment, and betel leaf cultivation (Rashid 2017). Meanwhile, the influx of Rohingya refugees from Myanmar since 2017 resulted in the loss of 3,238 ha. This influx induced deforestation in Ukhiya, The Forest Department (FD) and non-governmental organizations restored 2,600 ha area along with 600 ha replanted by Rohingya people by 2018 (Khan 2022). Even though afforestation programs are being initiated to restore these areas, coastal forests are still threatened by land erosion, illegal harvesting of plant resources, tropical natural calamities as well, and conversion of forest land into agricultural land (Chowdhury *et al.* 2020; Islam *et al.* 2021). Noakhali lost 322 ha of tree cover from 2000 to 2021 which led to emissions of 52.5 MT CO_2 in the atmosphere (GFW 2022). Nijhum Dwip Forest also lost average AGB per hectare (16%) from 44.36 $Mg.ha^{-1}$ in 2014 to 37.46 $Mg.ha^{-1}$ in 2020 (Figure 6a and Figure 6b).

Table 4 Aboveground biomass content in 2014 and 2020 in four different forest types (in parentheses below forest name) and the associated areas of the forest of Bangladesh

Forest type and union names	2014 (Mg.ha^{-1})	2020 (Mg.ha^{-1})
Sundarban Mangrove Forest		
(Natural Mangrove)		
Khulna Range	84.37	84.90
Sharankhola Range	98.12	102.36
Nalian Range	94.64	95.78
Chandpai Range	81.76	80.00
Average	*89.73*	*90.76*
Madhupur Sal Forest		
(Plain Land Sal Forest)		
Alokdia	99.87	99.87
Solakuri	108.58	102.75
Ausnara	108.88	100.29
Golabari	118.54	137.66
Mirzabari	111.24	95.35
Madhupur Paurashava	114.46	114.46
Arankhola	108.53	100.18
Average	*110.01*	*107.22*
Ukhia Hill Forest		
(Hill Forest)		
Jalia Palong	4.08	8.17
Palong Khali	3.03	3.25
Raja Palong	10.42	10.93
Ratna Palong	11.13	11.13
Haldia Palong	10.77	10.77
Average	*7.89*	*8.85*
Nijhum Dwip Forest ***(Mangrove Plantation)***	44.36	37.46

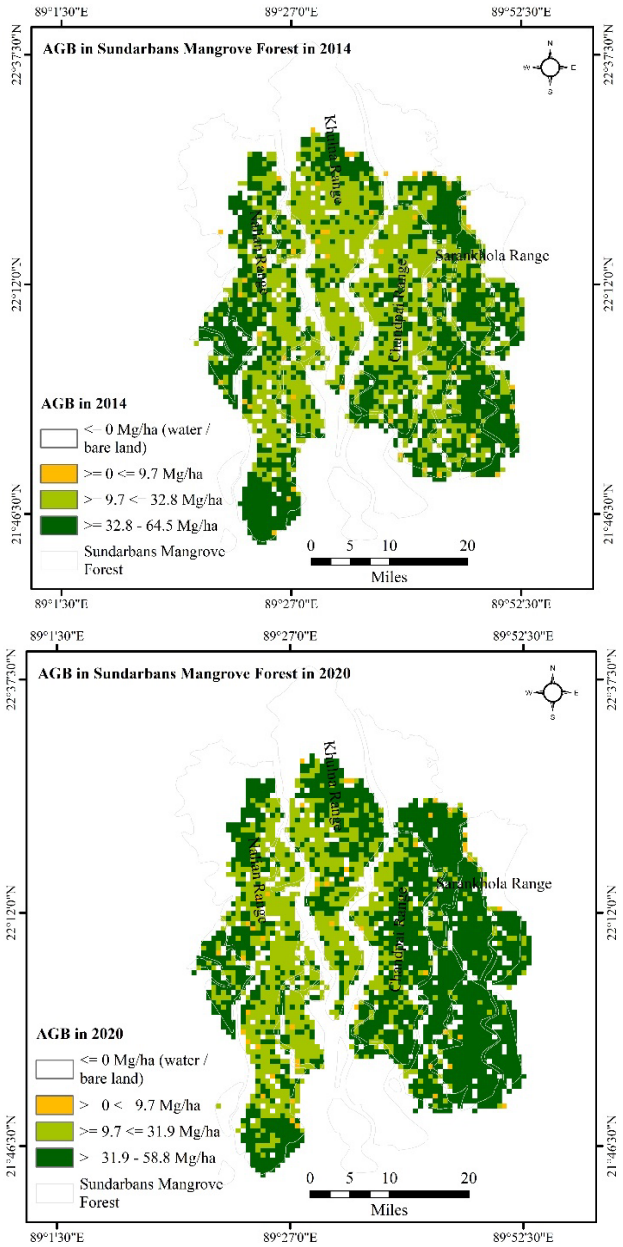

Figure 3 Spatial distribution of biomass (Mg.ha^{-1}) in the Sundarbans Mangrove Forest (a) 2014 (b) 2020. Each pixel represents a 30 x 30 m area

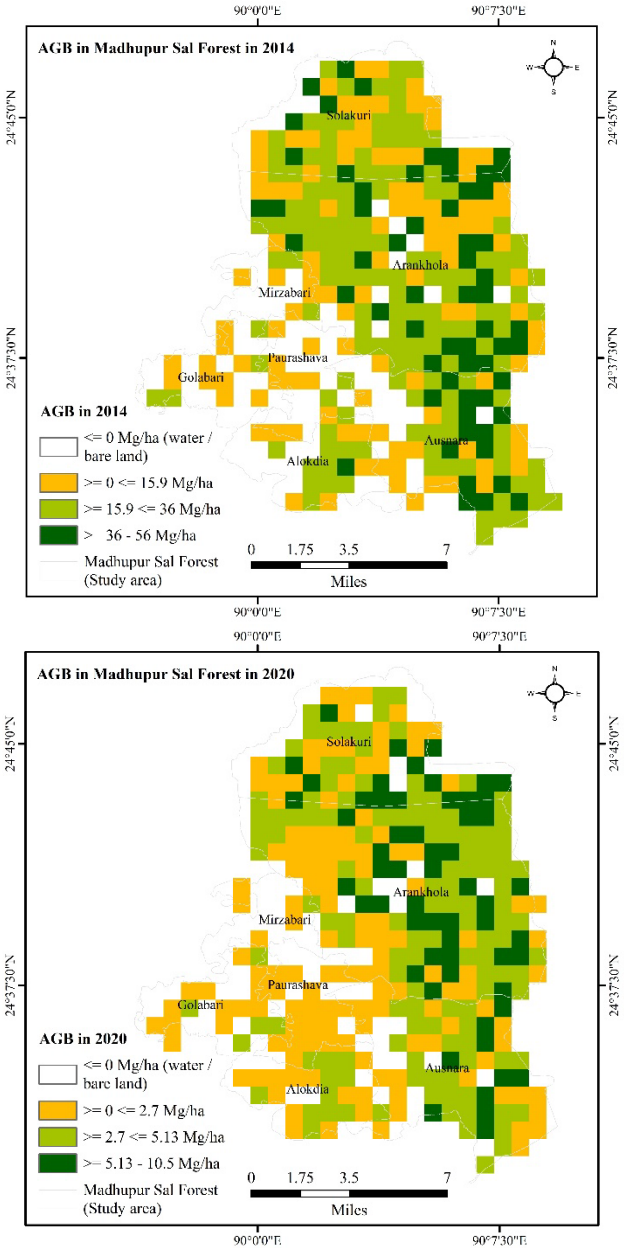

Figure 4 Spatial distribution of biomass (Mg.ha^{-1}) in Madhapur Sal Forest (a) 2014 (b) 2020

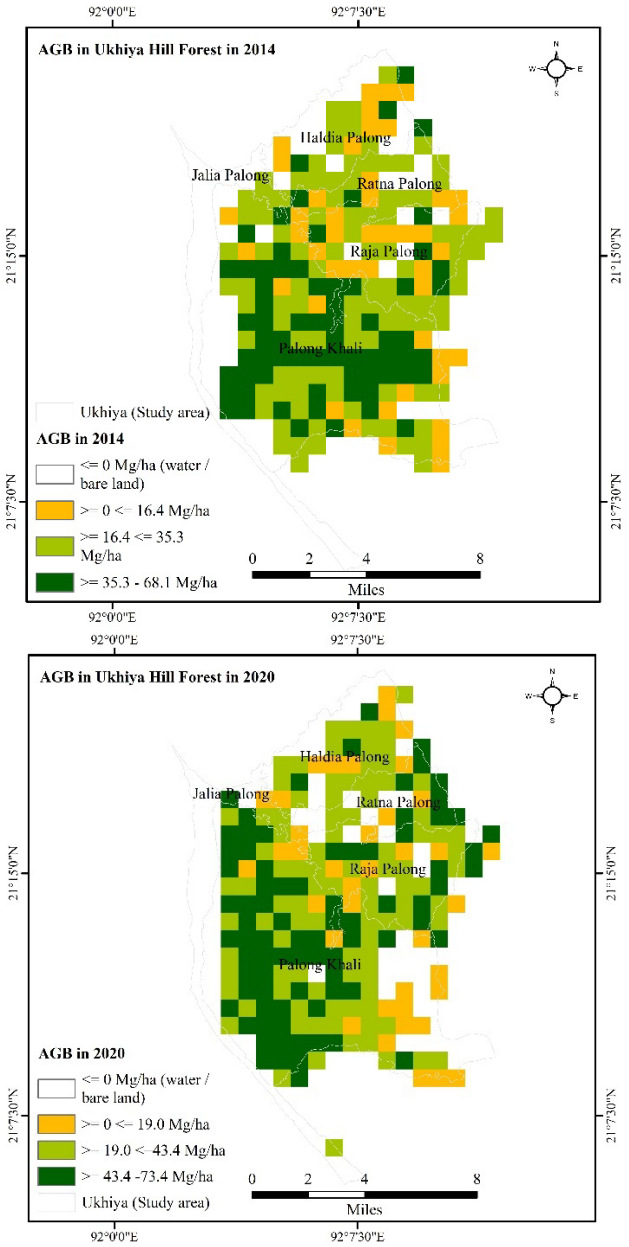

Figure 5 Spatial distribution of biomass (Mg.ha^{-1}) in Ukhia Hill Forest (a) 2014 (b) 2020

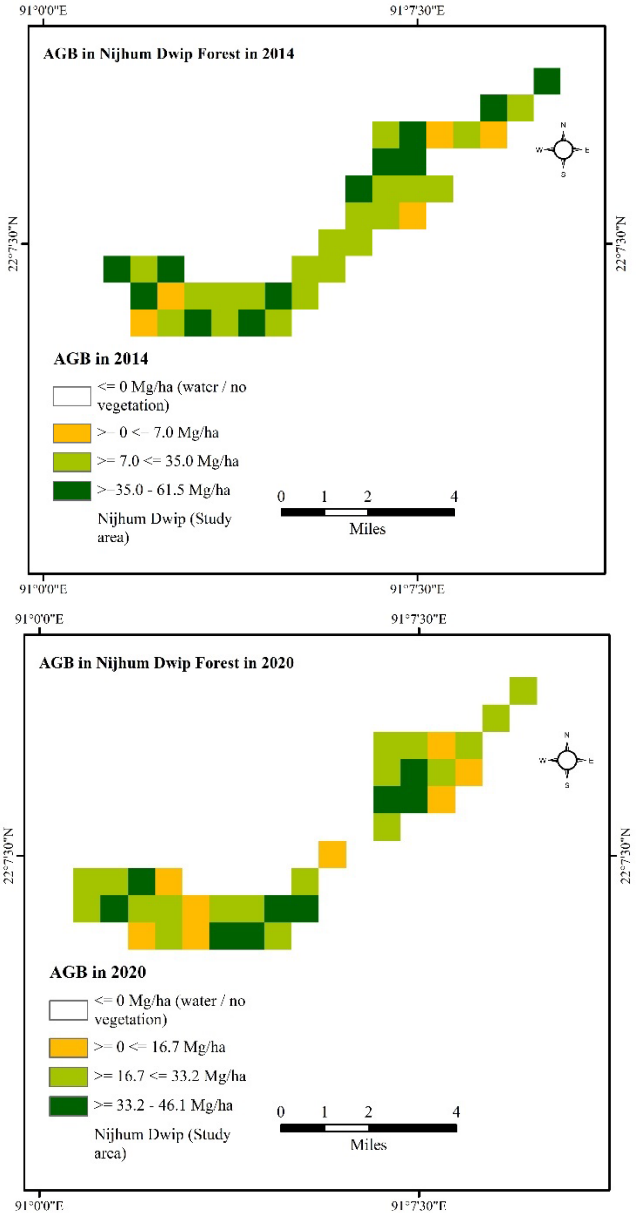

Figure 6 Spatial distribution of biomass (Mg.ha^{-1}) in Nijhum Dwip Forest (a) 2014 (b) 2020

In the four forests assessed in this study, the estimated average AGB in 2014 was about 63 Mg.ha^{-1} (minimum 3.03 Mg.ha^{-1} to maximum 118.54 Mg.ha^{-1}) and it dropped to 61 Mg.ha^{-1} (minimum 3.25 Mg.ha^{-1} to maximum 137.7 Mg.ha^{-1}) in 2020. The average minimum AGB stock was observed in the hill forest with estimated values of 7.89 Mg.ha^{-1} and 8.85 Mg.ha^{-1} while the average maximum stock reported was 110.01 Mg.ha^{-1} and 107.22 Mg.ha^{-1} during both years of estimation.

The average AGB stock in the forests of Bangladesh was 63 Mg.ha^{-1} in 2014 and 61 Mg.ha^{-1} in 2020, which are lower than other reported values of 145.47 Mg.ha^{-1} (BFD 2020), 159.5 to 360 Mg ha^{-1} (Rahman *et al.* 2015), 49 to 121 Mg.ha^{-1} (Alamgir and Turton 2013), 158 Mg.ha^{-1} (Brown *et al.* 2001), 93 Mg.ha^{-1} (Eggleston *et al.* 2006), 92 Mg.ha^{-1} (Brown 1997), 137 Mg.h^{-1} (DeFries *et al.* 2002), and 175.5 Mg.ha^{-1} (Mukul *et al.* 2014). However, the estimation is very close to a satellite-based study conducted by Saatchi *et al.* (2011), which reported a value was 70.5 Mg.ha^{-1}. Other inventory or harvest data-based studies observed similar values like Gibbs *et al.* (2007) reported 65 Mg ha^{-1}, 55 Mg.ha^{-1} by FAO (2010), and 62 Mg.ha^{-1} by Rahman (2021) (Table 5). In addition to differences in methods used for these assessments, this study only examined four representative forests in Bangladesh so does not capture the forest biomass across all the forested land in the country.

Table 5 Biomass estimations and methods for Bangladesh from different studies

Study	Study type	Biomass stock (Mg.ha^{-1})
Present study	Satellite (2014 image)	63
Present study	Satellite (2020 image)	61
BFD (2020)	Inventory	145.47
Rahman *et al.* (2015)	Inventory	159.5–360
FAO (2010)	Inventory	55
Alamgir and Turton (2013)	Inventory	49-121
Saatchi *et al.* (2011)	Satellite	70.5
Brown *et al.* (2001)	Harvest data	158
Rahman (2021)	Inventory	62
Gibbs *et al.* (2007)	Harvest data	65
Eggleston *et al.* (2006)	Harvest data	93
Brown (1997)	Inventory	92
DeFries *et al.* (2002)	Harvest data	137
Mukul *et al.* (2014)	Literature based modeling	175.5

This study found a low amount of biomass stock in the hill forests of Bangladesh compared with 115.6 Mg.ha^{-1} and 103.4 Mg.ha^{-1} reported values by Ullah and Al-Amin (2012) and Mukul *et al.* (2016). A huge variation in AGB stock in Madhupur Sal Forest compared with 153.9 Mg.ha^{-1} and 34.5 Mg.ha^{-1} was estimated by Kibria and Saha (2011) and Mukul *et al.* (2014). However, the AGB stock for Sundarbans Mangrove Forest reported values by Mukul *et al.* (2014) and Rahman (2021) as 88.2 Mg.ha^{-1} and 98.9 Mg.ha^{-1} which are very close to the present study. Similarly, the estimated biomass value of Nijhum Dwip Forest is also very close to the 19.6 Mg.ha^{-1} reported value by Mukul *et al.* (2014) (Table 6). The variations might be due to differences in methods, areas studied like north-eastern versus south-eastern hill forest areas, as well as month and year of satellite data acquisition. For example, NDVI concentration remains similar in a season (Tripathi *et al.* 2010) but changes between months with the lowest values in February and the highest in October (Islam and Mamun 2015). This study used satellite images for January and February i.e., the winter season, which might contribute to the lower estimation. Moreover, higher spatial resolution, such as that used in this study (30 m × 30 m), has limitations, such as the potential inability to estimate finer changes over time, the incapability to estimate biomass under dense canopies (Lu *et al.* 2012), variation in estimation due to sensor angles in challenging terrain with steep slopes, and the potential to be affected by moisture content variation of the surrounding atmosphere. The accuracy of these results can be increased in future studies by adding other data sources, such as extended band SAR, LiDAR, high spatial resolution, and auxiliary data for AGB estimates (Li *et al.* 2020).

Table 6 Aboveground biomass in different forest types of Bangladesh

Study	Hill Forest (Mg.ha^{-1})	Sundarbans Mangrove Forest (Mg.ha^{-1})	Madhupur Sal Forest (Mg.ha^{-1})	Nijhum Dwip Forest (Mg.ha^{-1})
Mukul *et al.* (2014 a)	49. 5	88.2	34.5	19. 6
Mukul *et al.* (2014 b)	103.4	-	-	-
Ullah and Al-Amin (2012)	115.6	-	-	-
Alamgir and Al-Amin (2007)	73.6	-	-	-
Shin *et al.* (2007)	92.0	-	-	-
Rahman *et al.* (2015)	-	98.9	-	-
Donato *et al.* (2011)	-	126.7	-	-
Kibria and Saha (2011)	-	-	153.9	-
BFD (2020)	24.03	49.28	31.36	21.79
Present study, 2020	8.85	90.76	107.22	37.46
Present study, 2014	7.89	89.73	110.01	44.36

CONCLUSION

This satellite-based study found considerable variation in the aboveground biomass among the four different forest types of Bangladesh. It showed that average AGB stock increased in Sundarbans Reserve Forrest and Ukhiya Hill Forest while decreasing in Nijhum Dwip Forest and Madhupur Sal Forest. Bangladesh has set a target to stop forest losses and increase forest cover from 22.37% to 24%, reforest 150,000 ha of coastal areas, and restore 137,800 ha of destroyed and 200,000 ha of degraded hill and Sal Forest by 2030 (MoEFCC 2021). This study helps to identify areas under human pressure and could be used to identify priority areas for action.

This study had a lower estimate of AGB than some other studies (e.g., 159.5 to 360 $Mg.ha^{-1}$ by Rahman *et al.* (2015)). These studies' estimates were very close to studies with similar inventory or harvest data-based methods (e.g., Saatchi *et al.* (2011)) but there was wide variation with other methods. Better agreement between methods and an improvement in data quality can be enhanced by the replication of the study and including ground truthing in the study areas, especially for the dominant species like *Nypa fruticans* for Sundarbans Reserve and Nijhum Dwip Forest or *Shorea robusta* for Madhupr Sal Forest. Bangladesh needs an accurate national level assessment of forests using both satellite and inventory based methods that can be used to improve forest management and aforestation planning to achieve nationally determined contribution (NDC) goals by 2030.

Our research will be useful in determining how the AGB biomass of forests under human pressure has changed over time. The results may be used to compare the change, which will help to determine the root reasons for the AGB change. Identifying these areas and the associated causes of the change can guide the organizations and agencies who are involved in protecting and managing these areas to develop targeted plans to reduce forest loss in the future.

REFERENCES

Akhtar, N., Uddin, M. K., & Tan, Y. (2022). Remote sensing-based changes in the Ukhia Forest, Bangladesh. *GeoJournal*, *87*(5), 4269-4287. https://doi.org/10.1007/s10708-021-10494-3

Akhter, M., Mahmood, H., Birigazzi, B., & Matieu, H. (2013). *Tree volume and biomass allometric equations of Bangladesh* (Inventory No. 10.13140/ RG.2.2.29295.97445) (p. 33). Dhaka, Bangladesh: Bangladesh Forest Department and Food and Agriculture Organization of the United Nations. https://www.researchgate.net/publication/351390832_Tree_volume_and_biomass_allometric_equation_in_Bangladesh. Accessed 8 August 2023.

Al Faruq, M. A., Zaman, S., & Katoh, M. (2016). Analysis of forest cover changes using Landsat satellite imagery: a case study of the Madhupur Sal forest in Bangladesh. *Journal of Forest Planning, 21*(2), 29-38.

Alamgir, M., & Al-Amin, M. (2007). Organic carbon storage in trees within different Geopositions of Chittagong (South) Forest Division, Bangladesh. *Journal of Forestry Research*, *18*, 174-180.

Alamgir, M., & Turton, S. M. (2013). Climate change and organic carbon storage in Bangladesh forests. *Climate change and plant abiotic stress tolerance*, 881-902.

Ali, I., Greifeneder, F., Stamenkovic, J., Neumann, M., & Notarnicola, C. (2015). Review of machine learning approaches for biomass and soil moisture retrievals from remote sensing data. *Remote Sensing*, *7*(12), 16398-16421.

Altrell, D., Saket, M., Lyckebäck, L., & Piazza, M. (2007). *National forest and tree resources assessment 2005-2007, Bangladesh* (Assessment) (p. 280). Dhaka, Bangladesh: Ministry of Environment and Forest (MoEF), Bangladesh and Food and Agriculture Organization of the United Nations (FAO). https://www.fao.org/forestry/15466-0af9a225183f27cf84c440a6a1bf90d6e.pdf. Accessed 2 August 2023.

Angelsen, A. (2009). *Realising REDD+: National strategy and policy options*. Bogor, Indonesia: Centre for International Forestry Research.

Aziz, A., & Paul, A. (2015). Bangladesh Sundarbans: Present Status of the Environment and Biota. *Diversity*, *7*(3), 242-269. https://doi.org/10.3390/d7030242

BFD. (2017). Natural Mangrove Forest (Sundarbans). *Bangladesh National Portal: Forest Department, Government of the People's Republic of Bangladesh*. Government Database. http://www.bforest.gov.bd/site/page/19d63ffe-01e1-4351-b85b-3b60811b87f7/-. Accessed 3 August 2023.

BFD. (2020). *Tree and Forest Resources of Bangladesh: Report on the Bangladesh Forest Inventory* (Assessment No. GCP/BGD/058/USA) (p. 213). Dhaka, Bangladesh: Forest Department, Ministry of Environment, Forest and Climate Change, Government of the People's Republic of Bangladesh. http://bfis.bforest.gov.bd/bfi/. Accessed 2 August 2023.

BMD. (2020). Bangladesh Climate Data Portal. BMD: Bangladesh Meteorological Department, Dhaka, Bangladesh. http://bmd.wowspace.org/team/homex.php. Accessed 10 August 2023.

Brown, S. (1997). *Estimating biomass and biomass change of tropical forests: a primer* (Vol. 134). Food & Agriculture Org.

Brown, S., Iverson, L., & Prasad, A. (2001). Geographical Distribution of Biomass Carbon in Tropical Southeast Asian Forests: A Database (NPD-068). Environmental System Science Data Infrastructure for a Virtual Ecosystem; Carbon Dioxide Information Analysis Center (CDIAC), Oak Ridge National Laboratory, Oak Ridge, TN (USA). https://doi.org/10.3334/CDIAC/LUE.NDP068

Chowdhury, M. S. H., & Koike, M. (2010). An overview on the protected area system for forest conservation in Bangladesh. *Journal of Forestry research*, *21*, 111-118.

Chowdhury, S. U., Foysal, M., Shahadat, O., Prince, N. U., Mohsanin, S., & Islam, M. T. (2020). Globally threatened shorebirds of Nijhum Dwip National Park and management implications. *Wader Study Group Bulletin*, *127*, 244-251.

Costello, L., Piazza, M., Iqbal, Z., Nur Siddiqui, B., Akhter, M., Siddiqui, R., & Henry, M. (2016). *Experiences in Field Missions to Locate the Plots of the 2005 National Forest Assessment (NFA) of Bangladesh* (Assessment No. GCP/GD/058/USAID) (p. 13). Dhaka, Bangladesh: Bangladesh Forest Department (BFD) and Food and Agriculture Organization of the United Nations (FAO). http://bfis.bforest.gov.bd/library/wp-content/uploads/2018/12/38.pdf. Accessed 2 August 2023.

Deb, J., Phinn, S., Butt, N., & McAlpine, C. (2018). Climate change impacts on tropical forests: identifying risks for tropical Asia. *Journal of Tropical Forest Science*, *30*(2), 182-194.

DeFries, R. S., Houghton, R. A., Hansen, M. C., Field, C. B., Skole, D., & Townshend, J. (2002). Carbon emissions from tropical deforestation and regrowth based on satellite observations for the 1980s and 1990s. *Proceedings of the National Academy of Sciences*, *99*(22), 14256-14261.

Donato, D. C., Kauffman, J. B., Murdiyarso, D., Kurnianto, S., Stidham, M., & Kanninen, M. (2011). Mangroves among the most carbon-rich forests in the tropics. *Nature geoscience*, *4*(5), 293-297.

Eggleston, H., Buendia, L., Miwa, K., Ngara, T., & Tanabe, K. (2006). 2006 IPCC guidelines for national greenhouse gas inventories.

FAO (Ed.). (2010). *Global forest resources assessment 2010: main report*. Rome: FAO. https://www.fao.org/3/i1757e/i1757e.pdf. Accessed 10 August 2023.

Field, C. B., Randerson, J. T., & Malmström, C. M. (1995). Global net primary production: combining ecology and remote sensing. *Remote sensing of Environment*, *51*(1), 74-88.

GFW. (2022). Forest Monitoring Designed for Action. *Global Forest Watch*. https://www.globalforestwatch.org/. Accessed 10 August 2023.

Gibbs, H. K., Brown, S., Niles, J. O., & Foley, J. A. (2007). Monitoring and estimating tropical forest carbon stocks: making REDD a reality. *Environmental research letters*, *2*(4), 045023.

Giri, C., Long, J., Abbas, S., Murali, R. M., Qamer, F. M., Pengra, B., & Thau, D. (2015). Distribution and dynamics of mangrove forests of South Asia. *Journal of environmental management*, *148*, 101-111.

Giri, C., Ochieng, E., Tieszen, L. L., Zhu, Z., Singh, A., Loveland, T., et al. (2011). Status and distribution of mangrove forests of the world using earth observation satellite data. *Global Ecology and Biogeography*, *20*(1), 154-159.

Hale, R. P., Wilson, C., & Bomer, E. (2019). Seasonal variability of forces controlling sedimentation in the Sundarbans National Forest, Bangladesh. *Frontiers in Earth Science*, 211.

Harris, N., & Gibbs, D. (2021). Forests absorb twice as much carbon as they emit each year. *World Resources Institute*. Forests. https://www.wri.org/insights/forests-absorb-twice-much-carbon-they-emit-each-year. Accessed 9 August 2023.

Hasan, M. E., Zhang, L., Mahmood, R., Guo, H., & Li, G. (2021). Modeling of Forest Ecosystem Degradation Due to Anthropogenic Stress: The Case of Rohingya Influx into the Cox's Bazar–Teknaf Peninsula of Bangladesh. *Environments*, *8*(11), 121. https://doi.org/10.3390/environments8110121

Henry, M., Iqbal, Z., Johnson, K., Akhter, M., Costello, L., Scott, C., et al. (2021). A multi-purpose National Forest Inventory in Bangladesh: design, operationalisation and key results. *Forest Ecosystems*, *8*(1), 12. https://doi.org/10.1186/s40663-021-00284-1

Hossain, F., & Moniruzzaman, Dr. Md. (2021). Environmental change detection through remote sensing technique: A study of Rohingya refugee camp area (Ukhia and Teknaf sub-district), Cox's Bazar, Bangladesh. *Environmental Challenges*, *2*, 100024. https://doi.org/10.1016/j.envc.2021.100024

Hossain, K. M., & Haider, R. M. (2020). *Assessment Report on Plantation/Reforestation 2019 at Rohingya Camp, Cox's Bazar, Final Draft*.

Hossain, K. T., Tanim, I. A., & Salauddin, M. (2016). Change detection of forest cover: a study Nijhum Dwip National Park, Hatiya, Noakhali. *Journal of Life and Earth Sciences, Jagannath University*, *2*(2), 54-90.

Hu, Z., Wu, G., Zhang, L., Li, S., Zhu, X., Zheng, H., *et al.* (2017). Modeling and Partitioning of Regional Evapotranspiration Using a Satellite-Driven Water-Carbon Coupling Model. *Remote Sensing*, *9*(1), 54. https://doi.org/10.3390/rs9010054

Islam, M. M., & Mamun, M. M. I. (2015). Variations of NDVI and its association with rainfall and evapotranspiration over Bangladesh. *Rajshahi University Journal of Science and Engineering*, *43*, 21-28.

Islam, Md. R., Khan, Md. N. I., Khan, Md. Z., & Roy, B. (2021). A three decade assessment of forest cover changes in Nijhum dwip national park using remote sensing and GIS. *Environmental Challenges*, *4*, 100162. https://doi.org/10.1016/j.envc.2021.100162

Islam, Md. Y., Nasher, N. M. R., Karim, K. H. R., & Rashid, K. J. (2023). Quantifying forest land-use changes using remote-sensing and CA-ANN model of Madhupur Sal Forests, Bangladesh. *Heliyon*, *9*(5), e15617. https://doi.org/10.1016/j.heliyon.2023.e15617

Islam, S., & Alam, A. R. (2021). Performance evaluation of FAO Penman-Monteith and best alternative models for estimating reference evapotranspiration in Bangladesh. *Heliyon*, *7*(7).

Khan, Ms. N. (2022, February 9). Involving Rohingya refugees in reforestation in Bangladesh to reduce disaster risks. *United Nations Office for Disaster Risk Reduction (UNDRR)*. Analysis. https://www.undrr.org/news/involving-rohingya-refugees-reforestation-bangladesh-reduce-disaster-risks. Accessed 10 August 2023.

Khan, N. A., & Millate-e-Mustafa, M. (2001, September 16). The State Of Forestry In Bangladesh. *The Bangladesh Observer*, p. 6. Dhaka, Bangladesh. https://ruchichowdhury.tripod.com/state_of_forestry_in_bangladesh.htm. Accessed 16 August 2023.

Kibria, M. G., & Saha, N. (2011). Analysis of existing agroforestry practices in Madhupur Sal forest: an assessment based on ecological and economic perspectives. *Journal of Forestry Research*, *22*, 533-542.

Köhl, M., Magnussen, S., & Marchetti, M. (2006). *Sampling methods, remote sensing and GIS multiresource forest inventory* (Vol. 2). Springer.

Li, Y., Li, M., Li, C., & Liu, Z. (2020). Forest aboveground biomass estimation using Landsat 8 and Sentinel-1A data with machine learning algorithms. *Scientific Reports*, *10*(1), 9952. https://doi.org/10.1038/s41598-020-67024-3

Littell, J. S., Oneil, E. E., McKenzie, D., Hicke, J. A., Lutz, J. A., Norheim, R. A., & Elsner, M. M. (2010). Forest ecosystems, disturbance, and climatic change in Washington State, USA. *Climatic change*, *102*, 129-158.

Lu, D., Chen, Q., Wang, G., Moran, E., Batistella, M., Zhang, M., *et al.* (2012). Aboveground Forest Biomass Estimation with Landsat and LiDAR Data and Uncertainty Analysis of the Estimates. *International Journal of Forestry Research*, *2012*, 1-16. https://doi.org/10.1155/2012/436537

Macdicken, K. G., Fao, R. eng, Jonsson, Ö., Pina, L. C. de, Maulo, S., Adikari, Y., *et al.* (2015). Global Forest Resources Assessment 2015: how are the world's forests changing? https://api.semanticscholar.org/CorpusID:134207584

Mahmood, H., Ahmed, M., Islam, T., Uddin, M. Z., Ahmed, Z. U., & Saha, C. (2021). Paradigm shift in the management of the Sundarbans mangrove forest of Bangladesh: Issues and challenges. *Trees, Forests and People*, *5*, 100094. https://doi.org/10.1016/j.tfp.2021.100094

Mahmood, H., Saha, C., Paul, N., Deb, S., Abdullah, S. M. R., Tanvir, Md. S. S. I., et al. (2021). The soil quality of the world's largest refugee campsites located in the Hill forest of Bangladesh and the way forward to improve the soil quality. *Environmental Challenges*, *3*, 100048. https://doi.org/10.1016/j.envc.2021.100048

Masson-Delmotte, V., Zhai, P., Pörtner, H.-O., Roberts, D., Skea, J., & Shukla, P. R. (2022). *Global Warming of 1.5 C: IPCC special report on impacts of global warming of 1.5 C above pre-industrial levels in context of strengthening response to climate change, sustainable development, and efforts to eradicate poverty*. Cambridge University Press.

MoEFCC. (2021). *Nationally Determined Contributions (NDCs) 2021 Bangladesh* (p. 37). Dhaka, Bangladesh: Ministry of Environment, Forest and Climate Change (MoEFCC), Government of the People's Republic of Bangladesh. https://unfccc.int/sites/default/files/NDC/2022-06/NDC_submission_20210826revised.pdf

Mollick, A. S., Rahman, M. K., Khan, M. N. I., & Sadath, M. N. (2018). Evaluation of good governance in a participatory forestry program: A case study in Madhupur Sal forests of Bangladesh. *Forest Policy and Economics*, *95*, 123-137.

Mukul, Sharif A, Biswas, S. R., Rashid, A. M., Miah, D., Kabir, E., Uddin, M. B., et al. (2014). A new estimate of carbon for Bangladesh forest ecosystems with their spatial distribution and REDD+ implications. *International Journal of Research on Land-use Sustainability*, *1*(1), 33-41.

Mukul, Sharif A, Herbohn, J., Rashid, A. M., & Uddin, M. B. (2014). Comparing the effectiveness of forest law enforcement and economic incentives to prevent illegal logging in Bangladesh. *International Forestry Review*, *16*(3), 363-375.

Mukul, Sharif Ahmed, Rashid, A. Z. M. M., & Khan, N. A. (2016). *Forest Protected Area Systems and Biodiversity Conservation in Bangladesh* (preprint). EARTH SCIENCES. https://doi.org/10.20944/preprints201611.0101.v1

Nishorgo Network. (2018). *Nijhum Dwip National Park* (Monitoring) (p. 2). Hatiya, Noakhali: USAID and Government of Bangladesh. https://nishorgo.org/project/nijhum-dweep-national-park/. Accessed 3 August 2023.

Nur, A., Nandi, R., Jashimuddin, M., & Hossain, M. A. (2016). Tree Species Composition and Regeneration Status of Shitalpur Forest Beat under Chittagong

North Forest Division, Bangladesh. *Advances in Ecology*, *2016*, 1-7. https://doi.org/10.1155/2016/5947874

Pan, Y., Birdsey, R. A., Fang, J., Houghton, R., Kauppi, P. E., Kurz, W. A., et al. (2011). A large and persistent carbon sink in the world's forests. *Science*, *333*(6045), 988-993.

Potter, C. S., Randerson, J. T., Field, C. B., Matson, P. A., Vitousek, P. M., Mooney, H. A., & Klooster, S. A. (1993). Terrestrial ecosystem production: A process model based on global satellite and surface data. *Global biogeochemical cycles*, *7*(4), 811-841.

Rahman, L. M. (2021). *Bangladesh National Convervation Strategy: Forest Resources* (Inventory) (p. 43). Dhaka, Bangladesh: Ministry of Environment, Forest and Climate Change, Government of the People's Republic of Bangladesh. http://bforest.portal.gov.bd/. Accessed 8 August 2023.

Rahman, M. M., Khan, M. N. I., Hoque, A. F., & Ahmed, I. (2015). Carbon stock in the Sundarbans mangrove forest: spatial variations in vegetation types and salinity zones. *Wetlands Ecology and Management*, *23*, 269-283.

Rahman, M., Nishat, A., & Vacik, H. (2009). Anthropogenic disturbances and plant diversity of the Madhupur Sal forests (Shorea robusta CF Gaertn) of Bangladesh. *International Journal of Biodiversity Science & Management*, *5*(3), 162-173.

Rashid, A. (2017). Literature Review of Gender dimensions in forest use, management, governance, land tenure arrangements and safeguards in support of REDD+ in Bangladesh. UN-REDD Bangladesh National Program.

Reddy, C. S., Pasha, S. V., Jha, C., Diwakar, P., & Dadhwal, V. (2016). Development of national database on long-term deforestation (1930–2014) in Bangladesh. *Global and Planetary Change*, *139*, 173-182.

Reza, A. A., & Hasan, M. K. (2019). Forest biodiversity and deforestation in Bangladesh: the latest update. *Forest degradation around the world*, 1-19.

Saatchi, S. S., Harris, N. L., Brown, S., Lefsky, M., Mitchard, E. T., Salas, W., et al. (2011). Benchmark map of forest carbon stocks in tropical regions across three continents. *Proceedings of the national academy of sciences*, *108*(24), 9899-9904.

Salam, M. A., Noguchi, T., & Koike, M. (1999). The causes of forest cover loss in the hill forests in Bangladesh. *GeoJournal*, *47*, 539-549.

Sarkar, S. K., Saroar, M., & Chakraborty, T. (2023). Navigating nature's toll: Assessing the ecological impact of the refugee crisis in Cox's Bazar, Bangladesh. *Heliyon*, *9*(7), e18255. https://doi.org/10.1016/j.heliyon.2023.e18255

Sellers, P., Meeson, B., Hall, F., Asrar, G., Murphy, R., Schiffer, R., et al. (1995). Remote sensing of the land surface for studies of global change: Models—algorithms—experiments. *Remote Sensing of Environment*, *51*(1), 3-26.

SER. (2022). Bangladesh: Mangrove Afforestation Programme of the National Forest Department. *Society for Ecological Restoration (SER)*. Analysis. https://www.ser-rrc.org/project/bangladesh-mangrove-afforestation-programme-of-the-national-forest-department/. Accessed 10 August 2023.

Shin, M. Y., Miah, M. D., & Lee, K. H. (2007). Potential contribution of the forestry sector in Bangladesh to carbon sequestration. *Journal of environmental management*, *82*(2), 260-276.

Sims, D. A., Luo, H., Hastings, S., Oechel, W. C., Rahman, A. F., & Gamon, J. A. (2006). Parallel adjustments in vegetation greenness and ecosystem CO2 exchange in response to drought in a Southern California chaparral ecosystem. *Remote Sensing of Environment*, *103*(3), 289-303. https://doi.org/10.1016/j.rse.2005.01.020

Sohel, Md. S. I., Islam, H. M. N., Newaz, K. Md. N., Khan, M. F. A., Sarker, G. C., & Bhuiyan, Md. S. R. (2023). Ecohydrological, climatic and tree architectural considerations for reforestation program using swamp vegetation of Bangladesh. *Ecohydrology & Hydrobiology*, *23*(1), 127-140. https://doi.org/10.1016/j.ecohyd.2022.12.005

Suri, M., Betak, J., Rosina, K., Chrkavy, D., Suriova, N., Cebecauer, T., et al. (2020). *Global Solar Atlas* (Assessment No. 149846) (p. 62). Washington DC: World Bank and Internation Finance Corporation. https://globalsolaratlas.info/map. Accessed 9 August 2023.

Tripathi, S., Soni, S. K., Maurya, A. K., & Soni, P. K. (2010). Calculating carbon sequestration using remote sensing and GIS. *Geospatial world*, 1-8.

Tucker, C. J. (1979). Red and photographic infrared linear combinations for monitoring vegetation. *Remote sensing of Environment*, *8*(2), 127-150.

Uddin, M. M., Mahmud, M. A. A., & Jannat, M. (2019). Impacts of mangrove plantations on land stabilization along the coastline in Bangladesh. *American Journal of Earth and Environmental Sciences*, *2*(1).

Ullah, M., & Al-Amin, M. (2012). Above-and below-ground carbon stock estimation in a natural forest of Bangladesh. *Journal of forest science*, *58*(8), 372-379.

Ullah, S. A., Tsuchiya, J., Asahiro, K., & Tani, M. (2022). Exploring the socioeconomic drivers of deforestation in Bangladesh: The case of Teknaf Wildlife Sanctuary and its surrounding community. *Trees, Forests and People*, *7*, 100167.

UNHCR. (2023, July 23). Rohingya Crises: Bangladesh. *The UN Refugee Agency*. Monitoring. https://data.unhcr.org/en/country/bgd. Accessed 4 August 2023.

Unni, N. M. (1983). Forest survey and management using remote sensing. *Proceedings of the Indian Academy of Sciences Section C: Engineering Sciences*, *6*, 209-232.

Yasmin, R., Wadud, M., Mandol, M., & Sharif, M. (2010). Tree diversity in the homestead and cropland areas of Madhupur Upazila under Tangail District. *J. Agrofor. Environ*, *4*(1), 89-92.

ORANGUTAN (*Pongo pygmaeus ssp. wurmbii*) RANGE PATTERN IN PUNGGUALAS, SEBANGAU NATIONAL PARK, CENTRAL KALIMANTAN, INDONESIA

ADVENTUS PANDA[1,2]*, ARI MEIDIDIT[3], OKTA SIMON[3], WAYAN TUNAS ARTAMA[4], DWI PRIYOWIDODO[5] AND TJUT SUGANDAWATY DJOHAN[6]

[1] *Doctorate Study Program, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*
[2] *Department of Biological Science, Faculty of Mathematics and Natural Science, University of Palangka Raya 73111, Indonesia*
[3] *Forum Orangutan Kalimantan Tengah (FORKAH Kalteng), Palangka Raya 73112, Indonesia*
[4] *One Health Colaborating Centre, Gadjah Mada University, Yogyakarta 55281, Indonesia*
[5] *Department of Parasitology, Faculty of Veterinary Medicine, Gadjah Mada University, Yogyakarta 55281, Indonesia,*
[6] *Laboratory of Ecology and Conservation, Faculty of Biology, Gadjah Mada University, Yogyakarta 55281, Indonesia*

Received 3 March 2023 / Revised 2 September 2023 / Accepted 5 September 2023

ABSTRACT

Punggualas is a logged-over forest in Sebangau National Park (SNP), serving as a primary stronghold for orangutan (*Pongo pygmaeus ssp. wurmbii)* population. Therefore, this study aimed to evaluate orangutan range patterns and distribution within Punggualas forest. The ranging data were collected inside the area of interest (AOI) from February 2015 to December 2019 (P1) and March 2020 to May 2022 (P2). The minimum convex polygon (MCP) area and Kernel Utilization Distribution (KernelUD) of orangutan were estimated using the adehabitatHR package in RStudio 4.2.3, with the Kernel Density (KD) tool in ArcGIS 10.5 used for visualization. The results showed that the largest range size, measuring 259.6 Ha, was observed in the mother-infant pairs throughout the investigation, surpassing the flanged male home range (HR) size of 250 Ha per year reported at the Natural Peat Laboratory, CIMTROP-UPR. The range pattern also varied significantly between the two study periods. During P1, the distribution area was positioned in the northern part of the AOI, shifting to the southeastern during. This indicated that *P. p. wurmbii* range pattern in Punggualas moved to the southeastern part of the grid trails, as shown by MCP and KernelUD. The probability of orangutans using random points across the study area, specifically for food, was adequate from the total basal area (m^2ha^{-1}) and tree species composition. The compression effect became evident as a potential determining factor contributing to rapid changes in range patterns during the study periods.

Keywords: epidemiology triad, kernel density estimation, minimum convex polygon, punggualas, sebangau national park

INTRODUCTION

The Bornean orangutan (*Pongo pygmaeus*) is comprised of three sub-species, namely *P. p. pygmaeus* (West Kalimantan and Sarawak, Malaysia), *P. p. wurmbii* (West Kalimantan and Central Kalimantan) and *P. p. morio* (East Kalimantan, extending north to include Sabah, Malaysia) (Ancrenaz *et al.* 2016). However, the conservation status of this charismatic wildlife on the IUCN Red List has reached the critically endangered (CR) level, indicating a severe and continuous population decline over the past 45 years. The contributing factor to this decline is habitat loss, which leads to habitat fragmentation and increases the number of poachers and killings of orangutan (Santika *et al.* 2017b; M. Voigt *et al.* 2018; Wich *et al.* 2012) and forest fires (Erb, Barrow, Hofner, Utami-Atmoko & Vogel 2018).

During the 1970 to 2000 period, Sebangau forest experienced logging concessions, with an influx of illegal activities in various forest areas, particularly in the Katingan Catchments, including Punggualas, until early 2005 when the government intervened with law enforcement actions. Both authorized logging concessions and

*Corresponding author, email: apanda@mipa.upr.ac.id

illegal logging activities have resulted in orangutan habitat loss and fragmentation in the Sebangau peat forest ecosystem. These past activities are evident in the form of semi-permanent railways and canal networks used to transport logs out of the forest, which are still visible in the areas or through high-resolution satellite imagery. During the logging concessions, the railways were used to access the targeted timber, making frequent return visits to log secondary species. However, canal is currently being used for alternative purposes such as granting access to non-timber forest products including jelutong tap, medicinal plants, or fishing harvesting in the interior forests. These developments have created the orangutan-human interface in Sebangau National Park (SNP).

Orangutan has the ability to adapt to human-dominated landscapes (Rayadin & Spehar 2015). However, these conditions are ideal for pathogenic organisms such as parasites, bacterial, and virus transmission, posing a threat to orangutan and human (Herrera, Chakraborty, Rushmore, Altizer & Nunn 2019; Nurcahyo, Konstanzová & Foitová 2017; Philippa & Dench 2019; Rondón *et al.* 2017). Several studies stated that the source of pathogenic transmission was from tourists, researchers, the local community (Woodford *et al.* 2003), and orangutan release, including translocation (Kilbourn *et al.* 2003; Mul *et al.* 2007). Furthermore, anthropogenic activities are increasingly implicated as drivers of pathogenic potential (Allen *et al.* 2017; Bengis *et al.* 2004; Daszak, Cunningham & Hyatt 2000; Han, Kramer & Drake 2016; Jones *et al.* 2008). The hypothesis of Maria Voigt *et al.* (2018) suggested that the absence of an explanation for reported orangutan deaths in their natural habitat, made it necessary to consider the existence and spread of deadly infectious diseases, as discovered in the group of apes in Africa. This leads to the consideration of the factors that determine the transmission of pathogenic agents such as parasites, bacteria, and viruses in wild orangutan.

Bordes, Morand, Kelt and Van Vuren (2009) reported that the important determinants of parasite transmission were host mobility and home range size. Mammals, such as orangutan, are host to a wide array of gastrointestinal parasites, often acquired through contact with infectious stages present in soil, feces, or vegetation, suggesting the influence of range behavior on their spread (Nunn, Thrall, Leendertz & Boesch 2011). The details on how range use behavior might influence the spread of fecally transmitted parasites are also described, specifically the term of "territoriality benefits" and the "fecal exposure" hypothesis. Furthermore, several field parasitologists have identified the important roles of home range in parasite dynamics (Foitová, Huffman & Wisnu 2009; Labes *et al.* 2010).

In this study, Burt home range definition as "that area traversed by the individual in its normal activities of food gathering, mating and caring for young" was used to prevent any misinterpretation (Burt 1943). These activities depend on the life stage, sex, and habitat of animals. Home range estimated by studies that are not very intensive, short, or restricted to a small area is considered to represent core areas (Singleton & Carel P. van Schaik 2001). This indicates that the core area of home range is the most frequently used by an animal.

A previous study showed that orangutans had fixed home range, spanning from 100 to 900 ha for females and up to 2,500 ha for adult males (Singleton, Knott, Morrogh-bernard, Wich & Schaik 2009). The peat swamp forests of Suaq Balimbing, Aceh, Sumatra, and Tanjung Puting National Park, Central Kalimantan, have the highest recorded home range. For example, a single female orangutan may require at least 1500 ha for self-support and their offspring, while males need more than 4000 ha. These ranges include a variety of habitats, offering unique benefits due to different fruiting patterns (Cattau, Husson, & Cheyne, 2015; Saputra, Perwitasari-Farajallah, Suci Utami-Atmoko, Ariyanto, & van Noordwijk, 2017). Several investigations have identified that the male range is significantly greater compared to females due to the food availability. Despite the increase in deforestation, the orangutan population has shown the opposite trend (Santika *et al.* 2017a; Maria Voigt *et al.* 2021, 2018; Wich *et al.* 2012).

The investigation into orangutan daily range size and pattern in Punggualas has been initiated by Rukmawardani & Imron (2021). The results study suggested understanding pattern and range

size positively benefit future forest rehabilitation. However, there is no information regarding the potential overlap between the vast area of orangutan and the subsistence activities of communities surrounding Punggualas. Therefore, this study aimed to estimate orangutan range size, pattern, and the determining factors influencing the range pattern shift in Punggualas forest, particularly in relation to food availability.

MATERIALS AND METHODS

Study locations

This study was conducted in Punggualas, a logged-over forest, covering approximately 1.320 Ha, located in the western part of SNP, as presented in Figure 1. This location is an active site for orangutan studies since 2010 and is allocated as a focal point of eco-tourism development for orangutans. From January to December 2018, tourist visit data to Punggualas reached a total of 260 visitors (Kartini, PEH TN Sebangau: Pers. Comm.).

Range Observations

Range data within the area of interest (AOI) was collected from Feb 2015 – Dec 2019 (P1) and Aug 2020 – May 2022 (P2). Due to the COVID-19 pandemic lockdown, the observations in 2020 were only conducted from August to September and continued from May to December 2021. A standardized method was used for orangutan studies, indicating the orangutans follow protocol (H. Morrogh-Bernard, Husson & McLardy 2002).

Behavioral data were collected during nest-to-nest focal animal follows, using two-minute instantaneous sampling to record activity and feeding. All observed behavior was recorded in 2-minute intervals and completed as the focal built night nest by a team of 2-3 personnel. Observations were terminated when individuals moved beyond the boundaries of the observation grid. Subsequently, names were assigned to individuals when consecutive days extended beyond four days, with their sex-age class as presented in the datasheets.

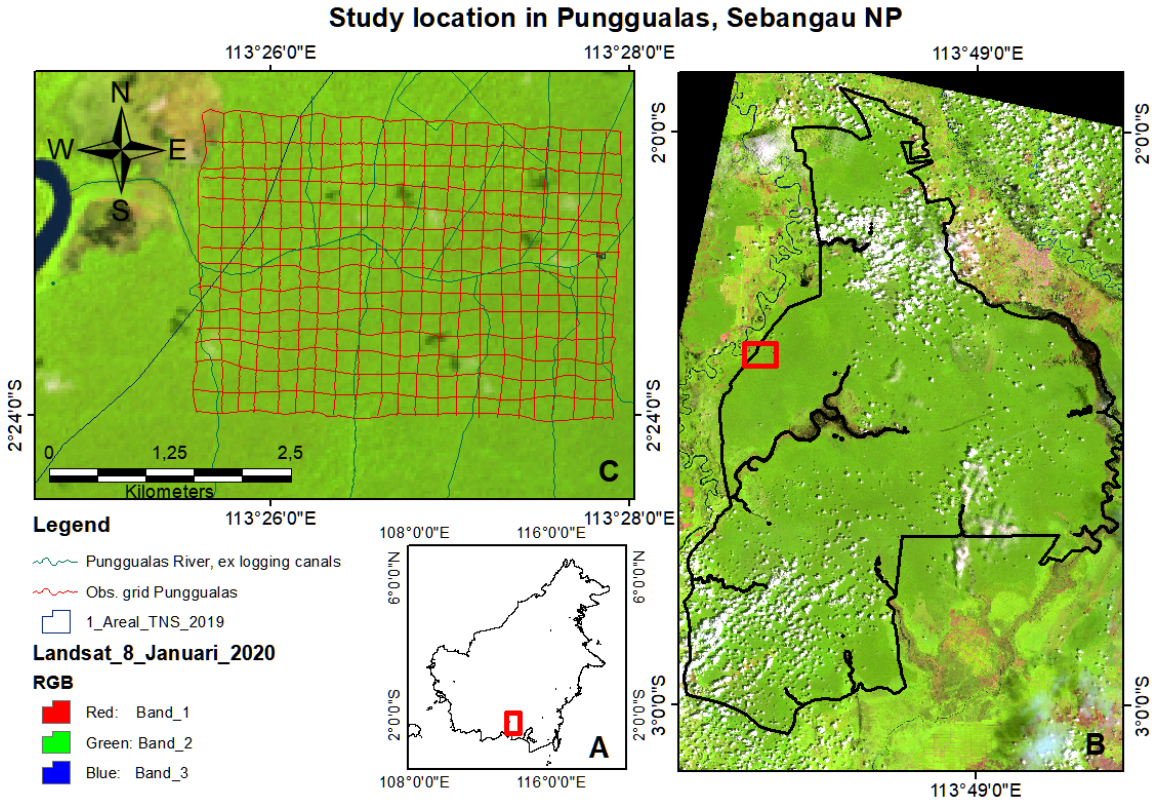

Figure 1 Map of the study area in SNP:(A) Borneo Island; (B) SNP; and (C) Orangutan observation grid in Punggualas

Range data were collected using a handheld GPS unit (Garmin GPS78) to record the location of the focal at 30-minute intervals. Regarding range size, the focus was extensively on nest-to-nest follows, without specifying additional criteria such as a minimum of 1000 hours followed for estimations. Subsequently, orangutan position points data were extracted, analyzed, and nest-to-nest observation of 21 orangutan were completed, as representative of three sex classes, consisting of two adolescent females (FA), five flanged-male (FM), and 13 mother-infant (MI), respectively. Table 1 describes individual estimated age, Home Range (HR) in (Ha), total hours followed, and habituation status.

Forest Block Sampling

A rapid assessment of the habitat structure was made to determine the comparativeness between the North and South Forest block, based on range results. The point quarter centered quadrat (PCQ) method was used to calculate basal tree area and important value index for each forest block during focal follows. A total of 20 points in the northern forest were assessed, while another 22 points were found in the southern points. The assessment commenced when the focal spent ≥ 10 minutes in a patch tree, as one observer continuously recorded the focal behavior, while the other two operated the PCQ method (Mitchell 2010). The patch tree was tagged during the assessment, followed by the documentation of canopy height and connectivity within a 10 m radius from the focal latest positions.

Data analysis

All range points, updated in each 30-minute interval, were conbined in the recapitulation matrix. Minimum convex polygon (MCP) and Kernel Utilization Distribution (KernelUD) were calculated using the adehabitatHR package in RStudio 4.2.3 (Posit 2023), where the core area was derived from KernelUD with a certain probability. Since this study did not infer specific focal, the matrix excluded range estimates for individuals per annum or seasonal. The distribution was visualized in ArcGIS 10.5 with Kernel Density (KD) tool, which allowed the differentiating of various parts of the animal range according to the intensity of use (Wartmann, Purves & van Schaik 2010). Vegetation data and calculation were carried out using Microsoft® Excel 2019, while graphical figure and color correction were performed using CorelDraw®2021. To identify the items associated with the highest loaded score for each parameter calculated from the PCQ method, Principal component analysis (PCA) was carried out using RStudio 4.2.3 (Posit 2023).

Table 1 Orangutan ID, estimated age, estimated Home Range (Ha), total hours followed, and their habituation status in the study periods

Phase	SC	Est_Age	Orangutan ID	Estimated HR (Ha)	Hours Followed	Remark
Phase-1	MI	24	Marlenda Martinus	241,82295	216	Habituated
Phase-1	MI	21	Jane Jack	192,84975	176	Habituated
Phase-1	FM	29	Brown	149,0408	152	Habituated
Phase-1	FM	22	Coded	123,5825	176	Habituated
Phase-1	MI	23	Vina Vino	123,565	144	Habituated
Phase-1	MI	21	Yulia Yani	108,43875	104	Habituated
Phase-1	MI	22	Nani Nina	92,56	192	Habituated
Phase-1	MI	21	Ibu Anak	84	26	Habituation
Phase-1	MI	26	Rere Reno	51,1972	120	Habituated
Phase-1	FM	21	Damang	37,16015	44	Habituated
Phase-1	FM	20	Kadir	8,49585	6	Habituated
Phase-1	MI	26	Anita_Anton	9	32	Habituated
Phase-2	FM	24	Brown	3	95	Habituated
Phase-2	AF	16	Adolescent_001	5	23	Habituation
Phase-2	AF	18	Adolescent_002	31,216686	99	Habituation
Phase-2	MI	25	Ibu_Anak	71,4011712	25	Habituation
Phase-2	MI	23	Ibu_Anak001	80,034119	83	Habituation
Phase-2	MI	20	IbuAnak003	51,125365	92	Habituation
Phase-2	MI	23	Ibu_anak_2020	26,548	44	Habituation
Phase-2	MI	20	Ibu_Anak_001	23	76	Habituation
Phase-2	FM	21	unk_flanged_male01	23,65411	34	Habituation

RESULTS AND DISCUSSION

Range Size

MCP area was calculated using a value of 80% to include the area used beyond the core home range.

This analysis yielded the estimated home range, in phases 1 and 2, as shown in Figure 2, with the range size of each individual varying from 20 to > 250 Ha. Despite the most representative sex classes, the mother-infant had the highest average range size, approximate (P1), exceeding 250 Ha (P2). A flanged male individual named Brown yielded 149.04 Ha (P1) and less than 5 Ha (P2). The mother-infant had a greater range size than the flanged-male in the same study periods, namely Kadir (FM) in Phase-1 and unk_flanged_male01(FM) in Phase-2. The varying results were attributed to the female behavior and range, which depended on reproductive state, particularly mating and caring for the young. The babysitting lasts for 6 to 8 years before the offspring become independent at the age of twelve (Scott, Knott & Susanto 2019). In these years, the mother imparts and mediates essential skills for the immature offspring (Noordwijk, Sauren, Morrogh-bernard, Atmoko & Schaik 2003). Secondly, the range use may reflect the patch sources, as fully discussed in the subsequent section.

The analysis showed relatively low range estimates for flanged-male but was not significantly small compared to previous studies. The results showed that male ranges are often larger than females, exceeding the grid trails of the study area (Wartmann *et al.* 2010). This indicated the tendency of female orangutans to stay close to their natal location (so-called philopatric), while male orangutan typically dispersed from this area. Based on the dataset, which showed the dyadic in most observations in both phases, also confirmed the co-existence of the philopatric.

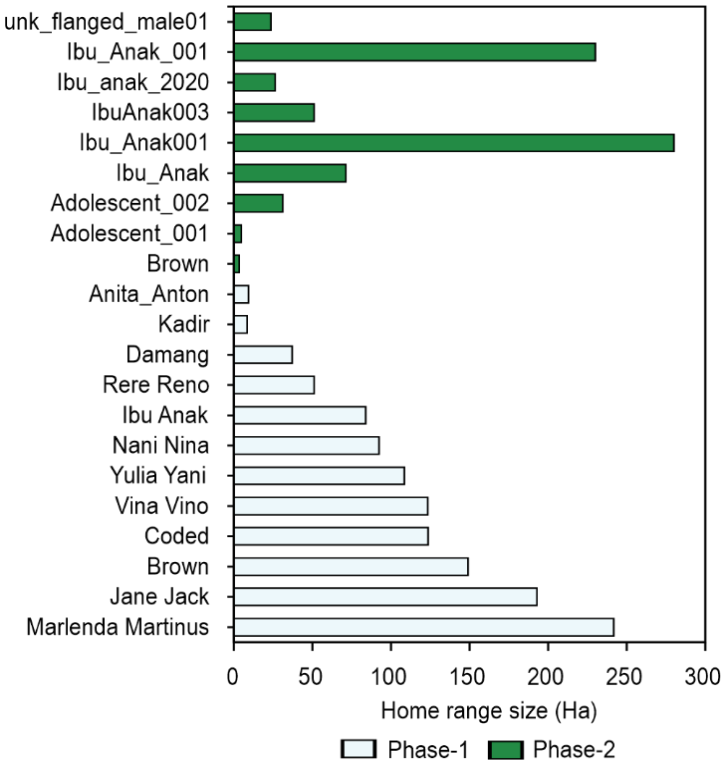

Figure 2 Home range size as per Sex-Classes (SC) in Phase-1 (2015-2019) and Phase-2 (2020-2022) derived from Minimum convex polygon calculations; names provided as appeared in GPS-code; Mother-Infant (MI), Flanged-Male (FM), and Adolescents Female (FA)

Figure 3 shows that the range overlap predominantly among mother-infant sex classes. As shown in Figure 3A, the 12 individuals in phase-1 from 2015 to 2019 were concentric, while in Figure 3B, observed individuals in phase-2 presented that the polygon was dispersed. Individual range that overlaps tend to oppose each other, as indicated by KernelUD, using the 'getverticehr' function for those in Figures 4A and 4B.

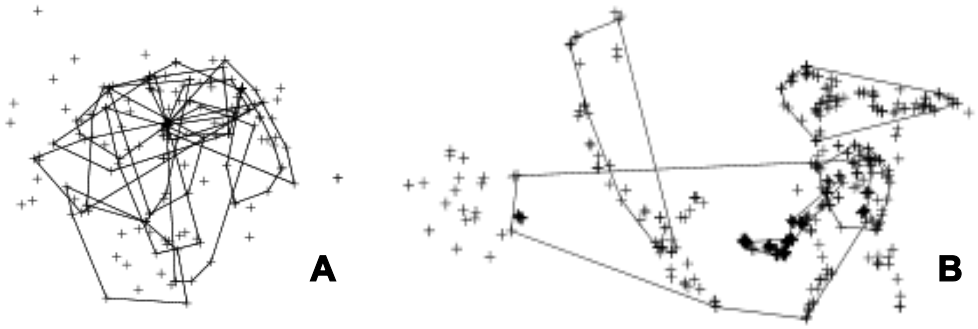

Figure 3 MCP per study periods; P1(A) and P2 (B)

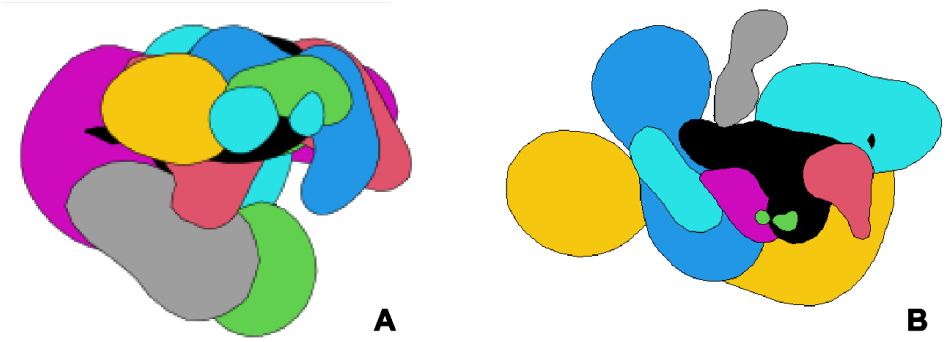

Figure 4 Kernel UD visualization using 'getverticehr' function for each observed individual during P1(A) and P2 (B)

An overlap is a common occurrence among *P. p. wurmbii* in the natural habitat (Wartmann *et al.* 2010). Previous investigations showed that apart from mother–infant dyads, *P. p. wurmbii* were semi-solitaire animals occupying highly overlapping home range. Female home range is assumed to be affected by ecological factors, reflecting the distribution of food sources. Meanwhile, male range use is a response to the distribution of female (Singleton *et al.* 2009; Suci *et al.* 2009), with the compression effect leading to orangutan favoring the fine-scale habitat (Helen C. Morrogh-Bernard, Husson, Harsanto & Chivers 2014; Helen Celia Morrogh-Bernard, Morf, Chivers & Krützen 2011).

According to individual levels, each subset was plotted based on their range size, as presented in Figures 5A and 5B, to determine the residency patterns, including 1) residents, who are consistently present for many years in a particular area, 2) commuters, observed regularly for several weeks or months each year for an extended period. This implied that commuters tended to result in home range with two separate core areas, namely multinuclear, and 3) wanderers, observed infrequently (or once) for at least 3 years, without returning to the area (Singleton & Carel P. van Schaik 2001). Based on this criterion, the only individual in Punggualas, who met the requirements is Brown, the 29-year-old Flanged Male. However, the identification of commuters and wanderers was not possible, particularly in phase-2, where new dyadic pairs were encountered during each sampling period.

The identification was not carried out because 1) the observation area was relatively small, and 2) a longer study period was required, indicating the need to determine home range stability. The datasets engage more *dyads*, with varying range sizes, among the two phases. This indicated that the use of distribution calculated showed the probabilities of where an animal might have been

found at any randomly selected time (Powell and Mitchell 2012). Furthermore, it showed that the greater the range size, the more likely an individual has been followed. The problem of home range stability can be solved through a long-term study in an area within defined boundaries set by the movements of the subjects (Singleton & Carel P. van Schaik 2001).

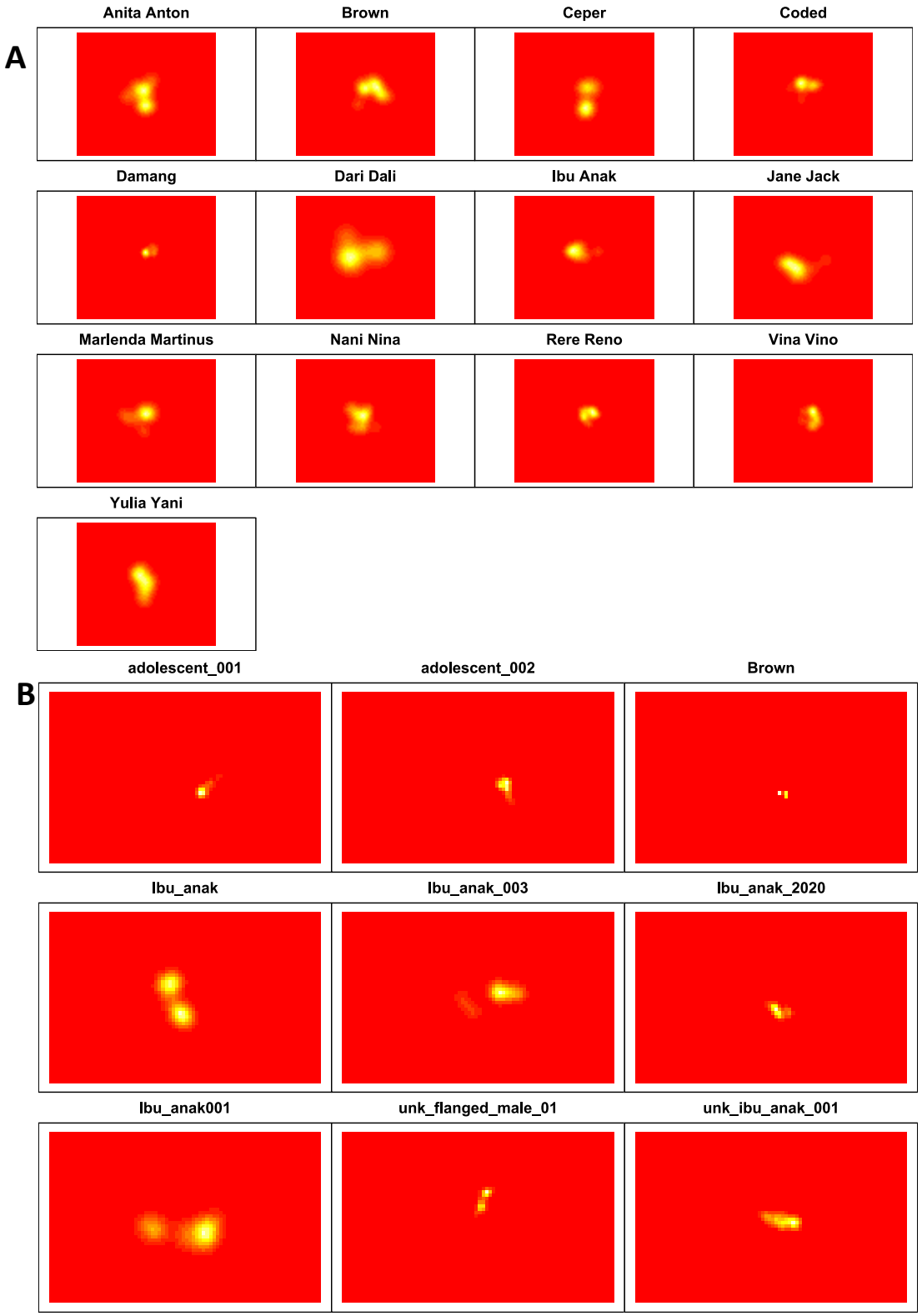

Figure 5 KernelUD as per individual orangutans; (A) Phase-1: 2015 – 2019; and (B) Phase-2: 2020 -2022

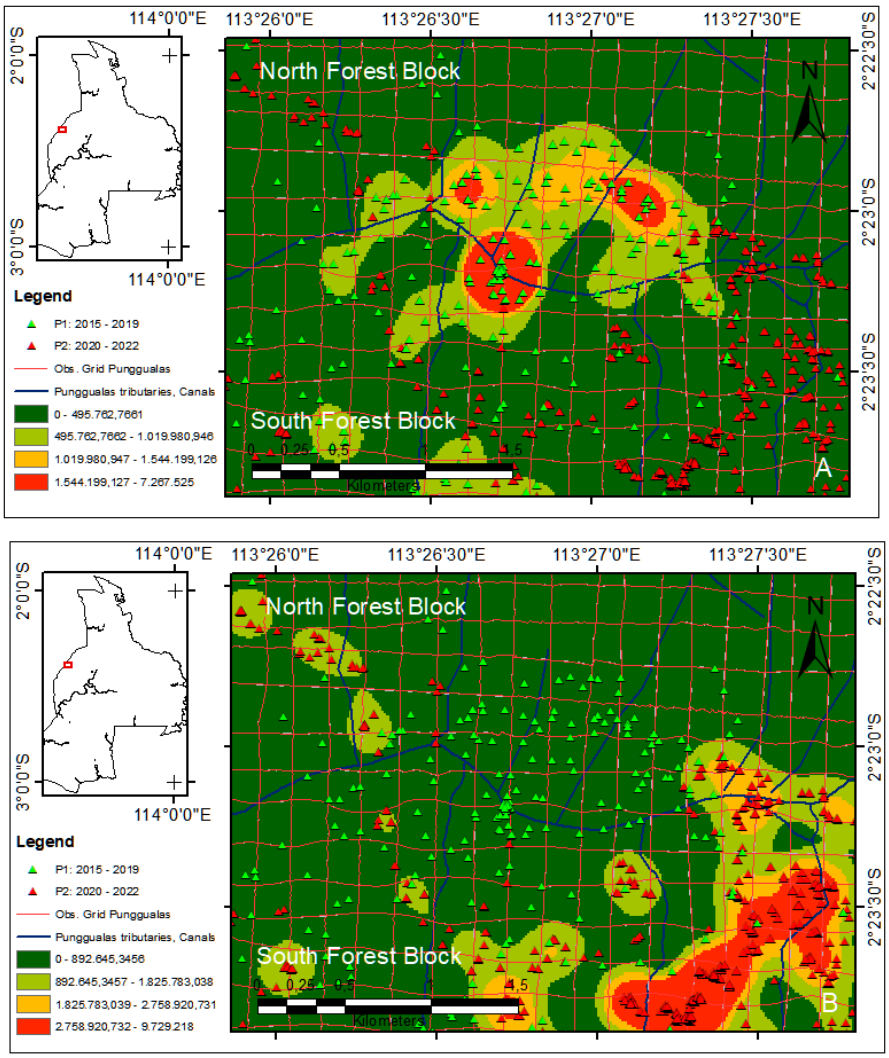

Figure 6 Maps constructed from the orangutan point positions, overlap with the Kernel density estimate: (A) 2015-2019; and (B) 2020 – 2022; North vs South delineated by Punggualas tributaries

Range Pattern

During P1, the distribution area was located within the northern part of the AOI and changed to the southeastern part in P2, as shown in Figure 6. Each red area showed the most overlap in forest areas, indicating a higher probability of hosting various individual normal activities such as food gathering and care for the young. In P1, the most overlap areas were shown by three zones, while a single red area dominated P2. However, a change in pattern was observed, indicating that the orangutan movement corresponded to the tree composition as a determining factor.

The mean basal area (m^2), trees/ha, and total basal area (m^2/ha) were calculated using vegetation data from PCQ. The result presented in Figure 7 showed that each forest block varies slightly, while the southern part tended to have a bigger mean basal area (m^2), total number of trees/ha, and total basal area (m^2/ha). This implied that the southern part of the AOI, featured a greater number of individual trees per ha, particularly *Cryptocarya crassinerva*, *Bluemeodendron tokbrai,* and *Campnospermae coriaceum*. However, the total basal area (m^2/ha) showed the same trend in each AOI, namely *Shorea* sp1.

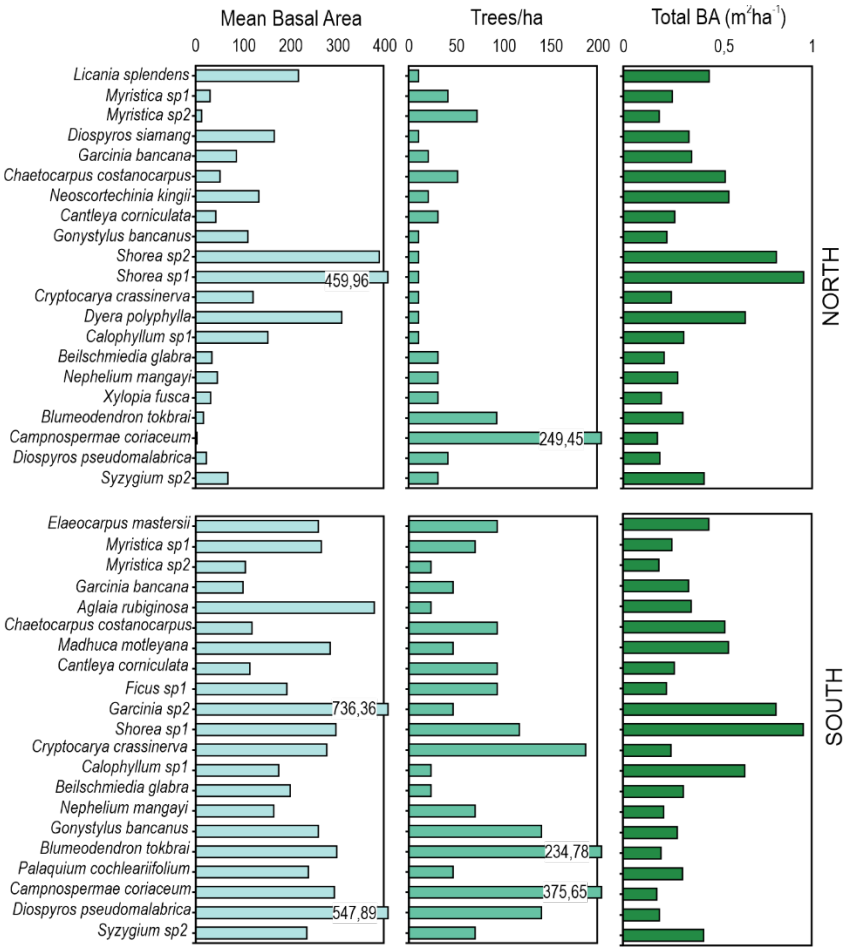

Figure 7 Mean basal area, number of trees/ha, and total basal area (m²/ha) per tree species in the north and south forest blocks

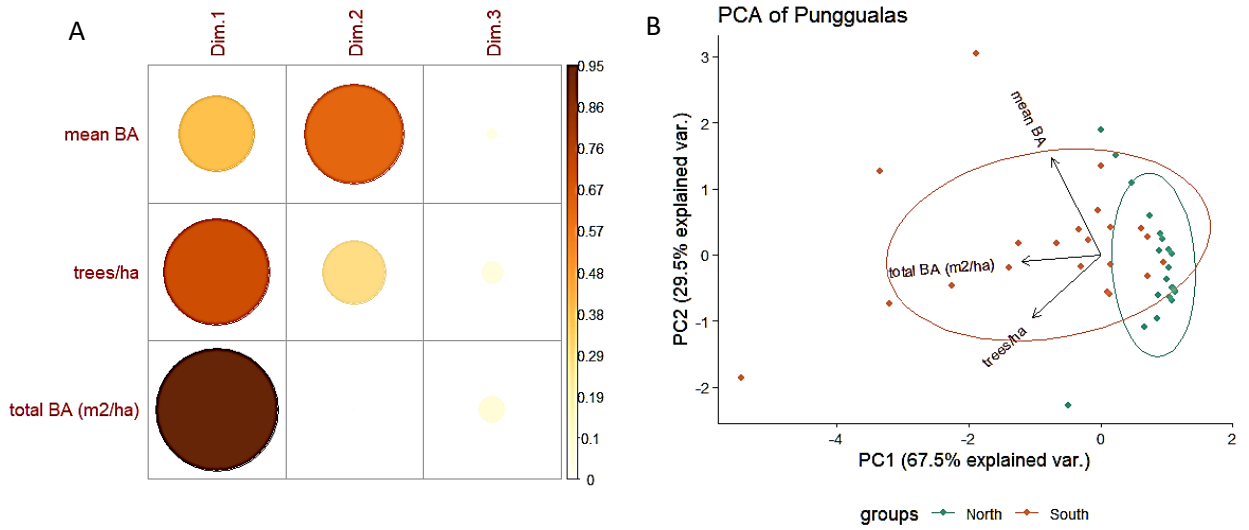

Figure 8 (A) The coefficient correlations mean BA, trees/ha, and total BA (m²/ha) as per dimension; and (B) Biplot Principal component analysis, depicts factor loadings and cluster

The first principal component was strongly correlated with a total BA (m^2/ha), followed by trees/ha and mean BA, respectively (Figure 8A). This indicated whether the North and South Forest blocks yielded, trees/ha, and whether mean BA contributed to the total BA (m^2/ha). PC1 can be considered a measure of total BA (m^2/ha), while PC2 coefficient value of mean BA (0.8388) was perpendicular to the total BA (m^2/ha) and opposed trees/ha. This showed that a higher mean BA value corresponded to a decrease in the contribution to the total variance of trees/ha and total BA (m^2/ha). As for trees/ha, the results produced bias due to only the measurement of each quadrant tree representative. Consequently, when standardized vegetation plots were deployed, different PCA loading biplots would be obtained.

Trees composed of different species such as *Campnospermae coriaceum* (Anacardiaceae), *Blumeodendron tokbrai* (Euphorbiaceae), *Garcinia* sp2 (Clusiaceae), *Cryptocarpa crassinerva* (Lauraceae) and *Diospyros* sp1 (Ebenaceae), were placed beyond elliptical. The result of the principal component analysis in Figure 8 showed that total BA (m^2/ha) loading had the highest eigenvalue. However, mean BA and trees/ha loadings were pointed out in other directions and the probability of orangutans using any random point over the grid trails was adequate from tree species composition.

Figure 9 showed data uniformity despite the differences among mean BA, trees/ha, and total BA (m^2/ha), and variation in tree species, the relative density, coverage, frequency, and important value. Using habitat classification (Morrogh-Bernard *et al.* 2014a), it was discovered that the orangutan used Tall connected A (TCA) and medium-connected (MC). TCA was characterized by the high stem density of large trees (>20 m tall) connected with high canopy (>75%). Meanwhile, MC was characterized by a few tall trees with a high stem density of medium-sized trees and a connected canopy at 10 – 20 m. The interlocked canopy was inadequate to support the range pattern-shifting idea. Recent observations have shown that natural tree falling has been spotted in a regularly visited grid in the Northern and Southern Forest block of Punggualas.

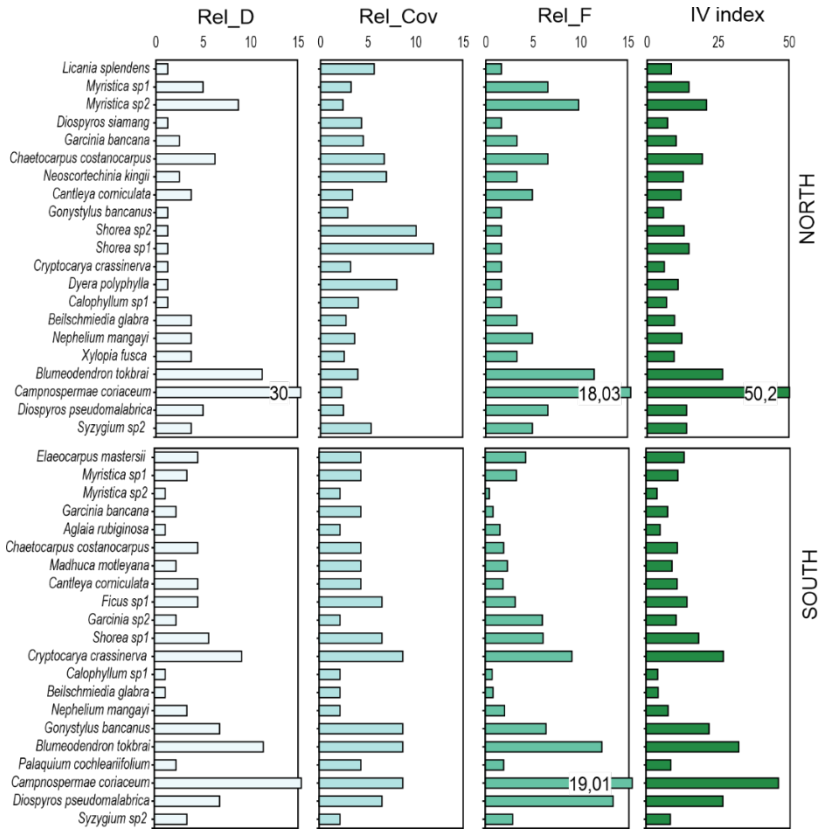

Figure 9 Relative density (%), coverage (%), frequency (%), and important value index (%) as per tree species derived from point-centered quarter method

In addition to tree composition, dietary preferences were also explored as another argumentation. In peat forests, orangutan spent half of the active time feeding compared to those in a mixed-dipterocarp forest. Observations showed that the focal being followed was frequently feeding on young leaves, flowers of *Mezzetia leptopoda* (Annonaceae), and immature fruits of *Nephelium lappaceum* (Sapindaceae). Moreover, recent observations also indicated that the orangutan diet included the flower of *Madhuca motleyana* (Sapotaceae), inner bark, termites, and liana. The fruits from *Willughbeia* sp1 and *cf. Lucinia* sp1, commonly woody lianas found in Punggualas, was observed from several dyad mother-infant and flanged-male.

Punggualas phenology data showed that the *Camnospermae coriaceum* generally had two fruiting periods yearly, first from October/November to February/March, followed by May to June. The general dietary pattern of orangutans during the study period did not always describe the fruit as their dietary preference because seeds, flowers, leaves, and bark were also included as fallback foods. Studies of the nutritional content and its relationship with the energy budget of orangutans in Tuanan, Central Kalimantan, found that young leaves had a higher protein content than fruits (Vogel *et al.* 2017). Basedon the results, confirm that variations in energy absorption were strongly influenced by the range of fruiting periods in the Sebangau habitat. A diet that depended on fibrous vegetation and bark confirmed that *P. p. wurmbii* shapes their mandibulae for dietary preferences (Traylor-Holzer *et al.* 2009). Detail observations in Ketambe Study Areas by (Hardus, Lameira, Menken & Wich 2012) regarding liana-derived resources such as fruit and leaves indicate the critical role of liana in the orangutan diet, specifically for orangutan population in the primary forest.

Table 2 List of observed tree/sapling, liana, and *Ficus* species showing parts eaten by orangutan during P1 and P2

Species	Family	Fruit	Seed	Flower	Leaves	Bark
Campnosperma coriaceum	Anacardiaceae	☐	☒	☐	☐	☐
Xylopia fusca	Annonaceae	☒	☒	☐	☐	☐
Xylopia malayana		☒	☒	☐	☐	☐
Dyera polyphylla	Apocynaceae	☒	☒	☒	☒	☒
Licania splendens	Chrysobalanaceae	☒	☒	☐	☒	☐
Callophylum hosei	Clusiaceae	☒	☐	☐	☒	☐
Callophylum sclerophyllum		☒	☐	☒	☐	☐
Callophylum sp3		☒	☒	☐	☐	☐
Garcinia banana		☒	☐	☐	☒	☐
Diospyros confertiflora	Ebenaceae	☒	☒	☐	☐	☐
Diospyros evena		☒	☐	☐	☐	☐
Diospyros pseudomalabrica		☒	☐	☒	☐	☐
Diospyros siamang		☒	☒	☐	☐	☐
Elaeocarpus mastersii	Elaeocarpaceae	☒	☒	☒	☒	☐
Neoscortechinia kingii	Euphorbiaceae	☒	☐	☒	☒	☐
Castanopsis foxworthyii	Fagaceae	☒	☒	☐	☒	☐
Lithocarpus conocarpus		☒	☒	☐	☐	☐
Litsea cf. resinosa	Lauraceae	☒	☒	☐	☒	☐
Phoebe zsp SE cf. grandis		☐	☐	☐	☒	☐
Koompassia malaccensis	Leguminosae	☐	☐	☐	☐	☒
Magnolia bintulensis	Magnoliaceae	☒	☒	☒	☒	☒
Dactylocladus stenotachys	Melastomaceae	☐	☐	☒	☐	☐
Aglaia rubiginosa	Meliaceae	☒	☐	☐	☒	☐
Parartocarpus venenosa	Moraceae	☒	☐	☒	☐	☐
Horsfidelia crassifolia	Myristicaceae	☒	☐	☒	☐	☐
Myristica sp2		☐	☐	☐	☐	☒
Ardisia sp2	Myrsinaceae	☒	☒	☐	☐	☐
Syzygium sp1	Myrtaceae	☒	☒	☐	☐	☐
Pittosporum sp1	Pittosporaceae	☐	☐	☐	☒	☐
Nephelium lappaceum	Sapindaceae	☒	☐	☐	☐	☐
Nephelium maingayi		☒	☐	☐	☐	☐

Table 2 (Continued)

Species	Family	Fruit	Seed	Flower	Leaves	Bark
Madhuca motleyana	Sapotaceae	☒	☐	☒	☒	☒
Palaquium cochlearifolium		☒	☐	☒	☐	☐
Palaquium pseudorastratum		☒	☒	☒	☐	☐
Palaquium sp		☒	☐	☐	☐	☐
Palaquium sp2		☒	☐	☐	☐	☐
Microcosm sp	Tiliaceae	☒	☐	☐	☒	☐
Willughbeia sp1	Apocynaceae	☒	☐	☐	☒	☐
Ziziphus angustifolia	Rhamnaceae	☒	☐	☐	☒	☐
cf. Lucinea sp1	Rubiaceae	☒	☐	☐	☒	☐
Ficus sp	Moraceae	☒	☒	☐	☐	☐
Ficus sp8		☒	☒	☐	☐	☐

Notes: recorded fruits, are grouped by maturity stages, e.g., immature, semi-mature, or mature fruit.

The observation made in Tuanan, Central Kalimantan, showed the importance of liana in adolescent orangutan (Saputra *et al.* 2017) during the absence of fruiting. The determining factor of liana density in LAHG-CIMTROP UPR Sebangau was highly correlated with tree crown size, shape, and height (Schofield 2015) due to the light penetration to the forest floor.

Another determining factor was found to be crowding, also known as compression. This occurred when orangutan was exposed to the disturbance in a portion of their range, mostly using parts that had not been logged or so-called mosaic, and began to use their home range differently. Since orangutan home range overlaps, many individuals made more biased use of the home range, resulting in population crowding into areas of undisturbed habitat, or 'refuges.' This phenomenon made male orangutans travel further away from the observation grid. To address this issue, additional perspectives were incorporated concerning anthropogenic activities in SNP.

Potential overlap between orangutan range and subsistence activities

Nontimber forest products, known for their aromatic fragrance, so-called gaharu, were mainly produced by the family Thymelaeaceae, specifically *Aquilaria beccariana* van Tiegh, and *Aquilaria filaria* (Oken.) Merr (Giesen 2015; Paoli, Peart, Leighton & Samsoedin 2001; Sitepu, Santoso, Siran & Turjaman 2011). These products were harvested from the Aquilaria genus that had been infected by a particular fungus. This study also documented the activities of villagers, mostly from Karuing, moving back-forth and to the forest, collecting gaharu. Meanwhile, their harvesting methods varied, including the use of a 1,20 m long iron stick pushed into the peat surface to detect the particular sound produced as the stick hit gaharu. After locating the suspected wood, the soil was dug, lifted out of the surface, and taken to the village to be weighed and exchanged for money. The aromatic essence of gaharu was economically beneficial in raw materials, crafted as the bracelet, and occasionally, tasbih.

Recent studies found that some of this aromatic wood was obtained from *Gonystylus bancanus* (Miq.) Kurz, of Thymelaeaceae. The wood is expensive and most wanted during logging concessions in Sebangau ecosystem. Verification has been made for a product claimed as gaharu (Nordahlia 2017), indicating that a product other than the plant genus *Aquilaria* is called pseudo-gaharu. Although the aromatic properties and mechanical structure, the wood are not similar to truly gaharu trees, *Aquilaria beccariana* van Tiegh, and *Aquilaria filaria* (Oken.) Merr.

This study showed the importance of considering some factors such as distance of access and number of days spent in the interior forest should be considered. Some villagers use the observation grid and also encounter the orangutan. According to local sources, these activities were held during the COVID-19 pandemic and continued unto the study in phase-2 (P2). Whether considered a coincidence or not, there was sufficient proof that access to natural resources in the interior forest of SNP contributed to the orangutan range pattern.

Consequently, the Park Authority has now prohibited this gaharu harvesting out of the forest. Recommendations for future study incorporate detailed observations on the orangutan-human interface, including the habitat structure profile, orangutan parasites burden, and the community access into the interior forest.

CONCLUSIONS

In conclusion, this study showed that *P. p. wurmbii* range pattern in Punggualas moved to the southeastern part of the grid trails, as indicated by MCP and KernelUD. The probability of orangutans using any random point over the area, specifically for food was adequate from tree species composition and total basal area (m^2/ha). Additionally, the compression effect became a potential determining factor regarding swift changes in range patterns within the study periods.

COMPETING INTERESTS

The authors declare that there are no competing interests. This study is part of the One-Health showcase, which aims to elucidate the interplay among orangutans, including their behavior, home range, population, intestinal parasites, microbiome, and habitat, referred to as the epidemiological triad in SNP. Therefore, some of the unpublished data will be indicated to support the current study argumentation.

REFERENCES

Allen, T., Murray, K. A., Zambrana-Torrelio, C., Morse, S. S., Rondinini, C., Di Marco, M., … Daszak, P. (2017). Global hotspots and correlates of emerging zoonotic diseases. *Nature Communications*, *8*(1), 1-10. doi: 10.1038/s41467-017-00923-8

Ancrenaz, M., Gumal, M., Marshall, A. J., Meijaard, E., Wich, S. A., & Husson, S. (2016). Pongo pygmaeus. The IUCN Red List of Threatened Species 2016e. In *Cambridge, UK: International Union for Conservation of Nature* (Vol. 8235). Retrieved from http://dx.doi.org/10.2305/IUCN.UK.2016-1.RLTS.T17975A17966347.en

Bengis, R. G., Leighton, F. A., Fischer, J. R., Artois, M., Mörner, T., & Tate, C. M. (2004). The role of wildlife in emerging and re-emerging zoonoses. *OIE Revue Scientifique et Technique*, *23*(2), 497–511. doi: 10.20506/rst.23.2.1498

Bordes, F., Morand, S., Kelt, D. A., & Van Vuren, D. H. (2009). Home range and parasite diversity in mammals. *American Naturalist*, *173*(4), 467-474. doi: 10.1086/597227

Burt, W. H. (1943). Territoriality and Home Range Concepts as Applied to Mammals Author (s): William Henry Burt Published by : American Society of Mammalogists Stable URL : *Journal of Mammalogy*, *24*(3), 346-352.

Cattau, M. E., Husson, S., & Cheyne, S. M. (2015). Population status of the Bornean orang-utan Pongo pygmaeus in a vanishing forest in Indonesia: The former Mega Rice Project. *Oryx*, *49*(3), 473-480. doi: 10.1017/S003060531300104X

Daszak, P., Cunningham, A. A., & Hyatt, A. D. (2000). Emerging infectious diseases of wildlife - Threats to biodiversity and human health. *Science*, *287*(5452), 443-449. doi: 10.1126/science.287.5452.443

Erb, W. M., Barrow, E. J., Hofner, A. N., Utami-Atmoko, S. S., & Vogel, E. R. (2018). Wildfire smoke impacts activity and energetics of wild Bornean orangutans. *Scientific Reports*. doi: 10.1038/s41598-018-25847-1

Foitová, I., Huffman, M. A., & Wisnu, N. (2009). Parasites and their impacts on orangutan health. In Serge A Wich, S. S. U. Atmoko, T. M. Setia, & C. P. van Schaik (Eds.), *Orangutans: Geographic Variation in Behavioral Ecology and Conservation* (pp. 157-170). New York, US: Oxford University Press.

Giesen, W. (2015). Utilising non-timber forest products to conserve Indonesia ' s peat swamp forests and reduce carbon emissions. *Journal of Indonesian Natural History*, *3*(2), 69-72.

Han, B. A., Kramer, A. M., & Drake, J. M. (2016). Global Patterns of Zoonotic Disease in Mammals. *Trends in Parasitology*, *32*(7), 565-577. doi: 10.1016/ j.pt.2016.04.007

Hardus, M. E., Lameira, A. R., Menken, S. B. J., & Wich, S. A. (2012). Effects of logging on orangutan behavior. *Biological Conservation*. doi: 10.1016/ j.biocon.2011.12.014

Herrera, J. P., Chakraborty, D., Rushmore, J., Altizer, S., & Nunn, C. (2019). The changing ecology of primate parasites: Insights from wild-captive comparisons. *American Journal of Primatology*. doi: 10.1002/ ajp.22991

Jones, K. E., Patel, N. G., Levy, M. A., Storeygard, A., Balk, D., Gittleman, J. L., & Daszak, P. (2008). Global trends in emerging infectious diseases. *Nature*, *451*(7181), 990–993. doi: 10.1038/nature06536

Labes, E. M., Hegglin, D., Grimm, F., Nurcahyo, W., Harrison, M. E., Bastian, M. L., & Deplazes, P. (2010). Intestinal parasites of endangered orangutans (Pongo pygmaeus) in Central and East Kalimantan, Borneo, Indonesia. *Parasitology*. doi: 10.1017/S0031182009991120

Mitchell, K. (2010). *Quantitative Analysis by the Point-Centered Quarter Method*. Retrieved from http://arxiv.org/abs/1010.3303

Morrogh-Bernard, H., Husson, S., & McLardy, C. (2002). Orangutan Data Collection Standardisation. In *Orangutan Culture Workshop*. San Anselmo, USA. Retrieved from https://www.aim.uzh.ch/dam/jcr:00000000-31fd-16f1-ffff-fffff64ca75e/method_standardisation_fina_II.pdf

Morrogh-Bernard, Helen C., Husson, S. J., Harsanto, F. A., & Chivers, D. J. (2014). Fine-scale habitat use by orang-utans in a disturbed peat swamp forest, central Kalimantan, and implications for conservation management. *Folia Primatologica*, *85*(3), 135-153. doi: 10.1159/000358251

Morrogh-Bernard, Helen Celia, Morf, N. V., Chivers, D. J., & Krützen, M. (2011). Dispersal Patterns of Orang-utans (Pongo spp.) in a Bornean Peat-swamp Forest. *International Journal of Primatology*, *32*(2), 362-376. doi: 10.1007/s10764-010-9474-7

Noordwijk, M. A. Van, Sauren, S. E. B., Morrogh-bernard, H. C., Atmoko, S. S. U., & Schaik, C. P. Van. (2003). Development of independence Sumatran and Bornean orangutans compared. In Serge A Wich, S. S. U. Atmoko, T. M. Setia, & C. P. van Schaik (Eds.), *Orangutan Geographic variation in behavioral ecology and conservation* (pp. 189-203). New York, US: Oxford University Press.

Nordahlia, A. (2017). *Timber technology bulletin* (Vol. 71).

Nunn, C. L., Thrall, P. H., Leendertz, F. H., & Boesch, C. (2011). The spread of fecally transmitted parasites in socially-structured populations. *PLoS ONE*, *6*(6), 1-10. doi: 10.1371/journal.pone.0021677

Nurcahyo, W., Konstanzová, V., & Foitová, I. (2017). Parasites of orangutans (primates: ponginae): An overview. *American Journal of Primatology*, *79*(6). doi: 10.1002/ajp.22650

Paoli, G. D., Peart, D. R., Leighton, M., & Samsoedin, I. (2001). An ecological and economic assessment of the nontimber forest product gaharu wood in Gunung Palung National Park, West Kalimantan, Indonesia. *Conservation Biology*, *15*(6), 1721-1732. doi: 10.1046/j.1523-1739.2001.98586.x

Philippa, J., & Dench, R. (2019). Infectious Diseases of Orangutans in their Home Ranges and in Zoos. In *Fowler's Zoo and Wild Animal Medicine Current Therapy, Volume 9* (Vol. 1). doi: 10.1016/b978-0-323-55228-8.00080-1

Posit, P. team. (2023). *RStudio: Integrated Development Environment for R*. Boston, MA.

Rayadin, Y., & Spehar, S. N. (2015). Body mass of wild bornean orangutans living in human-dominated landscapes: Implications for understanding their ecology and conservation. *American Journal of Physical Anthropology*, *157*(2), 339-346. doi: 10.1002/ajpa.22709

Rondón, S., Ortiz, M., León, C., Galvis, N., Link, A., & González, C. (2017). Seasonality, richness and prevalence of intestinal parasites of three neotropical primates (Alouatta seniculus, Ateles hybridus and Cebus versicolor) in a fragmented forest in Colombia. *International Journal for Parasitology: Parasites and Wildlife*, *6*(3), 202-208. doi: 10.1016/j.ijppaw.2017.07.006

Rukmawardani, E., & Imron, M. A. (2021). *Movement patterns and daily range of Bornean Orangutan (Pongo pygmaeus wurmbii) in Punggualas, Sebangau National Park*. Yogyakarta. Retrieved from https://etd.repository.ugm.ac.id/penelitian/detail/198238

Santika, T., Ancrenaz, M., Wilson, K. A. K. A., Spehar, S., Abram, N., Banes, G. L. G. L., … Meijaard, E. (2017a). First integrative trend analysis for a great ape species in Borneo. *Scientific Reports*, *7*(1). doi: 10.1038/s41598-017-04435-9

Santika, T., Ancrenaz, M., Wilson, K. A., Spehar, S., Abram, N., Banes, G. L., … Meijaard, E. (2017b). First integrative trend analysis for a great ape species in Borneo. *Scientific Reports*, *7*(1). doi: 10.1038/s41598-017-04435-9

Saputra, F., Perwitasari-Farajallah, D., Suci Utami-Atmoko, S., Ariyanto, T., & van Noordwijk, M. A. (2017). Monthly range of adolescent orangutans (Pongo pygmaeus wurmbii) based on fruit availability in tuanan orangutan research station, Central Kalimantan, Indonesia. *Biodiversitas*, *18*(4), 1445-1452. doi: 10.13057/biodiv/d180421

Schofield, K. (2015). Changes in forest structure and tree species composition after logging in tropical peat-swamp forest in Central Kalimantan, Indonesia. *University of Aberdeen*.

Scott, A. M., Knott, C. D., & Susanto, T. W. (2019). Are Male Orangutans a Threat to Infants? Evidence of Mother–Offspring Counterstrategies to Infanticide in Bornean Orangutans (Pongo pygmaeus wurmbii). *International Journal of Primatology*, *40*(3), 435-455. doi: 10.1007/s10764-019-00097-8

Singleton, I., & Carel P. van Schaik. (2001). Orangutan Home Range Size and Its Determinants in a Sumatran Swamp Forest. *International Journal of Primatology*, *22*(6), 877-911. doi: 10.1023/A

Singleton, I., Knott, C. D., Morrogh-bernard, H. C., Wich, S. A., & Schaik, C. P. Van. (2009). *C H A P T E R 13 Ranging behavior of orangutan females and social organization* (Serge A Wich, S. S. U. Atmoko, T. M. Setia, & C. P. van Schaik, Eds.). New York, US: Oxford University Press.

Sitepu, I. R., Santoso, E., Siran, S. A., & Turjaman, M. (2011). Fragrant Wood Gaharu: When the Wild Can No Longer Provide. In *Fragrant Wood Gaharu: When the Wild Can No Longer P R O V Ide* (Vol. 50). Jakarta, Indonesia: ITTO. Retrieved from http://search.ebscohost.com/login.aspx?direct=true&db=a9h&AN=77484929&site=ehost-live

Suci, S., Atmoko, U., Singleton, I., Noordwijk, M. A. Van, Schaik, C. P. Van, & Setia, T. M. (2009). C H A P T E R 15 Male – male relationships in orangutans. In Serge A Wich, S. S. U. Atmoko, T. M. Setia & C. P. van Schaik (Eds.), *Orangutan Geographic variation in behavioral ecology and conservation* (pp. 225-233). New York, US.

Traylor-Holzer, K., Lacy, R., Stephens, S., Wich, S. A., Byers, O., Leighton, M., … Meijaard, E. (2009). Orangutan population biology, life history, and conservation. *Orangutans*. doi: 10.1093/acprof:oso/9780199213276.003.0022

Vogel, E. R., Alavi, S. E., Utami-Atmoko, S. S., van Noordwijk, M. A., Bransford, T. D., Erb, W. M., … Rothman, J. M. (2017). Nutritional ecology of wild Bornean orangutans (Pongo pygmaeus wurmbii) in a peat swamp habitat: Effects of age, sex, and season. *American Journal of Primatology*, *79*(4). doi: 10.1002/ajp.22618

Voigt, M., Wich, S. A., Ancrenaz, M., Meijaard, E., Abram, N., Banes, G. L., … Kühl, H. S. (2018). Global Demand for Natural Resources Eliminated More Than 100,000 Bornean Orangutans. *Current Biology*, *28*(5). doi: 10.1016/j.cub.2018.01.053

Voigt, Maria, Kühl, H. S., Ancrenaz, M., Gaveau, D., Meijaard, E., Santika, T., … Struebig, M. J. (2021). Deforestation projections imply range-wide population decline for critically endangered Bornean orangutan. *BioRxiv*, (July 16), 1-24.

Voigt, Maria, Wich, S. A., Ancrenaz, M., Meijaard, E., Abram, N., Banes, G. L., … Kühl, H. S. (2018). Global Demand for Natural Resources Eliminated More Than 100,000 Bornean Orangutans. *Current Biology*, *28*(5), 761-769.e5. doi: 10.1016/j.cub.2018.01.053

Wartmann, F. M., Purves, R. S., & van Schaik, C. P. (2010). Modelling ranging behaviour of female orang-utans: A case study in Tuanan, Central Kalimantan, Indonesia. *Primates*, *51*(2), 119-130. doi: 10.1007/s10329-009-0186-6

Wich, S.A., Gaveau, D., Abram, N., Ancrenaz, M., Baccini, A., Brend, S., … Meijaard, E. (2012). Understanding the Impacts of Land-Use Policies on a Threatened Species: Is There a Future for the Bornean Orang-utan? *PLoS ONE*, *7*(11). doi: 10.1371/journal.pone.0049142

NUTRIENT PROFILE OF BLACK SOLDIER FLY LARVAE (*Hermetia illucens*): EFFECT OF FEEDING SUBSTRATE AND HARVEST TIME

MARIA R. WEKO, FREDERIUS BAO, MARIA E. EGA, HENDRIKA MIA, KLARA S. I. UNA, MARCELINA VIANA, LODOVIKUS WALE, CATOOTJIE L. NALLE*, YACOB M. BURITNABAN, ABNER TONU LEMA, HELDA AND ASRUL

Animal Husbandry Department, State Polytechnic of Agriculture Kupang, Kupang 85228, Indonesia

Received 14 February 2023 / Revised 30 May 2023 / Accepted 30 May 2023

ABSTRACT

The objective of this study was to asses the effect of different feeding substrates (FS), harvesting times (HT), and the interaction between FS and HT on the chemical composition of black soldier fly larvae (BSFL). The experiment used a 4 x 2 factorial arrangement with two main factors, FS (T1, T2, T3, T4), and HT (15-d and 20-d). Thus, there were eight treatment combinations, all together with five replications. The results show that there was no interaction (P>0.05) between FS and HT on dry matter (DM), crude protein (CP), crude lipid (CL), phosphorus (P), gross energy (GE), and amino acid contents. The ash content of BSFL grown on T2 media and harvested on day 15 was higher (P<0.05) than those grown on T2 media and harvested on day 20. The calcium (Ca) content of BSFL grown on all media and harvested on day 20 was higher (P<0.05) than those harvested on day 15. In conclusion, combining fruit wastes and tofu by-products produced BSFL with high CP content but low CL, ash, Ca, and P contents. In addition, BSFL grown on all substrates media and harvested on day 15 had better CP, Ca, and P contents. The dispensable amino acid of BSFLs fed with T3 diets was the best. The lowest body weight gain was produced by feeding a substrate containing a high percentage of rice bran. The findings indicate that the best nutrient composition of BSFL as animal feed would be achieved in early harvest time (15-d) and grown in heterogeneous feeding substrates.

Keywords: black soldier fly larvae, growing media, maggot, nutrient, proximate

INTRODUCTION

Protein sources for poultry diets generally come from both plant protein sources (PPS) and animal protein sources (APS). Generally, the utilization of PPS in poultry diet formulations is comparatively higher than APS. The Indonesian poultry feed industry still depends on PPS and APS from overseas, including meat and bone meal, corn gluten meal, and soybean meal (SBM). Argentina, Brazil, the USA, Paraguay, and India are the countries that produce and supply SBM for Indonesia (Natalia *et al.* 2019). Regarding meat and bone meal, Indonesia imports this ingredient from Australia, New Zealand and Canada. As reported by Dimiyati (2021), the volume of imports of PPS increased from 57.30% in 2015 to 84% in 2020. Meanwhile, the volume of imported meat and bone meal range from 0.63 to 1.05 million tons per year, so the economic value reaches IDR5.5-9.2 trillion (Dimiyati 2021). The price of these feed ingredients frequently fluctuates, depending on the exchange value of the US Dollar towards IDR.

Recently, the limited availability of SBM in the international market triggered the increase in prices of SBM. This limited availability of SBM in the international market was caused by the increased importation of SBM by China. Meanwhile, local soybeans are available but highly limited, so it is not a feasible option for feed millers. An increase in SBM prices will certainly have an impact on increasing the local compound feed prices.

*Corresponding author: catootjielusjenalle@gmail.com

The cost of feed ingredients reached 83-89% of the total cost of broiler production, and 84 to 89% of the total cost of the laying hens (Natalia *et al.* 2019). Therefore, efforts to find alternative proteins must be made to overcome the issue of dependence on imported protein sources, as to finally reduce production costs. The black soldier fly (BSF) (*Hermetia illucens*) is one of the organisms studied for its characteristics and nutrient content. This fly originated in America and subsequently spread to subtropical and tropical regions of the world (Čičková *et al.* 2015). Indonesia's tropical climate is ideal for cultivating black soldier fly larvae (BSFL). Regarding cultivation, BSFLs are very easy to develop on a mass-production scale and do not require special equipment. Patterson *et al.* (2021) reported that the BSFL meal could be included in the laying hen's diets up to 16% without any negative effects on growth performance and egg production.

The crude protein (CP) content in BSFL was quite high, around 12.9 to 78.8%, with crude lipid content of 29 to 32%, 4.8 to 5.1% calcium (Ca), and 0.60 to 0.63% phosphorus (P) (Bondari & Sheppard, 1987; Bosch *et al.* 2014; Hopkins *et al.* 2021; Lu *et al.* 2022). The amino acid content of the BSFL, especially leucine, lysine, and valine, was superior to other protein sources such as SBM and fish meal, while methionine and tryptophan of BSFL were almost similar to that of SBM (Lu *et al.* 2020).

However, the nutritional value, survival, and performance of BSFL are influenced by various factors, such as the type and composition of the growing substrates and harvesting times (Barragan-Fonseca *et al.* 2017; Ewald *et al.* 2020; Broeckx *et al.* 2021; Lan *et al.* 2022). The growing media generally used for BSFL production are municipal waste, agro-industrial waste, and manure and feces (Supriyatna *et al.* 2016a; Dortmans *et al.* 2017; Broeckx *et al.* 2021). Dortmans *et al.* (2017) and Broeckx *et al.* (2021) reported that BSFL grew well in a substrate rich in protein and available carbohydrates. On the other hand, Tschirner and Simon (2015) found that the BSFLs fed with organic substrates high in fiber produced BSFL with higher CP content (52.3%) compared to those fed with substrates high in protein content with BSFL CP content of only 40%. In addition, the larva may consume the waste easily when the waste has undergone some microbial decomposition process and is in the form of pasta or liquid.

Most published data regarding the chemical composition of BSFL was obtained from single substrate or two different substrates (Tschirner & Simon 2015; Supriyatna *et al.* 2016a; Lan *et al.* 2022). The production of BSFL from a combination of substrates is limited. In addition, the chemical composition of BSFL that has been reported is mostly obtained from BSFL harvested on day 20. The published data of BSFL chemical composition harvested on day 15 is highly limited. Based on the above explanation, research has been conducted to evaluate the effect of different feeding substrates (FS) and harvesting times (HT) on the chemical composition and growth performance of BSFL.

MATERIALS AND METHODS

Feed Ingredients

The main ingredients used in this experiment are fruit wastes (banana and papaya) and vegetable wastes (mustard greens and water crest) obtained from a local traditional market, tofu by-products purchased from a local tofu market, and liquid palm sugar, sago (*Putak* meal), and rice bran obtained from a local distributor.

Experimental Design and Diet Formulation

The experiment was designed using a 4 x 2 factorial design, with the first main factor being feeding substrates (FS: T1, T2, T3, and T4) and the second main factor being harvested time (HT: 15 days and 20 days). Each treatment was replicated five times (10 kg/replication). The experimental diets were as follows:

Table 1 Growing media of black soldier fly larvae (BSFL)

Ingredients	Feeding Substrate			
	T1	T2	T3	T4
	……..% as fed.....			
Fruits wastes (50% banana and 50% papaya)	24.4	24.4	-	-
Vegetable wastes (50% water crest and 50% mustard greens)	-	-	75	-
Tofu by-product	61	7	-	92.4
Rice bran	7	56	17.4	-
Sago meal	-	5	-	-
Effective Micro-organism (EM4)	0.3	0.3	0.3	0.3
Liquid palm sugar	0.3	0.3	0.3	0.3
Clean Water	7	7	7	7
Total	100	100	100	100
Calculated analysis (% as fed)				
Crude protein (%)	16.07	11.43	4.03	22.81
Crude lipid (%)	3.54	4.31	2.65	4.56
Crude fiber (%)	8.11	10.64	15.56	6.56
Ash (%)	3.70	8.87	17.19	3.67
Calcium (Ca, %)	5.53	4.51	27.54	8.32
Phosphorus (P, %)	2.76	2.91	2.18	0.22

Black Soldier Fly Larvae (BSFL) Production

BSFL was produced at three main stages, including (i) fermentation, (ii) BSF catching and mating, and (iii) the growing period. 1) *Fermentation:* All ground ingredients, free from harmful materials and inorganic elements, were weighed according to feeding substrate formulation. The ingredients were mixed until homogenous with a moisture level of 70 to 80% and fermented for seven days in a room. 2) *BSF catching, mating, and egg deposition:* on day seven, the fermented substrates were moved into a round plastic container and placed in a sheltered cavity to invite the BSF to mate and lay eggs. 3) *Growing period:* Once the larvae have appeared, the feeding substrates containing larvae were removed to the BSF housing for growing. 4) The BSF larvae (BSFL) were fed and grown till days 15 and 20 during the experiment. The feed given to the BSFL is 50 g/day per replication (container). The determination of feed given to BSFL per day (50 g) is referred to Supriyatna *et al.* (2016b). The authors reported that the feed needed for the best growing of one larva was 100 mg/day on a dry weight basis.

For the growing period, the BSFL used for growth performance data was placed separately from the BSFL for chemical composition data. There were 60 larvae per replication used for growth performance data.

Sample preparation

Black soldier fly larvae (BSFL) were harvested on day 15 (Figure 1a) and day 20 (Figure 1d). The BSFL from each treatment was removed from the leftover growing media, mixed with water, and then screened. The BSFL was then weighed to obtain the total wet weight. Then the BSFL was oven-dried (at 60^{o} C, Memmert) for four days, crushed with a stone mortal, ground with a sample mill (0.5 mm screen size), and sub-sampled, packed, and labeled (Figure 1). The BSFL sample was sent to the laboratory for chemical analysis.

(a) 15-days-old BSFL (b) oven dried BSFL (c) Ground BSFL

(d) 20-days-old BSFL (e) oven dried BSFL (f) Ground BSFL

Figure 1 Sample Preparation of Black Soldier Fly Larvae. (a) Black Soldier Fly Larvae (BSFL) (15-days-old); (b) Oven-Dried BSFL (15-days-old); (c) Ground BSFL (15-days-old); (d) BSFL (20-days-old); (e) Oven-Dried BSFL (20-days-old); (f) Ground BSFL (20-days-old);

Chemical Analysis

The dried BSFL samples were analyzed for their dry matter (DM), crude protein (CP, crude lipid (CL), crude fiber (CF), Neutral Detergent Fiber (NDF), Acid Detergent Fiber (ADF), gross energy (GE), Ca, P, and amino acids. All the chemical analyses were performed at BPT Ciawi Bogor Laboratory. The DM content was determined by using AOAC method No. 930.15 (AOAC 2005). The nitrogen content was analyzed using AOAC 2001.1 (AOAC 2005), while AOAC 942.5 (Van Soest Method; AOAC 2005) was used to determine NDF and ADF contents. The starch content of sago was analyzed using the titration method. An automatic PARR Bomb Calorimeter was used to measure the GE level. The Ca content was analyzed using an Atomic Absorption Spectrophotometer (AAS, Flame Varian 220). The Ca analysis was conducted as described by Nalle *et al.* (2021): the sample was weighed and put in the muffle furnace for three hours (550^{o} C). Then, the ash was destructed using acid, solubilized, and pipetted to measure at AAS with the wavelength of 422,7 nm (Nalle *et al.* 2021). The spectrophotometer method (UV VIS Agilent Cary 100) was used to determine the content of P. The wavelength of the spectrophotometer used was 400 nm (AOAC 2012). The amino acid content was determined using High-Performance Liquid Chromatography (HPLC, ICI Instrument/Shimadzu SCL-10A/Shimadzu CBM 20A) as described by Nalle *et al.* (2019).

Statistical Analysis

The chemical composition of dried BSFL samples obtained from this experiment was analyzed using the two-way analysis of variance (ANOVA) referring to the General Linear Model procedure of SAS (SAS OnDemand). Meanwhile, body weight gain data were analyzed using the one-way analysis of variance (ANOVA) referring to the General Linear Model procedure of SAS (SAS On Demand).

RESULTS AND DISCUSSION

Chemical Composition of Black Soldier Fly Larvae

Table 2 describes the chemical composition of BSFL fed with different feeding substrates and

harvested at different times. Statistical analysis showed that no interaction ($p > 0.05$) between FS, and HT was observed in the parameter of DM, CP, CL, P, and GE. There was an interaction ($p < 0.05$) between feeding substrate (FS), harvesting time (HT) for ash, and Ca content of BSFL. The ash content of BSFL grown in T2 media and harvested on day 15 was higher ($p < 0.05$) than those grown in T2 media and harvested on day 20. The ash content of BSFL reared in T1, T3, and T4 media and harvested on day 15 and day 20 was similar ($p > 0.05$). The present result partly agreed with Liu *et al.* (2017), who reported that there the ash content of late pupae was higher (10.2%) than the ash content of 14-day-old BSFL (8.3%). The range of BSFL ash content in the present study was 9.60 to 17.6%, which was in the range reported by Seyedalmoosavi *et al.* (2022).

The difference ($p < 0.05$) in Ca content was only found between BSFL grown in T3 media harvested on day 15 and those grown in T3 media and harvested on day 20. The tendency of higher Ca content in 20-day-old BSFL was probably due to the bone and teeth of BSFL having grown well. In a review by Seyedalmoosavi *et al.* (2022), it was reported that BSFL required minerals for the skeleton formation and the formation of other structural tissues (e.g., teeth). Furthermore, it was explained that although protein and chitin are the main components of the exoskeleton of most insect species, BSFL has a so-called mineralized exoskeleton which explains the high Ca content.

Statistical analysis showed that except for DM content, the main factor, FS affected ($p < 0.05$ to $p < 0.001$) the chemical composition of BSFL meal. The CP content of BSFL fed with T1 experimental diet was similar ($p > 0.05$) to the CP content of BSFL from the T2 diet; however, the CP content of T2 was higher ($p < 0.05$) than the CP content of BSFL fed with T3 and T4 diets. It is interesting to note that the T3 feeding substrate with the lowest CP content (Table 1) produced the BSFL with lower CP content ($p < 0.05$) compared to T1 and T2 treatment diets (Table 2). This could probably be due to the presence of higher antitrypsin and lectin contents in the tofu by-product used in the T3 treatment diet, which inhibits CP digestibility. Isanga and Zhang (2008) explained that tofu by-products contained 30 to 50% trypsin inhibitor and caused a negative effect on CP digestibility. Another possibility was that the lower mineral and vitamin contents in the T3 diet led to low nutrient metabolism.

It is interesting to note also that of the CP content of BSFL from T3 diets (high in fiber but low in protein) had similar ($p > 0.05$) CP content with BSFL grown in T4 diets (high protein substrates and low in fiber). The present result agreed with Tschirner and Simon (2015), who reported that the BSFLs given organic materials rich in fiber produced high CP BSFL. According to Tschirner and Simon (2015), the larva may consume the waste easily when the waste has been degraded by microbes and in the form of pasta. The present result indicated that the BSFL was tolerable to a high-fiber diet and could digest the fiber. Kim *et al.* (2011) reported that the BSFL digestion system contained several micro-organisms that produced lignin-cellulase, which degrades lignin into simple sugar. Supriyatna and Ukit (2016) reported that BSFL could degrade organic wastes because of the presence of cellulolytic produced by cellulolytic bacteria, especially *Bacillus sp.*

The range of CP content of BSFL obtained in the present study was 32.7 to 42.9% (as fed). The CP content of BSFL in the present study was lower than the CP content of BSFL reported by Tschirner and Simon (2015), i.e., 37 to 52.3%. The difference was probably due to the difference in the method, including the type of growing media and the harvested time. Growing media used by Wardhana (2016) included palm kernel meal, cow feces, pig feces, chicken excreta, fruit wastes, and other organic wastes. In addition, the BSFL was harvested on day 25. The growing media used by Tschirner and Simon (2015) was a mixture of middling (control group), dried distillers' grains with soluble (protein group), and dried sugar beet pulp (fiber group).

Regarding the second main factor, the CP content of BSFL harvested on day 15 was comparable ($p > 0.05$) to those harvested on day 20. The FS x HT interaction did not affect ($p > 0.05$) the CP content of BSFL.

It was expected that the CL content of BSFL fed with T4 and T2 diets should be higher than the CL content of BSFL grown in the T1 and T3 diets (Table 1 and Table 2) because of their high CL content in the diets. The reality showed that the CL of BSFL fed with the T3 diets was higher ($p < 0.05$) than in those fed with the T2 and T4 diets, while the CL content of T1, T2, and T4 was

similar ($p > 0.05$). Thus, this study proved that the CL content of BSFL did not relate to the CL content of the grown substrates.

The CL of BSFL harvested on day 20 was higher ($p < 0.05$) than those harvested on day 15. This result indicated that as the age of BSFL increased, the CL content of BSL increased. No interaction ($p > 0.05$) between FS and HT was found in the CL content of BSFL in all treatment diets.

The ash content of BSFL fed with T4 diets was higher ($p < 0.05$) than those fed with T1, T2, and T3 diets. The Ash content of BSFL was similar ($p > 0.05$) between T1 and T2 when harvested on day 20. The BSFL grown in T2 diets and harvested on day 15 had higher ($p < 0.05$) ash content compared to those who were grown in the same feeding substrate but harvested on day 20. On the other hand, the BSFL fed with T3 and T4 diets and harvested on day 20 had higher ($p < 0.05$) respective ash content than those that were grown in the same feeding substrate but harvested on day 20. The comparison was difficult to be made due to the limitation of published data related to the present study.

Table 2 shows the ash content of BSFL grown in the T4 diets was higher ($P<0.05$) than the ash content of BSFL grown in T3. This was an unacceptable result since the ash content of T4 diets was very low (Table 01) but produced BSFL with high ash content. The ash content of the T4 and T1 diets was quite similar (Table 1), but these media produced BSFL high in ash content (Table 2). The present study indicated that the ash content of BSFL was not affected by the ash content of the grown substrates.

The Ca content of the BSFL fed with T3 and T4 diets were higher ($p < 0.05$) than those grown in T1 and T2 diets. This could be due to the higher Ca content of the T3 and T4 diets than the T1 and T2 diets (Table 1). The P content of BSFLs fed with T3 was lower ($p < 0.05$) than those fed with T1, T2, and T4. The lowest P content of BSFL in T3 diets is related to the lowest P content of T3 diets (Table 1).

Table 2 Chemical composition of black soldier fly larvae (BSFL) fed with different feeding substrates and harvested at different times

Feeding Substrate	Harvesting time (day)	Dry Matter	Crude Protein	Crude Lipid	Ash	Ca	P	Gross Energy
		……% as fed……..……						(MJ/kg DM)
T1	15	93.4	39.6	29.9	10.7^{cd}	1.65^{d}	1.42	23.7
	20	94.8	36.9	36.1	9.83^{d}	2.16^{cd}	1.00	24.9
T2	15	94.1	42.9	20.7	13.2^{b}	2.92^{bc}	1.27	21.6
	20	92.4	40.0	33.0	9.60^{d}	2.21^{cd}	0.94	24.4
T3	15	94.5	32.7	36.9	12.9^{bc}	3.68^{b}	0.87	23.9
	20	94.4	33.6	35.9	14.4^{b}	5.06^{a}	0.83	23.5
T4	15	94.7	37.8	28.3	16.4^{a}	4.87^{a}	1.20	22.3
	20	93.8	34.5	30.8	17.6^{a}	5.09^{a}	1.16	22.2
SEM		0.617	1.75	2.99	0.815	0.295	0.086	0.67
Main Effects								
Feeding Substrate (FS)								
T1		94.1	38.3^{ab}	32.9^{ab}	10.2^{c}	1.91^{b}	1.21^{a}	24.3^{a}
T2		93.5	41.5^{a}	26.8^{b}	11.4^{c}	2.57^{b}	1.11^{a}	23.0^{ab}
T3		94.5	36.2^{c}	36.4^{a}	13.7^{b}	4.37^{a}	0.85^{b}	23.7^{a}
T4		94.3	36.1^{bc}	29.5^{b}	16.9^{a}	4.98^{a}	1.18^{a}	22.2^{b}
SEM		0.437	1.24	2.12	0.576	0.209	0.061	0.484
Harvesting Time (HT, day)								
15d		94.2	38.25	28.9^{b}	13.3	3.28	1.19^{a}	22.9
20d		94.0	36.27	33.9^{a}	12.9	3.63	0.98^{b}	23.8
SEM		0.309	0.876	1.50	0.407	0.148	0.043	0.25
Probability P> F								
FS		NS	***	*	***	***	**	*
HT		NS	NS	*	NS	NS	**	NS
FS x FA		NS	NS	NS	*	*	NS	NS

Notes: Different superscripts at the same column indicate significant differences ($P < 0.05$); * = significantly different at $P < 0.05$; *** = significantly different at $P < 0.001$; NS = not significantly different ($P > 0.05$); SEM = Standard Error of Mean.

Table 3 describes the indispensable amino acid (IAA) profile of BSFL fed with different feeding substrates and harvested at different times. Except for isoleucine, the interaction between FS and HT did not affect ($p > 0.05$) the indispensable amino acid content of BSFL. The content of isoleucine in BSFs fed with T3 and T4 diets and harvested on day 20 was similar ($p>0.05$) to those fed with the same feeding substrates but harvested on day 15. There was a tendency that as the age increased, the IAA content of BSFL increased. However, the statistical analysis showed that only the content of isoleucine in BSFL fed wth T1 and T2 diets and harvested on day 20 was higher ($p < 0.05$) than those fed with the same feeding substrates but harvested on day 15. This result partially indicated that the isoleucine content of BSFL increased as the age of BSFL increased.

Feeding substrate affected ($p < 0.05$ to <0.01) the content of leucine, phenylalanine, and valine content of BSFL. The leucine content of the BSFLs fed with T2 diet was lower ($p < 0.05$) than those fed with three other treatment diets. The difference in leucine and phenylalanine content of BSFL was probably due to the difference in leucine and phenylalanine content and the digestibility of feeding substrates. The phenylalanine content of BSFL grown in T4 feeding substrates was higher ($p < 0.05$) than those grown in T3 diets. No differences ($p > 0.05$) in valine content were observed in BSFLs fed with T1 and T2 diets and between T3 and T4.

Regarding the second main factor, the statistical analysis showed that the content of leucine, lysine, phenylalanine, and valine of 20d BSFL was higher ($p < 0.05$) than the respective content of leucine, lysine, phenylalanine, and valine of 15d BSFL. These results indicated that it is better to harvest the BSFL on day 20 because the indispensable amino acid profile of 20-day-old BSFL is better than 15-day-old BSFL. In addition, the results indicated that as the age of BSFL increased, the indispensable amino acid content increased. The comparison was difficult to be made because of the limitation of published data related to the present study.

Table 3 Indispensable amino acid (IAA) profile of black soldier fly larvae (BSFL) fed with different feeding substrates and harvested at different times

Feeding Substrate	Harvesting time (day)	Arg	His	Isoleu	Leu	Lys	Meth	Phen	Thr	Val
		……..% as fed……								
T1	15	1.97	1.20	1.51^{b}	3.03	2.57	0.46	1.47	1.48	2.29
	20	2.31	1.29	1.69^{a}	3.23	2.65	0.51	1.51	1.48	2.39
T2	15	2.03	1.19	1.49^{b}	3.01	2.57	0.55	1.38	1.66	2.25
	20	2.08	1.27	1.75^{a}	3.18	2.64	0.51	1.50	1.49	2.39
T3	15	2.13	1.19	1.62^{ab}	3.05	2.53	0.59	1.37	1.57	2.11
	20	2.23	1.25	1.69^{a}	3.20	2.64	0.64	1.54	1.67	2.29
T4	15	2.21	1.36	1.63^{a}	3.22	2.53	0.59	1.55	1.71	2.16
	20	2.15	1.26	1.65^{a}	3.20	2.59	0.54	1.54	1.58	2.17
SEM		0.103	0.040	0.043	0.042	0.047	0.056	0.033	0.07	0.05
Main Effects										
Feeding Substrate (FS)										
T1		2.14	1.25	1.61	3.13^{a}	2.61	0.49	1.49^{ab}	1.48	2.34^{a}
T2		2.05	1.23	1.62	3.09^{b}	2.61	0.53	1.44^{b}	1.58	2.32^{a}
T3		2.18	1.22	1.66	3.13^{a}	2.59	0.61	1.45^{b}	1.62	2.20^{b}
T4		2.18	1.31	1.64	3.22^{a}	2.56	0.57	1.55^{a}	1.64	2.17^{b}
SEM		0.072	0.029	0.030	0.290	0.033	0.039	0.023	0.052	0.033
Harvesting Time (HT, day)										
15d		2.09	1.24	1.57^{b}	3.08^{b}	2.55^{b}	0.55	1.44^{b}	1.60	2.21^{b}
20d		2.19	1.27	1.69^{a}	3.21^{a}	2.63^{a}	0.55	1.52^{a}	1.56	2.31^{a}
SEM		0.051	0.020	0.021	0.021	0.023	0.028	0.016	0.036	0.023
Probability P> F										
FS		NS	NS	NS	*	NS	NS	*	NS	*
HT		NS	NS	***	***	*	NS	**	NS	**
FS x FA		NS	NS	*	NS	NS	NS	NS	NS	NS

Notes: Different superscripts at the same row indicate significant differences ($P < 0.05$); * = significantly different at $P < 0.05$; *** = significantly different at $P < 0.001$; NS = not significantly different ($P > 0.05$); SEM = Standard Error of Mean. Arg = Arginine; His = Histidine; Iso = Isoleucine; Leu = Leucine; Lys = Lysine; Meth = Methionine; Phen = Phenylalanine; Thr = Threonine; Val = Valine.

Regarding the dispensable amino acids (DAA), published data showed that the DAA contents are now considered important in poultry diet formulation. The calculation of DAA such as alanine, aspartic acid, glutamic acid, glycine, serine, and proline in poultry diet had been reported by previous researchers (Awad *et al.* 2015; Siegert & Rodehutscord, 2018; Hofmann *et al.* 2020). According to Awad *et al.* (2015), in which low-protein diets fortified with DAA (glycine, glutamic acid, proline, alanine, and aspartic acid) produce similar body weight gain with the group of birds which were given the balanced nutrition diet (positive control). They also reported that the addition of glycine improved the feed conversion ratio of birds. Thus, it is essential to have a database of the dispensable amino acid profile for each feed ingredient, including BSFL, used in poultry diets. Table 4 describes the profile of DAA of BSFL fed with different feeding substrates and harvested at different times.

Table 4 shows no interaction ($p > 0.05$) between FS and HT was found for the DAA content of BSFL. Regarding the first main factor (FS), the significance ($p < 0.01$ to 0.001) was in the aspartic acid, cysteine, glutamic acid, and proline of BSFL. The aspartic acid and glutamic acid content of BSFLs fed with T3 diets were higher ($p < 0.05$) than the aspartic acid and glutamic acid content of BSFLs fed with T1, T2, and T4 diets. The cysteine content of BSFLs with fed T3, and T4 diets were higher ($p < 0.05$) than those fed with T1 and T2 diets. The present results indicated the feeding substrate containing a high percentage of vegetable wastes (T3) produced the best dispensable amino acid profiles.

Regarding the second main factor, the statistical analysis showed that the content of aspartic acid and serine of 20d BSFL was higher ($p < 0.05$) than the content of aspartic acid, glycine, glutamic acid, and proline of 15d BSFL. These results indicated that it is better to harvest the BSFL on day 20 because the indispensable amino acid profile of 20-day-old BSFL were better than 15-day-old BSFL. In addition, the results indicated that as the age of BSFL increased, 15-day-old indispensable amino acid content increased. The comparison was difficult to be made because of the limitation of published data related to the present study.

Table 4 Dispensable amino acid (DAA) profile of black soldier fly larvae (BSFL) fed with different feeding substrates and harvested at different times

Feeding Substrate	Harvesting time (day)	Ala	Asp	Cys	Glu	Tyr	Ser	Gly	Pro
		……..% as fed……..							
T1	15	0.66	3.51	0.30	4.85	1.11	0.62	1.11	1.08
	20	0.71	3.81	0.31	4.92	1.37	0.71	1.13	1.13
T2	15	0.37	3.06	0.36	4.87	1.07	0.58	1.04	1.13
	20	0.72	3.40	0.32	4.90	1.36	0.75	1.14	1.16
T3	15	0.72	3.53	0.44	4.75	1.16	0.72	1.18	1.42
	20	0.86	3.85	0.49	4.76	1.212	0.78	1.17	1.24
T4	15	0.81	3.87	0.44	5.01	1.15	0.75	1.19	1.38
	20	0.78	4.13	0.41	5.16	1.22	0.77	1.23	1.24
SEM		0.044	0.083	0.035	0.077	0.137	0.052	0.0471	0.063
Main Effects									
Feeding Substrate (FS)									
T1		0.73	3.23^{c}	0.34^{b}	4.89^{b}	1.22	0.67	1.09	1.14^{b}
T2		0.69	3.66^{b}	0.31^{b}	4.89^{b}	1.24	0.67	1.12	1.11^{b}
T3		0.79	4.00^{a}	0.43^{a}	5.09^{a}	1.19	0.76	1.21	1.31^{a}
T4		0.79	3.69^{b}	0.47^{a}	4.76^{b}	1.19	0.75	1.18	1.33^{a}
SEM		0.031	0.059	0.025	0.054	0.097	0.036	0.033	0.045
Harvesting Time (HT, day)									
15d		0.73	3.49^{b}	0.39	4.87	1.12	0.67^{b}	1.13	1.25
20d		0.77	3.79^{a}	0.38	4.94	1.29	0.75^{a}	1.17	1.19
SEM		0.022	0.041	0.017	0.038	0.068	0.026	0.023	0.031
Probability P> F									
FS		NS	***	***	**	NS	NS	NS	**
HT		NS	***	NS	NS	NS	*	NS	NS
FS x FA		NS	NS	NS	NS	NS	NS	NS	NS

Notes: Different superscripts at the same row indicate significant differences ($P < 0.05$); * = significantly different at $P < 0.05$; *** = significantly different at $P < 0.001$; NS = not significantly different ($P > 0.05$); SEM = Standard Error of Mean. Ala = alanine; Asp = aspartic acid; Cys = Cysteine; Glu = glutamic acid; Tir = Tyrosine; Ser = Serine; Gly = glycine; Pro = proline

The IAA and DAA content observed in the present study were lower than those reported by the previous researchers (Lu *et al.* 2022). The difference was probably due to the difference in methodology, especially for feeding substrate and harvested times.

Body Weight Gain (BWG) of Black Soldier Fly Larvae

Statistical analysis showed that the body weight gain (BWG) of BSFL harvested on day 20 was affected ($p < 0.05$) by the feeding substrates. This is in line with Kinasih *et al.* (2018), who reported that the body weight of BSFL was affected by the feeding substrates. Body weight gain of 15d-BSFL was not measured, so it is not presented in this article. As can be seen in Table 5, the BWG of BSFL fed with T2 diets (CP 11.43% and CF 10.64%) ($p < 0.05$) were lower than the BWG of BSFL fed with T1, T3, and T4 diets during the experiment. The difference in BWG may be due to the difference in nutrient composition, palatability, and digestibility of the feeding substrates.

It is interesting to note that the BWG of BSFL fed with T3 diets (CP 4.03% and CF 15.56%) (Table 5) was higher ($p < 0.05$) than the BWG of BSFL fed with T2 (CP11.43% and CF 10.64). Even though the feed intake was not recorded, the high BWG of BSFLs fed with T3 diets was probably due to the high feed intake in this treatment diet. The feed intake was not documented in this experiment because the media used was semi-liquid, so it was difficult to measure. The separation of BSFL and media should have been conducted by water so it would affect the weight of the feed. However, the palatability of the feeding substrate was conducted by observing the leftover of each treatment. The comparison was difficult to be made due to the limitation of publication related to the present research.

The BWG of BSFL fed with T3 diets (CP 4.03% and CF 15.56%) was similar ($p > 0.05$) to those fed with T4 (CP 22.81%; CF 6.56%) and T1 (CP 16.07%; CF 8.11%) diets. The reasons behind these results were: i) the BSFL could degrade fiber so the nutrients needed for growing were fulfilled; ii) the antinutrient level, especially protease inhibitors, in T3 diets was probably lower than in T1 and T4 diets. So, the nutrient digestibility in the T3 diets might be higher than in the T1 and T4 diets. As can be seen in Table 1, T1 and T4 diets contain a high amount of tofu by-products. The tofu by-product was made by soybean seeds, so the by-product may contain protease inhibitors that could inhibit protein digestibility since soybean contain trypsin inhibitory (Aviles-Gaxiola *et al.* 2018). As a consequence, the amount of nutrients absorbed would be low as well, which finally led to a low growth rate. Beniers (2021) reported that trypsin was located in the posterior part of the BSFL midgut, and the trypsin inhibitor may decrease its activity. However, the present result was not in agreement with Kinasih *et al.* (2018), who reported that BSFLs fed with tofu dreg and chicken feed had higher development time compared to those given horse manure and vegetable wastes. The difference was probably due to the difference in methodology.

Supriyatna and Ukit (2016) reported that the BSFL were able to digest organic wastes cellulolytic enzyme, which was produced by cellulolytic bacteria, especially *Bacillus* spp. in the BSFL digestive organ. Felicia and Suhartono (2021) reported that the whole body of BSFL contained proteolytic, amylolytic, chitinolytic, and cellulolytic activities. These enzymes play an important role in nutrient digestion (protein, starch, chitin, and cellulose). Kim *et al.* (2011) extracted gut BSFL and found several enzymes which were high amylase, lipase, and protease activities, trypsin-like protease activity of leucine arylamidase, α-galactosidase, β-galactosidase, α-mannosidase, and α-fucosidase.

Table 5 Growth performance of 20-day-old black soldier fly larvae (BSFL) (g) fed different organic substrates

Variable	Treatment				SEM	*p-value*
	T1	T2	T3	T4		
Initial body weight (g)	0.071	0.074	0.074	0.064		
Final body weight (g)	0.191	0.155	0.201	0.165		
Body weight gain (g)	0.122^{a}	0.082^{b}	0.127^{a}	0.102^{ab}	0.008	0.013

Notes: Different superscripts at the same row indicate significant differences (P < 0.05); SEM = Standard Error of Mean.

The present result did not agree with Spranghers (2020), who reported that the BWG of BSFL will increase as the dietary protein content increases. The difference was probably due to the difference in the feed ingredient used. Each feed ingredient contains a different level of nutrients and anti-nutrients. The experimental diet used by Spranghers (2020) was a chicken diet that was low in anti-nutritional factors. In this experiment, the authors used a self-mixing diet which consists of mostly organic wastes containing high anti-nutrients. The author also found that the shortage of lysine and methionine did not decrease the growth rate of BSFL.

CONCLUSION

Different feeding substrates and harvesting times produced different chemical compositions and amino acid profiles of black soldier fly larvae. The combination of fruit wastes and tofu by-products produced BSFL with high crude protein but low crude lipid, ash, calcium, and phosphorus. The use of tofu by-products as feeding substrates produced BSFL with the lowest crude protein content. Black soldier fly larvae fed with nearly all treatment diets and harvested on day 15 had higher crude protein, calcium, and phosphorus content. The dispensable amino acid profile of Black Soldier Fly Larvae fed with the substrates containing a high percentage of vegetable waste was the best. Feeding substrates containing a high amount of rice bran produced the lowest body weight gain.

REFERENCES

AOAC. 2005. Official Methods of Analysis of AOAC International. 18th Ed. Arlington (US): Association of Official Agricultural Chemists.

AOAC. 2012. Official Methods of Analysis, Association of Official Analytical Chemist 19th edition, Washington D.C., USA.

Aviles-Gaxiola S, Chuck-Hernandez C, and and Sald´ıvar SOS. 2018 Inactivation methods of trypsin inhibitor in legumes: A Review. J Food Sci 83(1): 17-29.

Awad EA, Zulkifli I, Soleimani AF, Loh TC. 2015. Individual non-essential amino acids fortification of a low-protein diet for broilers under the hot and humid tropical climate. Poult Sci 94: 2772-2777.

Barragan-Fonseca1 KB, Dicke M, van Loon JJA. 2017. Nutritional value of the black soldier fly (*Hermetia illucens* L.) and its suitability as animal feed – a review. J Insect Food Feed 3(2): 105-120.

Beniers JJA. 2021 Enzyme localization in the digestive tract of black soldier fly larvae, by freeze-substitution and glycol methacrylate inclusion. Master Thesis. Laval University, Canada.

Bondari K, Sheppard DC. 1987. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner), Aquaculture Res 18(3):209-220 Available from: https://doi.org/10.1111/j.1365-2109.1987.tb00141.x

Bosch G, Zhang S, Dennis GABO, Wouter HH. 2014. Protein quality of insects as potential ingredients for dog and cat foods. J Nut Sci. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4473158/pdf/S2048679014000238a.pdf

Čičková H, Newton, GL, Lacy RC, Kozánek M. 2015. The use of fly larvae for organic waste treatment. Waste Management 35: 68-80.

Dimiyati F. 2021. Pemerintah Sebut Impor Bungkil Kedelai Masih Sangat Besar. [cited December 10, 2022]. Available from https://www.poultryindonesia.com/en/pemerintah-sebut-impor-bungkil-kedelai-masih-sangat-besar/

Ewald N, Vidakovic A, Langeland M, Kiessling A, Sampels S, Lalander C. 2020. Fatty acid composition of black soldier fly larvae (*Hermetia illucens*) – Possibilities and limitations for modification through diet. Waste Manegement 10: 40-47.

Felicia, Suhartono MTW. 2021. Enzyme activitties of black soldier *(Hermetia illucens)* larvae. [cited on December 10, 2023). Available from http://repository.ipb.ac.id/handle/123456789/105979

Hofmann H, Siegert W, Naranjo VD, Rodehutscord M. 2020. Effects of supplemented non essential amino acids and non protein nitrogen on growth and nitrogen excretion characteristics of broiler chickens fed diets with very low crude protein concentrations. Poult Sci 99: 6848.

Hopkins I, Newman LP, Gill H, Danaher J. 2021. The influence of food waste rearing substrates on black soldier fly larvae protein composition: a systematic review. Insects 12(7): 608 Available from**:** https://doi.org/10.3390/insects12070608

Kim W, Bae S, Park K, Lee S, Choi Y, Han S, Koh Y. 201. Biochemical characterization of digestive enzymes in the black soldier fly, *Hermetia illucens* (*Diptera: Stratiomyidae*). J Asia Pac Entomol 14: 11-14.

Kinasih I, Putra RE, Permana AD, Gusmara FF, Nurhadi MY, Anitasari RA. 2018. Growth performance of black soldier fly larvae (*Hermetia illucens*) Fed on

Some Plant Based Organic Wastes. Hayati J Biosc *25*(2): 79. https://doi.org/10.4308/hjb.25.2.79

Lan PTP, Quan NH, Ngoan LD, Hong TTT and Tram NDQ. 2022. Amino acid and fatty acid compositions of black soldier fly larvae (*Hermetia illucens*) fed by Tofu by-products in Viet Nam. Lives Res Rural Dev 34(84).

Lu S, Taethaisong N, Meethip W, Surakhunthod J, Sinpru B, Sroichak T, Archa P, Thongpea, Paengkoum P. 2022. Nutritional composition of black soldier fly larvae (*Hermetia illucens* l.) and its potential uses as alternative protein sources in animal diets: a review. Insects 13:831. Available from: https://doi.org/ 10.3390/insects13090831

Nalle CL, Angi AH, Supit MAJ, Ambarwati S. 2019. Aflatoxin and ochratoxin a contamination in corn grains and sago (*putak* meal) from different sources for poultry in West Timor, Indonesia. Int J Poult Sci 18(8):353-360 Available from: https://dx.doi.org/10.3923/ ijps.2019.353.360

Natalia H, Huda RN, Gautama RG, Pradityo PS, Wijayanti S. 2019. Perkembangan pemasukan bahan pakan asal tumbuhan (BPAT). Direktorat Pakan, Ditjen Peternakan dan kesehatanj Hewan, Kementerian Pertanian Republik Indonesia.

Patterson PH, Acar N, Ferguson AD, Trimble D, Sciubba HB, Koutsos EA. 2021. The impact of dietary Black Soldier Fly larvae oil and meal on laying hen performance and egg quality. Poult Sci 100(8): 1-8.

Permana AD, Putra JENRAE. 2018. Growth of Black Soldier Fly (*Hermetia illucens*) larvae fed on spent coffee ground. IOP Conference Series: Earth Env Sci 187: 012070. Available from: https://iopscience.iop.org/article/10.1088/1755-1315/187/1/012070/pdf

Siegert W, Rodehutscord M. 2018. Non essential amino acids – the forgotten nutrients? World's Poult Sci J. Proceeding The XV th European Poultry Conference. 53-P61.

Spranghers T. 2020. Amino acid composition and requirements of black soldier fly larvae. Report. Bioboost. November 4 2020. [cited on February 2023]. Available from; https://www.bioboosteurope.com/assets/files/BIOBOOST-Report-Amino-acid-composition-and-requirements-of-BSF-larvae.pdf

Supriyatna A, Ukit. 2016a. Screening and isolation of cellulolytic bacteria from gut of black soldier fly larvae (*Hermetia illucens*) feeding with rice straw. J Bio Bio Edu 8(3): 314-320.

Supriyatna A, Manurung R, Esyanti RR, and Putra RE. 2016b. Growth of Black Soldier Larvae fed on cassava peel wastes, an agriculture waste. J Ent Zoo Studies 4(6): 161-165.

Tschirner M, Simon A. 2015. Influence of different growing substrates and processing and the nutrient composition of black soldier fly larvae destined for animal feed. J Insects Food Feed 1(4): 249-259.

BIOTROPIA Vol. 30 No. 3, 2023: 308 - 317 DOI: 10.11598/btb.2023.30.3.1919

EFFECT OF THINNING ON GROWTH AND WOOD PRODUCTION OF NATURALLY REGENERATED 8-YEAR-OLD *ACACIA MANGIUM* WILLD. PLANTATION ON ABANDONED MINING AREA, SOUTHERN THAILAND

JETSADA WONGPROM[1*], SOMPORN MAELIM[2], WASAN CHANDAENG[1], SAKHAN TEEJUNTUK[2], MONTATHIP SOMMEECHAI[2] AND DECHA DUANGNAMON[3]

[1]*Forestry Research Center, Faculty of Forestry, Kasetsart University, Bangkok 10900, Thailand*
[2]*Department of Silviculture, Faculty of Forestry, Kasetsart University, Bangkok 10900, Thailand*
[3]*Andaman Coastal Research Station for Development, Faculty of Fisheries, Kasetsart University, Bangkok 10900, Thailand*

Received 10 March 2023 / Revised 22 August 2023 / Accepted 22 August 2023

ABSTRACT

Thinning is an important practice for promoting growth and maintaining forest plantation for wood production from the remaining trees. In this study, thinning was carried out in a naturally regenerated 8-year-old *Acacia mangium* plot in the Phangnga Forestry Research Station. Three thinning schemes, with 175 (T_1), 300 (T_2) and 600 (T_3) remaining trees/ha, were compared with the control (no thinning) of 831 trees/ha. The diameter at breast height (DBH) and height (H) of the trees were measured. The differences in growth, current annual increment (CAI), aboveground biomass, and stem volume (V) were analyzed. We observed that the thinning of *A. mangium* increased the growth rate, with the DBH being clearly affected by thinning. CAI_{DBH} increased significantly, with the DBH class of thinned *A. mangium* plots also improving after thinning. The stem volume and aboveground biomass of T_3 plot was similar to the control plot after thinning. In addition, the number of large saw logs was the highest in T3 plot. The large saw logs can be used for multi-utilization and have a high value. These results suggest that thinning can promote stem growth, and increase the proportion of large saw logs in naturally regenerated *A. mangium* stands.

Keywords: abandoned mining area, aboveground biomass, *Acacia mangium*, growth, merchantable volume, thinning

INTRODUCTION

Acacia mangium Willd. is a fast-growing multipurpose tree species and is usually found in tropical plantations (Hegde *et al.* 2013). It has been widely planted for soil improvement of degraded lands (Martpalakorn 1990, Majid *et al.* 1998), as it is a nitrogen fixing tree and can supply nutrients back into the forest floor via litter decomposition processes (Fisher & Binkley 2000). *A. mangium* has thus been widely planted in Southeast Asia in commercial plantations (Nambiar & Harwood 2014). A density of approximately 1,100 stem/ha or a spacing of 3 m x 3 m is commonly used while planting *A. mangium*. A high density is usually recommended for trees grown in short-rotation periods (Saharjo 2006), with the rotation for *A. mangium* being between 5-8 years to be used in wood chip and pulp production (Huong *et al.* 2020b). On the other hand, older large trees, usually around 15-year-old, are used in furniture making and to obtain sawn wood (Yahya 1993).

Thinning is a silviculture practice to increase tree growth and stem volume of the remaining trees (Yahya *et al.* 2011; Beadle *et al.* 2013), as well as to improve the stem form and wood quality (Pérez & Kanninen 2005). It is commonly practiced in fast-growing trees species such as *Eucalyptus*, *A. auriculiformis*, and *Acacia* hybrid (Hung *et al.* 2019; Huong *et al.* 2020a). As a management practice in a plantation, thinning is used to reduce the number of trees in a stand, so as to increase the crown space between the remaining trees, to reduce the crown and root competition, and to increase growth. In addition,

*Corresponding author, email: fforjdw@ku.ac.th

thinning can help to control pests and diseases, which in turn can increase the earned incomes before the final harvest (Onyekwelu *et al.* 2011). However, as a downside, the stem volume and aboveground biomass of thinned plantation can decrease.

Past mining activity has had an extreme impact on the soil quality and has severely affected the adjoining ecosystems through the loss of soil structure and nutrient depletion (Thaiutsa & Rungruangsilp 1990; Maiti 2013). The Phangnga Forestry Research Station is located on one such abandoned mining area. The reclamation of mining area was undertaken by planting *A. mangium*, given its growth and high aboveground biomass compared to other fast-growing or native tree species (Martpalakorn 1990). Additionally, *A. mangium* has reportedly been recommended to rehabilitate degraded lands with a high survival rate (Majid *et al.* 1998) to restore the soil properties, forest structure, and nutrient cycling (Wongprom *et al.* 2020; Wongprom *et al.* 2022; Staporn *et al.* 2022).

Seeds of *A. mangium* can accumulate in the soil and forest floor (Saharjo 2006), with the seedlings being highly dense after clear-cutting. A high tree density can result in the reduction of growth rate and yield, accompanied by high mortality due to competition. *A. mangium* plantations mainly focus on producing wood with a short-rotation period but the management of *A. mangium* for the production of large saw logs has been rarely studied. Timber production from natural forests is expected to decline, and as such, *A. mangium* plantations can play an important role in maintaining the commercial supply of wood. Thinning is recommended for trees used in timber and sawn wood production, with larger trees having the potential to increase the income earned (Onyekwelu *et al.* 2011).

The objectives of this study were to identify the effects of thinning on the growth and wood production of a naturally regenerated *A. mangium* plantation on an abandoned mining area. Thinning was applied to stands which were established through natural regeneration after clear-cutting of the *A. mangium* plantation. These results can be used to manage *A. mangium* stand in the Phangnga Forestry Research Station and other such degraded sites located in southern Thailand.

MATERIALS AND METHODS

Study site

The plantation is located on an abandoned area, previously under tin mining, at the Phangnga Forestry Research Station (8° 46' 5" N, 98° 16' 7" E), Takuapa district, Phangnga province, in southern Thailand (Fig. 1). Tin mining was done through the gravel pumping method. The post tin mining land forms could be mainly divided into sand, clay, and gravel areas. The soil nutrients and organic matter levels after tin mining were very low and the soil pH was strongly acidic (Anunsiriwat 1986). However, soil nutrients and organic matter content of this area were improved after the establishment of *A. mangium*, especially the topsoil level. The soil properties of this area are shown in Table 1 (Wachrinrat *et al.* 2002). The area receives an annual rainfall of 3,566 mm, with the rainy season spanning from April to October and dry season occurring during the months from November to March. The mean temperature was around 27.1°C and relative humidity around 83% (Wachrinrat *et al.* 2002).

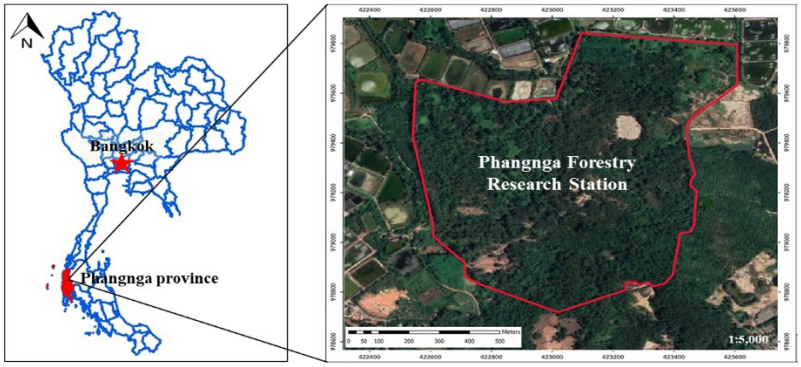

Figure 1 Location of the study sites in Phangnga province, southern Thailand

Table 1 Soil properties of *A. mangium* plantation in clay area at Phangnga Forestry Research Station

Soil depth (cm)	Sand (%)	Silt (%)	Clay (%)	Soil texture	pH	OM (%)	Avai. P (ppm)	Exchangeable bases (ppm)		
								K	Ca	Mg
0-7	10.40	30.72	58.88	clay	5.0	3.84	1.01	62.2	153.44	54.44
7-32	0.40	24.72	74.88	clay	4.9	0.91	0.03	38.4	11.68	67.34

Note: OM = organic matter, Avai. P = available phosphorus, K = potassium, Ca = calcium, Mg = magnesium.

In 1987, *A. mangium* was planted at a spacing of 4 m x 4 m, to reclaim the clayey soil (Wachrinrat *et al.* 2002). However, a clear cutting of *A. mangium* plantation was done for wood utilization when the trees were 14-year-old. After the area was cleared by cutting, the site was prepared by burning the branches and other parts of the trees. *A. mangium* seedlings were then allowed to naturally regenerate in the area and this resulted in a high density stand.

Methods

The experiment plots were located in an 8-year-old naturally regenerated *A. mangium* plantation. We observed that the crown cover of *A. mangium* stand was closed leading to a strong crown competition, which meant that thinning was needed for plantation management. A randomized completely block design (RCBD) with three replications was used in a plot of size 20 m x 20 m. Three different thinning schemes were used and included 175 (T_1), 300 (T_2), and 600 (T_3) remaining trees per ha which were compared with a control of 831 trees/ha. A low thinning method was used in T_1, T_2, and T_3 plots. The low thinning scheme was used to remove the suppressed and poor crown trees (Hawley 1947). In this study, any small and irregular trees were removed in the thinned plots. In addition, a good stem form of the co-dominant and intermediate trees in the T_1, T_2, and T_3 plots were also removed to determine the spacing and tree density.

The diameter at breast height (DBH) and height (H) of remaining trees were measured for a period of three years. The DBH and H of each treatment was calculated as a mean among replications (n = 3). The current annual increment (CAI) of H and DBH were calculated using the equations:

$CAI_{DBH} = (DBH_2 - DBH_1)/ t_2-t_1$
$CAI_H = (H_2 - H_1)/ t_2-t_1$

Where:

H_1 = tree heights (m) at times t_1
H_2 = tree heights (m) at times t_2t
DBH_1 = diameters (cm) at times t_1
DBH_2 = diameters (cm) at times t_2
t_1 = beginning times of each period
t_2 = ending times of each period.

Allometric equations of the 11-year-old *A. mangium* trees were developed to estimate the aboveground biomass and stem volume, as the estimation equation previously reported for woodchip was for trees within an age bracket of 4-5 years in Thailand (Peawsa-ad & Viriyabuncha 2002). In this study, trees sampled from seven different DBH classes (8.4 to 34.9 cm DBH) were cut and separated as logs according to their respective heights binned under 0-0.3 m, 0.3-1.3 m, 1.3-2.3 m, etc., i.e., at every 1.0 m increment from the bottom to top. The tree height and diameter of the logs were measured. The fresh weights of stem, branch, and leaf components were determined in the field and representative samples were taken from each tree to determine the dry weight. The stem, branch and leaf samples were oven-dried at 80°C for 48 h to obtain a constant weight.

The aboveground biomass, i.e., stem (W_S in kg), branches (W_B in kg), and leaf (W_L in kg), of *A. mangium* was estimated using the allometric equations derived using destructive sampling as follows:

$W_S = 0.0199*(DBH^2*H)^{0.9828}$, ($R^2 = 0.98$)
$W_B = 0.0001*(DBH^2*H)^{1.3345}$, ($R^2 = 0.99$)
$W_L = 0.0009*(DBH^2*H)^{0.9773}$, ($R^2 = 0.94$)
$W_T = W_S+W_B+W_L$,

Where:

W_T = total aboveground biomass
DBH = diameter at breast height (cm)
H = total height (m)

The volume of each log was calculated using the Smalian's formula:

$$V = (BA_1+BA_2)/2 \times L$$

Where:

V = log volume (m^3)
BA_1 = upper cross section area of the log
BA_2 = lower cross section area of the log
L = length of the log

The total stem volume (V_T) and merchantable volume (V_M) was estimated using the equation derived from destructive sampling. The merchantable volume was set at a top end diameter > 10.0 cm, as determined by the local wood sawmill;

$V_T = 0.0395*(DBH) - 0.3369$, ($R^2 = 0.98$)
$V_M = 0.0403*(DBH) - 0.4030$. ($R^2 = 0.98$)

Allometric equations for DBH and stem-log types were developed from seven representative trees (with DBH between 8.4 to 34.9 cm). Sawlog types were categorized as either small (V_{SSL}; diameter of log (D) $10.0 < D \leq 15.0$ cm and as a percentage of merchantable volume), medium (V_{MSL}; $15.0 < D \leq 20.0$ cm), or large (V_{LSL}; D > 20.0 cm). V_{SSL} and V_{LSL} were determined by establishing the respective allometric equations. V_{MSL} was determined as the difference between the merchantable volume (100%) and the sum of V_{SSL} and V_{LSL}. The sum of V_{SSL}, V_{MSL}, and V_{LSL} was equal to V_M.

The log components of each tree were estimated as a percentage of the merchantable volume (V_M = 100%) according to the following equations:

$V_{SSL} = 418.76e^{-0.137*(DBH)}$, ($R^2 = 0.97$)
$V_{LSL} = 8.9603e^{0.0677*(DBH)}$, ($R^2 = 0.77$)
$V_{MSL} = 100 - (V_{SSL} + V_{LSL})$
$V_M = V_{SSL} + V_{MSL} + V_{LSL}$,

where V_M is the merchantable volume (m^3).

Data Analysis

Growth performance, as indicated by DBH, H, CAI_{DBH} and CAI_H, the aboveground biomass of trees, stem volume, merchantable volume, and saw logs among the various treatments was compared using SPSS 16.0. A one-way analysis of variance (ANOVA) followed by Tukey HSD was used to determine the differences between means at a 5% probability level.

RESULTS AND DISCUSSION

Tree growth

Thinning had a positive influence on the growth of *A. mangium*, although the DBH and H were not found to be significantly different ($p > 0.05$) during the initial stages of development. The parameters measured during the initial stages of growth and development after thinning are listed in Table 2. After thinning for one year, the DBH and H of trees in the T_1 treatment increased rapidly compared with the control plot and were significantly different ($p < 0.05$). It has been previously reported that *A. mangium* grows well after an early thinning (Yahya 1993). Heavy thinning is an important factor influencing the DBH with its incremental change significantly and positively correlated with the thinning intensity (Mäkinen & Isomäki 2004; Juodvalkis *et al.* 2005). In this study, the small and suppressed trees were removed by thinning. This resulted in a structured stand, with a marked increase in measured DBH. The size distribution moved towards normality and then became positively skewed, and was affected by low intensity thinning. Larger DBH values were found in thinned plots, while trees with smaller DBH were frequently found in the unthinned plot (Fig. 2).

CAI_{DBH} was the highest in the T_1 plot, followed by T_2, T_3, and control, respectively. The density of *A. mangium* significantly influenced the CAI_{DBH} after thinning during the first and second years, but no significant difference was observed in the third year. CAI_{DBH} of trees in the thinned plots peaked during the second year. However, CAI_{DBH} tended to decrease and was not significantly different among treatments after thinning for three years. A rapid reduction in CAI_{DBH} from 2.47 cm/cm/yr to 1.03 cm/cm/yr under thinning for 1-3 years in the T_1 plot indicates a strong competition between the remaining trees. A dense *A. mangium* canopy was observed after thinning for three years. A crown competition within the stand led to the death of small and suppressed trees. A high relative mortality rate of 21.98% was observed in the unthinned plot.

CAI_H was significantly different after the thinning for one year, but was not significantly different after thinning for 2-3 years. This indicates that the increase in height was affected

by thinning only during the early period. We observed that the thinning intensity only slightly affected the increase in height and is similar to results reported previously for many plantations (Wanthongchai & Sahunalu 2002; Mäkinen & Isomäki 2004; Cicek *et al.* 2013; Rytter 2013).

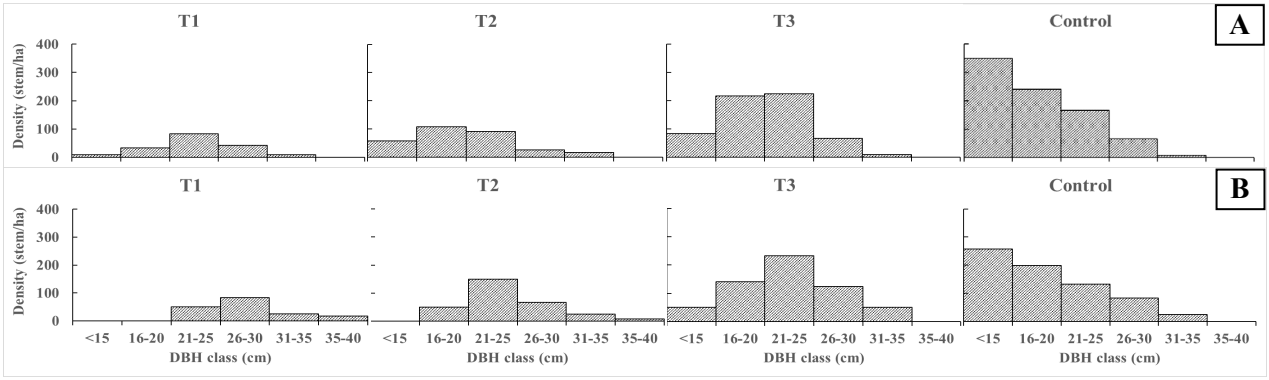

Figure 2 Distribution of DBH classes of the thinned *A. mangium* during the initial stages of development (A) and after three years of thinning (B) at the Phangnga Forestry Research Station

Table 2 Diameter at breast height (DBH), height (H), and current annual increment (CAI) of the thinned *A. mangium* plots after thinning for one, two, and three years at the Phangnga Forestry Research Station

Treatment	DBH (cm)	H (m)	CAI	
			DBH (cm)	H (m)
Before thinning				
T_1	21.94 ± 1.95	24.18 ± 0.53	-	-
T_2	19.91 ± 1.20	23.56 ± 0.76	-	-
T_3	19.47 ± 1.57	23.17 ± 0.71	-	-
control	17.24 ± 2.23	21.79 ± 2.59	-	-
F- value	2.58^{ns}	3.40^{ns}	-	-
After thinning for one year				
T_1	25.14 ± 1.96^{b}	26.22 ± 0.72^{b}	2.47 ± 0.26^{c}	1.20 ± 0.04^{c}
T_2	22.34 ± 1.16^{ab}	24.84 ± 0.47^{b}	1.80 ± 0.24^{b}	0.87 ± 0.13^{b}
T_3	21.22 ± 1.49^{ab}	24.38 ± 0.64^{b}	1.40 ± 0.27^{b}	0.67 ± 0.14^{b}
control	17.56 ± 3.03^{a}	22.29 ± 1.18^{a}	0.64 ± 0.25^{a}	0.54 ± 0.12^{a}
F- value	7.13^{*}	12.57^{**}	26.74^{**}	19.14^{**}
After thinning for two years				
T_1	27.65 ± 2.52^{c}	27.19 ± 1.00^{b}	2.51 ± 0.74^{c}	0.96 ± 0.33
T_2	24.30 ± 1.25^{bc}	25.69 ± 0.50^{b}	1.96 ± 0.13^{bc}	0.85 ± 0.10
T_3	22.85 ± 1.39^{b}	25.10 ± 0.56^{b}	1.63 ± 0.11^{b}	0.72 ± 0.10
control	18.33 ± 3.19^{a}	22.94 ± 1.74^{a}	0.77 ± 0.27^{a}	0.65 ± 0.62
F- value	8.98^{**}	8.15^{**}	9.90^{**}	0.47^{ns}
After thinning for three years				
T_1	28.65 ± 2.55^{b}	27.58 ± 0.99^{b}	1.03 ± 0.13	0.39 ± 0.10
T_2	25.28 ± 1.15^{b}	25.99 ± 0.40^{b}	0.98 ± 0.14	0.38 ± 0.15
T_3	23.71 ± 1.12^{ab}	25.48 ± 0.44^{ab}	0.86 ± 0.30	0.29 ± 0.14
control	18.93 ± 3.36^{a}	23.08 ± 1.70^{a}	0.60 ± 0.22	0.14 ± 0.10
F- value	9.62^{**}	9.82^{**}	2.36^{ns}	3.64^{ns}

Notes: * = significantly different at $p < 0.05$; ** = significantly different at $p < 0.01$; ns = not significant at $p > 0.05$ and letters a, b and c in the same column indicate significant differences at $p < 0.05$ and $p < 0.01$, as determined by Tukey HSD.

Aboveground biomass

The aboveground biomass among treatments was significantly different after thinning for years 1-3 (Table 3). In the thinned plots, the aboveground biomass from all parts of a tree was the highest for T_3 plot, while that for T_1 plot was the lowest. However, the aboveground biomass of the T_3 and control plots was similar. Differences in density can significantly affect the production of plantation. This is evident from our observation that the aboveground biomass estimated for the T_3 (169.89 t/ha) and control (185.74 t/ha) plots was relatively high, but was lower than that of a 10-year-old *A. mangium* plantation (1,050 stem/ha) planted on degraded lands in Indonesia, with value of 241.10 t/ha (Hardiyanto *et al.* 2004).

Stem biomass contributed the most to the main productivity, accounting for approximately 81-84% of the total aboveground biomass. On the other hand, the contribution of leaf component to the biomass pool was the lowest (4%). Leaf production of *A. mangium* is not the main objective of a commercial plantation. However, the contributions of leaf biomass and leaf litter were reported to be the most significant in terms of nutrient return to the forest floor (Nambiar & Hardwood 2014; Wongprom *et al.* 2022), as *A. mangium* leaf is rich in nutrient concentration, especially nitrogen. It therefore plays an important role in increasing the soil nutrients, improving the soil properties (Wongprom *et al.* 2020), and promoting nutrient supply for stand growth (Hardiyanto & Nambiar 2014). Previously, it has been reported that the wood production in an *Acacia* plantation is strongly correlated with the soil nutrients (Harwood *et al.* 2017). This observation was similar to Huong *et al.* (2020b), who found that four rotations in *A. auriculiformis* plantation resulted in high growth and production compared to the first rotation.

Table 3 Aboveground biomass of the 8-year-old *A. mangium* under various thinning durations and intensities

Treatment	Aboveground biomass (ton/ha)			
	W_S	W_B	W_L	W_T
After thinning for one year				
T_1	52.02 ± 8.53^a	8.16 ± 1.94^a	2.65 ± 0.47^a	62.83 ± 10.94^a
T_2	69.03 ± 6.17^a	10.34 ± 1.79^{ab}	3.49 ± 0.35^a	82.86 ± 8.33^a
T_3	119.95 ± 21.04^c	16.34 ± 4.33 ^{ab}	5.99 ± 1.13^b	142.28 ± 24.84^b
control	140.77 ± 10.70^c	17.22 ± 0.11^{ab}	5.03 ± 0.43^b	163.02 ± 11.05^b
F- value	33.68**	8.49*	28.03**	30.62**
After thinning for two years				
T_1	61.99 ± 12.05^a	10.60 ± 2.99^a	3.21 ± 0.67^a	75.80 ± 15.71^a
T_2	83.16 ± 7.08^a	13.67 ± 2.40^{ab}	4.27 ± 0.41^a	101.10 ± 9.77^a
T_3	141.67 ± 22.63^b	21.05 ± 5.16^b	7.17 ± 1.24^b	169.89 ± 27.01^b
control	156.38 ± 7.16^b	20.61 ± 0.91^b	7.75 ± 0.25^b	185.74 ± 6.54^b
F- value	33.38**	7.05*	26.51**	30.78**
After thinning for three years				
T_1	66.65 ± 13.09^a	11.77 ± 3.41^a	3.47 ± 0.73^a	81.89 ± 17.24^a
T_2	90.42 ± 5.14^a	15.05 ± 2.57^{ab}	4.64 ± 0.34^a	110.11 ± 7.91^a
T_3	152.53 ± 19.82^b	23.27 ± 4.66^b	7.75 ± 1.09^b	183.55 ± 25.54^b
control	161.46 ± 6.61^b	21.79 ± 1.00^b	7.92 ± 0.28^b	191.17 ± 6.15^b
F- value	42.41**	8.01*	30.89**	37.63**

Notes: * = indicates a significant difference at $p < 0.05$; ** = significantly different at $p < 0.01$; letters a, b, and c in the same column indicate significant difference at $p < 0.05$, as determined by Tukey HSD. W_S, W_B, W_L, and W_T are the stem, branches, leaf biomass, and total aboveground biomass, respectively.

Stem volume, merchantable volume, and sawlogs

After thinning for three years, the stem and merchantable volumes of the thinned and control *A. mangium* plots were significantly different ($p<0.01$) (Table 4). In addition, the production of small, medium, and large saw logs was also significantly different among the treatments. However, stem and merchantable volumes of the T_3 and control plots were similar. Thinning intervention positively affected the diameter of logs. Heavy thinning in the T_1 plot resulted in a high proportion of large saw logs (67% of merchantable volume). The proportion of large saw logs in the thinned plots was higher than both the medium and small sized saw logs. In contrast, the proportion of small saw logs was high in the unthinned plot (25%), while those in the T_1, T_2, and T_3 was 9, 14 and 16%, respectively. This indicates that thinning increased the merchantable volume and the number of large saw logs. For 9-year-old *A. auriculiformis* plantation (1,333 stem/ha), the medium sized saw logs formed the major proportion of saw logs obtained from the unthinned plot (Huong *et al.* 2020a). Thinning is important for improving the DBH class of a stand as well as the merchantable volume and is usually used as a plantation management strategy to increase growth and utilization.

For 9 and 10 year plantations, the current annual increment of volume (CAI_V) was found to be significantly different ($p<0.01$), with the T_3 plot having the highest CAI_V. However, CAI_V of the T_3 and unthinned plots was similar for the 11-year-old plantation. Low thinning resulted in a significant increase of stand stem volume (Fig. 3). After thinning for one and two years, CAI_V of the T_1 and T_2 plots increased, while the values were not different for the unthinned plot. Heavy and moderate thinning intensity had a significant influence on the increment in stem volume of the *A. mangium* plot, given that the trees had a positive response to thinning.

Table 4 Stem volume, merchantable volume, and saw logs of the thinned 11-year-old *Acacia mangium* stand under various thinning intensities

Treatment	Stem volume (m^3/ha)	Merchantable volume (m^3/ha)	Sawlogs (m^3/ha)		
			Small	Medium	Large
T_1	135.83 ± 16.79[a]	127.92 ± 17.37[a]	11.25 ± 1.75[a] (9%)	31.25 ± 6.75[a] (24%)	85.42 ± 21.11[a] (67%)
T_2	187.92 ± 6.71[b]	180.99 ± 13.65[b]	25.75 ± 5.88[b] (14%)	47.83 ± 10.07[a] (27%)	107.41 ± 23.46[a] (59%)
T_3	353.83 ± 23.76[c]	326.19 ± 23.73[c]	51.00 ± 3.54[c] (16%)	101.08 ± 7.44[b] (31%)	174.16 ± 24.56[b] (53%)
control	351.10 ± 22.33[c]	298.44 ± 15.27[c]	74.60 ± 10.69[d] (25%)	95.49 ± 7.07[b] (32%)	128.33 ± 12.57[a] (43%)
F-value	145.57**	92.44**	165.70**	51.05**	7.54*

Notes: * = indicates a significant difference at $p < 0.05$; ** = significantly different at $p < 0.01$; letters a and b in the same column indicate significant difference at $p < 0.05$, as determined by Tukey HSD. The numbers in brackets are percentage of saw log types estimated for each treatment.

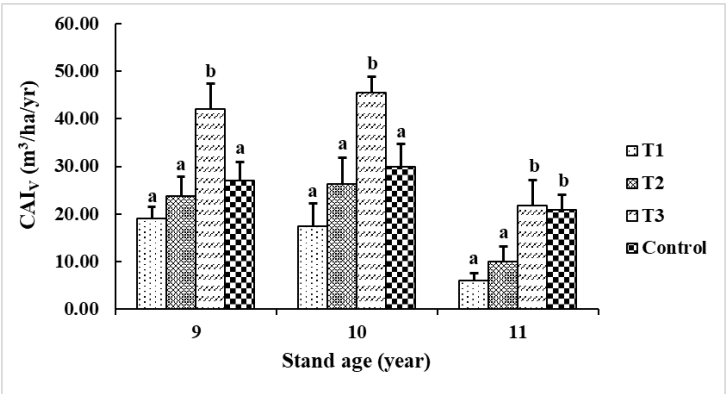

Figure 3 The current annual increment of stem volume (CAI_V) of the thinned 9 to 11-year-old *A. mangium* plots under various thinning durations

In southern Thailand, the local sawmills have a high demand for large saw logs to make wood furniture, as these products fetch a higher value compared to woodchips. In Thailand, during the year 2021, the timber imported was approximately 220,000 m^3 (Royal Forestry Department 2021). As such, fast-growing tree plantations can reduce the import bill related to import of timber wood, while also reducing the illegal cutting of natural forests. Thinning can be a valuable management strategy in plantations to increase the domestic timber production. A short rotation period (4-7 years) is generally used for the production of fuelwood and woodchips from *A. mangium* plantations.

The growth, aboveground biomass, and stem volume estimates reported in this study are based on a plantation established on poor soil conditions in an abandoned mining area where soil nutrients and organic matter were very low (Anunsiriwat 1986; Thaiutsa & Rungruangsilp 1990). However, *A. mangium* was able to grow well in this area as the site receives a high amount of rainfall (more than 3,500 mm/yr), with a rainfall of more than 2,500 mm/yr being suitable for the optimum growth of *A. mangium* (National Research Council 1983). The DBH and H of the T_2 plot (24.30 cm and 25.69 m, respectively) in the 10-year-old *A. mangium* plantation was similar to that of *A. mangium* planted in West Java, Indonesia, with a DBH, H, and density of 27.81 cm, 25.12 m, and 225 stem/ha, respectively (Heriansyah *et al.* 2007). In addition, the estimated total aboveground biomass and stem volume in this study was not different from that of other areas with a similar stand density. For example, the aboveground biomass estimated for the lightly thinned 11-year-old *A. mangium* plantation, with a density of 600 stem/ha was similar to that of an *A. mangium* plantation in southeastern, Vietnam (Cuong *et al.* 2020).

CONCLUSION

In this study, we presented the results of thinning on the growth of *A. mangium* trees in a naturally regenerated 8-year-old plantation in the Phangnga Forestry Research Station, with three different thinning schemes, which were compared with the control (unthinned plot). The growth and production of the remaining trees after thinning were observed to be affected by thinning. After thinning, the DBH and CAI_{DBH} of trees in the thinned plots were found to be significantly higher than those in the control plot. However, tree height was only slightly affected by thinning. A reduction in CAI_{DBH} was observed after thinning for three years, possibly resulting from a stronger competition for resources. The total aboveground biomass and stem volume in the lightly thinned T_3 plot was similar to that of the control plot. The thinning intensity significantly affected the growth and productivity of the *A. mangium* stand. CAI_V of the T_3 plot was relatively higher than that of the T_1 and T_2 plots. However, the large saw logs obtained from the T_3 and unthinned plots was significantly different, suggesting that thinning should be done for promoting stem growth and to obtain large sized timber wood.

ACKNOWLEDGEMENTS

This work was partially funded by the National Research Council of Thailand through the Knowledge Hub for Integrated Economic Trees: Plantation Establishment, Management, Utilization and Industry. We thank Dr. Atsushi Sakai and Asst. Prof. Dr. Kobsak Wanthongchai for their comments and help in improving this paper. We are grateful to the staff at the Phangnga Forestry Research Station for their assistance during the field work.

REFERENCES

Anunsiriwat A. 1986. Biomass production and some soil properties of mine spoils at Bangmuang, Takua Pa, Phangnga [Dissertation]. Retrieved from Kasetsart University.

Beadle CL, Trieu DT, Wood CE. 2013. Thinning increase saw-log values in fast-growing plantations of Acacia hybrid in Vietnam. J Trop For Sci 25: 42-51.

Cicek E, Yilmaz F, Ozbayram KA, Efe M, Yilmaz M, Usta A. 2013. Effects of thinning intensity on the growth of narrow-leaved ash (*Fraxinus angustifolia* sub sp. *oxycarpa*) plantations. Turk. J Agric For 37: 97-104.

Cuong L, Hung B, Bolanle-Ojo OT, Xu X, Thanh N, Chai L, Legesse N, Wang J, Thang B. 2020. Biomass and carbon storage in an age-sequence of *Acacia mangium* plantation forest in Southeastern region, Vietnam. For Syst 29: e009.

Fisher RF, Binkley D. 2000. Ecology and management of forest soils. John Wiley & Sons, New York.

Hardiyanto EB, Anshori S, Sulistyono D. 2004. Early results of site management in *Acacia mangium* plantations at PT Musi Hutan Persada, South Sumatra, Indonesia. In: NAMBIAR EKS, Tiarks A, Toma T, Ranger J, editors. Site Management and Productivity in Tropical Plantation Forests. Proceedings: 2001 Jul in Congo and 2003 Feb in China. Center for International Forestry Research, Bogor. p. 93-108.

Hardiyanto EB, Nambiar EKS. 2014. Productivity of successive rotations of *Acacia mangium* plantations in Sumatra, Indonesia: impacts of harvest and inter-rotation site management. New For 45: 557-75.

Harwood CE, Nambiar EKS, Dinh PX, Toan LX, Quang LT. 2017. Managing wood production from small grower acacia hybrid plantations on eroded soils in central Vietnam. Aust For 80(5): 286-93.

Hawley RC. 1947. The practice of silviculture. John Wiley and Sons, Inc. New York.

Hegde M, Palanisamy K, Yi JS. 2013. *Acacia mangium* Willd. - A fast growing tree for tropical plantation. J For Sci 29: 1-14.

Heriansyah I, Miyakuni K, Kato T, Kiyono Y, Kanazawa Y. 2007. Growth characteristics and biomass accumulation of *Acacia mangium* under different management practices in Indonesia. J Trop For Sci 19: 226-35.

Hung Tt, Almeida AC, Eyles A, Ratkowsky D, Lam VT, Mohammed C. 2019. Maximising growth and sawlog production from *Acacia* hybrid plantations in Vietnam. New For 50: 785-804.

Huong VD, Mendham DS, Beadle C, Hai NX, Close DC. 2020a. Growth, physiological responses and wood production of an *Acacia auriculiformis* plantation in southern Vietnam following mid-rotation thinning, application of phosphorus fertiliser and organic matter retention. For Ecol Manage 472: 118211.

Huong VD, Nambiar EKS, Hai NX, Ha KM, Dang NV. 2020b. Sustainable management of *Acacia auriculiformis* plantations for wood production over four successive rotations in south Vietnam. Forests 11: 550.

Juodvalkis A, Kairiukstis L, Vasiliauskas R. 2005. Effects of thinning on growth of six tree species in north-temperate forests of Lithuania. Eur J For Res 124: 187-92.

Maiti SK. 2013. Ecorestoration of the Coalmine Degraded Lands. Springer, India.

Majid NA, Muhamad N, Paudyal BKZ, Shebli BT. 1998. Survival and early growth of *Acacia mangium*, *Ceiba pentandra* and *Casuarina equisetifolia* on sandy tin tailings. Pertanika J Trop Agric Sci 21: 59-65.

Mäkinen H, Isomäki A. 2004. Thinning intensity and growth of Norway spruce stands in Finland. Forestry 77: 349-64.

Martpalakorn M. 1990. Tree species trials on mined spoils at Amphoe Takuapa, Changwat Phangnga [Dissertation]. Retrieved from Kasetsart University.

National Research Council. 1983. Mangium and other fast-growing Acacias for the humid tropics. National Academy Press, Washington, DC.

Nambiar EKS, Harwood CE. 2014. Productivity of acacia and eucalypt plantations in Southeast Asia. 1. Bio-physical determinants of production: opportunities and challenges. Int For Rev 16: 225-48.

Onyekwelu JC, Stimm B, Evans J. 2011. Plantation forestry. In: Gunter S, editors. Silviculture in the Tropics. Springer, Berlin. p. 399-454.

Pérez D, Kanninen M. 2005. Effect of thinning on stem form and wood characteristics of Teak (*Tectona grandis*) in a humid tropical site in Costa Rica. Silva Fenn 39: 217-25.

Peawsa-Ad K, Viriyabuncha C. 2002. Growth and aboveground biomass of *Acacia mangium* Willd. In: Silvicultural Research Report. Silviculture Research Division, Royal Forest Department, Bangkok. p. 130-46.

Royal Forestry Department. 2021. Forestry statistics data 2021. Royal Forestry Department, Bangkok.

Rytter L. 2013. Growth dynamics of hardwood stands during the precommercial thinning phase-Recovery of retained stems after competition release. For Ecol Manage 302: 264-72.

Saharjo BH. 2006. The role of industrial forest plantations in supporting pulp and paper industries: A case study in south Sumatra Indonesia. In: Suzuki K, Katsuaki I, Shobu S, Satohiko S, editors. Plantation Technology in Tropical Forest Science. Springer-Verlag, Tokyo. p 213-20.

Staporn D, Marod D, Wongprom J, Diloksumpun S. 2022. Drivers of native species regeneration in the process of restoring a dry evergreen forest from exotic tree plantations in northeastern Thailand. Forests 13: 1321.

Thaiutsa B, Rungruangsilp C. 1990. Impacts of tin mining on soil properties. Thai J For 9: 73-82.

Wachrinrat C, Sahunalu P, Sungpalee W, Nualngam S, Meunpong P, Srikongpan R, Thawornwong L, Thaiutsa B. 2002. Effect of *Acacia mangium* Willd. plantation in tin-mine soils on yield and soil characteristics at Bangmuang Forestry Student Training Station, Takuapa, Phangnga. In: 7th Silvicultural Seminar. Proceedings: 2001 Dec 12-14. Kasetsart University Bangkok. p. 261-71.

Wanthongchai K, Sahunalu P. 2002. Effect of thinning on growth and yield of *Eucalyptus camaldulensis* Dehnh. plantation. Thai J For 19-21: 9-20.

Wongprom J, Poolsiri R, Diloksumpun S, Ngernsaengsaruay C. 2020. Soil properties and tree composition in a 27-year-old *Acacia mangium* Willd. plantation on abandoned mining area at Phangnga Forestry Research Station. Biotropia 27: 125-33.

Wongprom J, Poolsiri R, Diloksumpun S, Ngernsaengsaruay C, Tansakul S, Chandaeng W. 2020. Litterfall, litter decomposition and nutrient return of rehabilitated mining areas and natural forest in Phangnga Forestry Research Station, southern Thailand. Biotropia 29: 69-80.

Yahya AZ. 1993. Growth of *Acacia mangium* during three years following thinning: preliminary results. J Trop For Sci 6: 171-80.

Yahya AZ, Saaiffudin KA, Hashim MN. 2011. Growth response and yield of plantation-grown Teak (*Tectona grandis*) after low thinning treatments at Pagoh, Peninsular Malaysia. J Trop For Sci 23: 453-59.

THE CHEMICAL CHARACTERISTICS OF ARABICA AND ROBUSTA GREEN COFFEE BEANS FROM GEOPARK RINJANI, INDONESIA

ZAINURI[1*], DEWA NYOMAN ADI PARAMARTHA[1], AMIRA FATINAH[1], RINI NOFRIDA[1], NOVIA RAHAYU[1], INES MARISYA DWI ANGGRAINI [1] AND QABUL DINANTA UTAMA[1]

[1]*Food Science and Technology Study Program, Faculty of Food Technology and Agroindustry, University of Mataram, Mataram 83125, Indonesia*

Received 8 March 2023 / Revised 26 July 2023 / Accepted 12 September 2023

ABSTRACT

Green coffee beans are also called unroasted coffee beans. The chemical composition of green coffee beans plays a vital role in determining the final product's aroma. The main objective of this research was to characterize the chemical properties of Arabica and Robusta green coffee beans grown in different regions of Geopark Rinjani, Lombok Island, Indonesia. The water, ash, protein, carbohydrate, total solids, and caffeine contents, total acidity, and pH were determined. Data were analyzed by analysis of variance. The results revealed significant differences in the moisture contents of Arabica beans from Sembalun, Sajang, and Sapit and Robusta beans from Rempek, Selelos, and Genggelang. Additionally, the ash contents of Arabica Sajang and Arabica Sapit showed notable differences compared to other samples. However, the total fat contents of Arabica Sajang, Arabica Sapit, Robusta Rempek, and Robusta Genggelang did not exhibit significant variations. On the other hand, significant differences were observed in the protein contents of all samples, particularly between Arabica Sembalun and Arabica Sapit, compared to Arabica Sajang, Robusta Rempek, Robusta Genggelang, and Robusta Selelos. Robusta coffee beans appeared to have a slightly lower pH than Arabica beans. The latter exhibited consistent acidity in the range of 0.20–0.21, whereas the former showed higher acidity levels (0.23–0.25). Arabica beans had a lower caffeine content, averaging 1.09%, whereas Robusta beans exhibited an average caffeine content of 2.09%. This research provides valuable insights into the chemical composition of green coffee beans from different species and locations within Geopark Rinjani, contributing to a better understanding of the factors influencing the aroma and quality of coffee.

Keywords: arabica coffee, geopark rinjani, green coffee bean, robusta coffee

INTRODUCTION

There are 130 coffee species identified worldwide but only two are responsible for most of the global coffee trade. *Coffea arabica* L. and *Coffea canephora* account for approximately 60–65% and 35–40% of the global coffee production, respectively. The coffee industry is a substantial contributor to the global economy, with an annual production of approximately 10 million tons and an income of approximately USD 200 million. This industry also supports the livelihoods of millions of smallholder farmers, who account for approximately 60% of coffee farms in tropical regions (Cassamo *et al.* 2022).

The quality of coffee beans is primarily determined by their physical and chemical properties, which are closely linked to the coffee species/genotype, microclimate conditions during the fruit and bean maturation process, soil characteristics, and agricultural crop management practices. Environmental conditions during fruit development also substantially affect the final bean quality (Ahmed *et al.* 2021). Shade and altitude positively affect coffee bean quality and are vital in producing high-quality coffee. Higher altitudes and shade at lower altitudes may enhance the physical and chemical properties of the beans as a result of the lower temperatures and higher air humidity. These climate conditions can extend the fruit and bean maturation period,

*Corresponding author, email: zainuri.ftp@unram.ac.id

increasing the accumulation of sugars and aroma-related phenolic compounds. In addition, Mendes *et al.* (2022) reported that beans grown at higher altitudes tend to have higher levels of sugars, lipids, amino acids, trigonelline, and chlorogenic acid isomers, leading to higher-quality coffees compared to beans from lower altitudes. By contrast, coffees of the same genotype grown at lower altitudes have lower quality and lower levels of the abovementioned compounds.

The chemical composition of green coffee beans plays a vital role in determining the final product's aroma. The Maillard reaction, which occurs during roasting, is the primary process that generates aroma through a reaction between amino acids and reducing sugars, forming nitrogenous heterocycles and brown melanoidins. This non-enzymatic browning generates many volatile compounds that shape the sensory experience of coffee. Arabica has a sweet, caramel-like aroma when roasted, whereas Robusta has an earthy, spicy aroma. Arabica contains higher levels of sucrose, which are essential for developing organoleptic properties and enhancing aroma formation in coffee (Liu *et al.* 2019). In addition, the caffeine content varies among the different cultivars and species. This variability can also be seen in other parts of the coffee plant, with the highest levels of caffeine found in the beans, flowers, and leaves. Additionally, it was reported that younger plant tissues contain more caffeine than mature parts (Dessalegn *et al.* 2008).

The main objective of this study was to characterize the chemical properties of Arabica and Robusta green coffee beans grown in different regions of Geopark Rinjani, located on the island of Lombok, Indonesia. This is an important geological and natural heritage site that UNESCO has recognized as a Global Geopark. The Geopark Rinjani-Lombok covers five districts/cities: North Lombok, East Lombok, Middle Lombok, West Lombok, and Mataram City. The altitude of the region where samples were collected was between 500 and 1500 meters above sea level (masl) (Figure 1). The parameters analyzed were the moisture, ash, protein, carbohydrate, total solid, and caffeine contents, total acidity, and pH. This is the first report on the chemical composition of green coffee beans from this region.

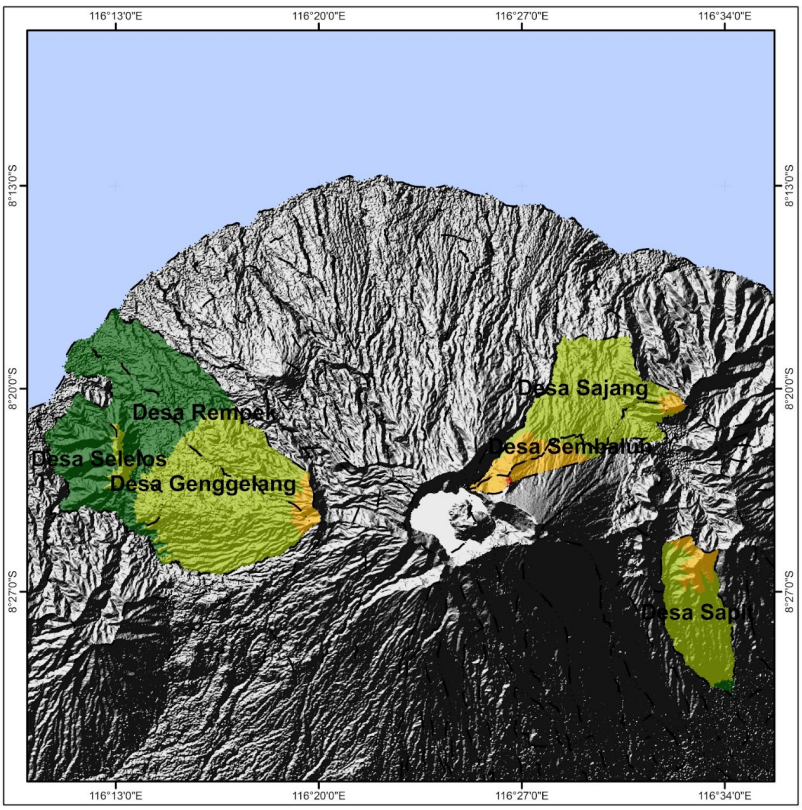

Figure 1 Distribution map of Arabica and Robusta green coffee bean plantations in Geopark Rinjani, Lombok Island, Indonesia. The different colors indicate different altitudes: dark green (0–500 m), light green (500–1500 m), and light brown (1500–2500 m).

MATERIALS AND METHODS

Materials

Arabica green bean samples were collected from various coffee-growing locations in Geopark Rinjani, Lombok, Indonesia. The Arabica coffee beans were from the Sembalun, Sajang, and Sapit regions, East Lombok. Meanwhile, Robusta green beans were collected from the Rempek, Selelos, and Genggelang regions, North Lombok Regency. Other materials used in this research were distilled water, Kjeldahl tablets, H_2SO_4, 0.1 N NaOH, a standard caffeine solution, Na_2CO_3, chloroform, a catalyst, boric acid, a phenolphthalein indicator, and diethyl ether. The tools used included Soxhlet extraction equipment, a drying oven (UNB 400, Memmert), a pH meter, a chromameter (MSEZ), a spectrophotometer, a weighing scale, and a blender.

Sample preparation

All green beans went through natural processing by drying for 2–3 weeks. The green beans were ground using a blender. Then, the ground coffee was sifted using a 40-mesh sieve. Fine powders from Arabica and Robusta green bean samples were packaged in aluminum foil Ziplock bags (approximately 100 g per sample per package) for further analysis.

Research design

A randomized block design (RBD) was used with a grouping of six samples, with the single factor being green beans from villages around Geopark Rinjani. Each sample comprised four replicates, resulting in 24 experimental units. The observed parameters were pH (AOAC, 1980), moisture content (AOAC, 1996), total fat content (AOAC, 2005), ash content (AOAC, 2000), protein content (AOAC, 2005), carbohydrate (by difference) (AOAC, 2005), total solid content (AOAC, 2005), total acidity (AOAC, 2005), and caffeine content (AOAC, 2005).

RESULTS AND DISCUSSION

Moisture content

The moisture content of coffee beans is essential as it can affect the taste, aroma, and overall quality of the coffee. Figure 2 shows that the moisture content of the Arabica samples (collected from Sembalun, Sajang, and Sapit) was significantly different compared to that of the Robusta samples (collected from Rempek, Selelos, and Genggelang). The Arabica sample from Sembalun was significantly different from those from Sajang and Sapit. However, there were no significant differences in the moisture contents of the Robusta samples.

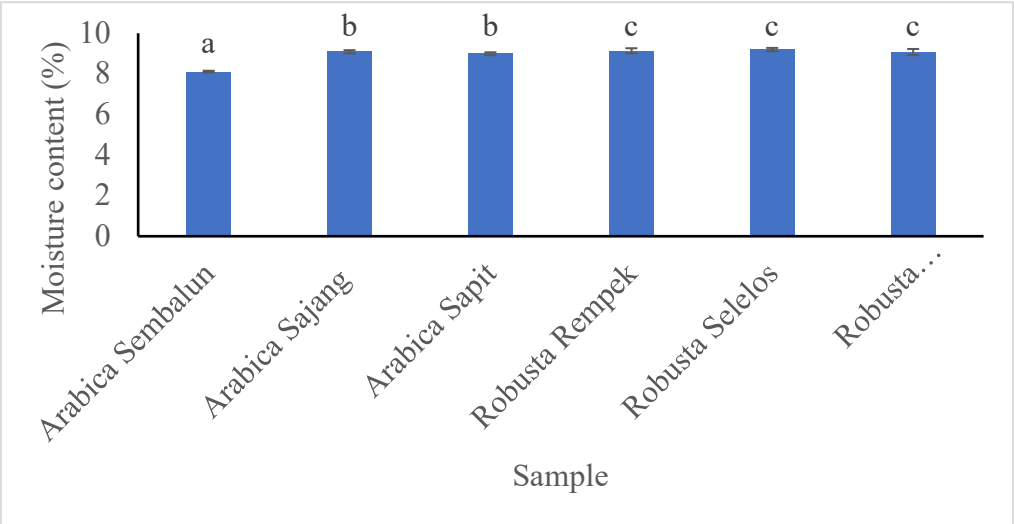

Figure 2 The moisture content of Arabica and Robusta green beans from different regions of Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$).

For the Arabica samples, the water content ranged from 8.11% to 9.09%, with the highest value corresponding to the Sajang sample. However, the water content in the Robusta samples was slightly higher, ranging from 9.07% to 9.20%. The highest value was found in the Selelos sample. The moisture contents of the Arabica and Robusta green beans met the requirement in the Indonesian standard (SNI 01-2907-2008), which states that the maximum moisture content of the green beans should be 12%.

It is essential to monitor the water content of green coffee beans as it can affect various aspects of coffee quality, such as flavor and aroma during storage and roasting. In addition, Reh *et al.* (2006) reported that the moisture content is considered the essential quality factor for green coffee, as it affects fermentation and mold growth during storage and transportation, potentially causing undesirable flavors or the formation of mycotoxins. The water content of the coffee bean can also vary depending on the specific growing conditions, processing methods, and storage conditions (Collazos-Escobar *et al.* 2020). The variability in the moisture content of the Arabica samples may be related to different environmental conditions in each planting area where the samples were obtained and also to differences during processing.

Ash content

The ash contents of the Arabica and Robusta green coffee beans were measured, and the results are shown in Figure 3. The ash content ranged from 4.5072% to 5.5182% for the Arabica samples, with the lowest value observed in the Sajang sample. The ash content of Arabica Sajang and Arabica Sapit was significantly different compared to that of other samples. However, the ash contents of Robusta Rempek, Robusta Selelos, and Robusta Genggelang were not significantly different.

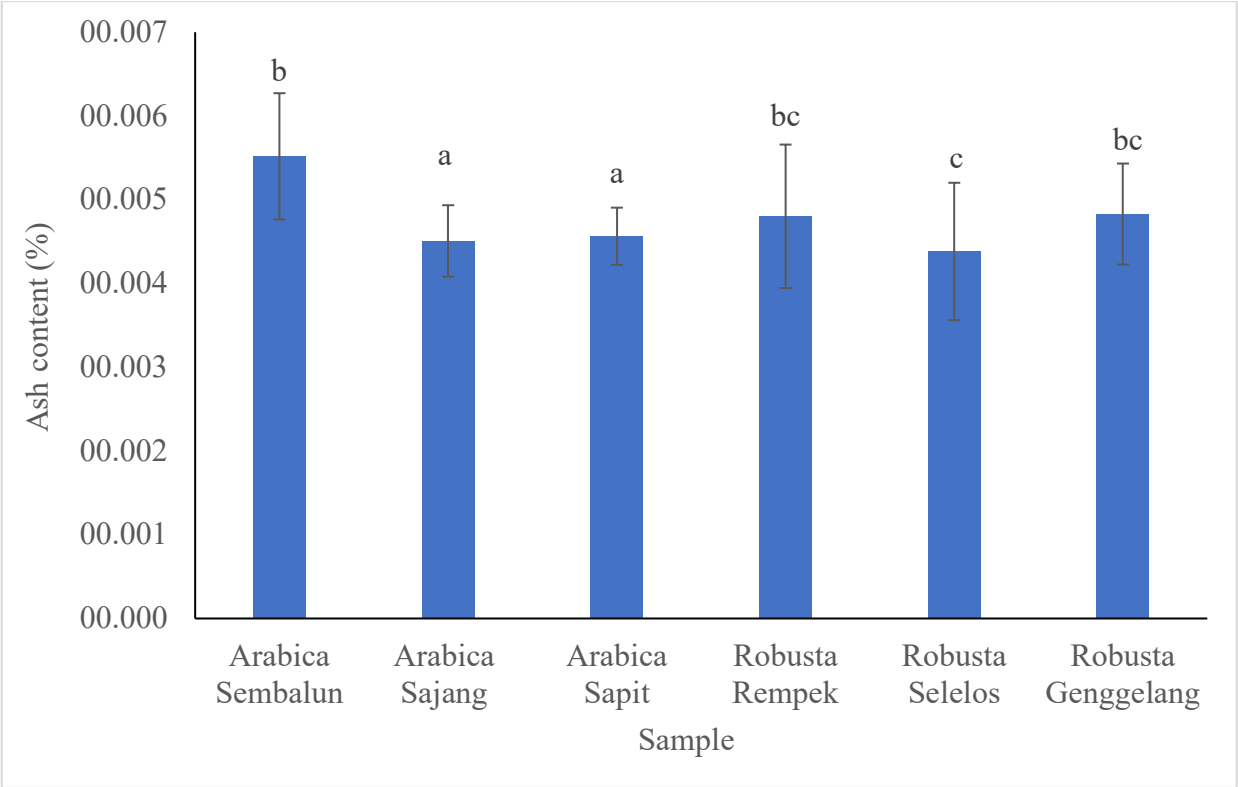

Figure 3 The ash content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$).

The ash content of coffee beans is an essential factor to consider as it can provide information on the quality and processing of the beans. A high ash content may indicate the presence of impurities, whereas, a low ash content may indicate under-roasting or poor-quality beans. Pizarro *et al.* (2004) reported that the ash content in coffee beans reflects the mineral content in the beans. A high ash content may indicate a high mineral content. The high ash content in the Arabica sample from Sembalun may be associated with the mineral content of the soil in the planting area because this is the closest area to Mount Rinjani, a volcano that erupted. Additionally, impurities and residual silver skin may also affect the ash content. Differences in the origin of raw materials, such as the maturity of the beans and environmental factors that may be related to chemical characteristics or other aspects of the soil are external factors that affect the ash content in coffee beans.

Total fat content

The lipid component in coffee beans is primarily found in the endosperm. Although the Indonesian National Standard does not use fat content to assess the quality of green beans, lipids are closely associated with coffee quality, and their content may differ among coffee cultivars.

Figure 4 shows the variations in the total fat contents of the different coffee species and growing areas for the same species. Arabica Sembalun had the lowest total fat content (9.86%), whereas Robusta Selelos had the highest total fat content (18.97%). The data also showed that the total fat contents of Arabica Sajang, Arabica Sapit, Robusta Rempek, and Robusta Genggelang were not significantly different. Robusta coffee beans are known for their strong and bold flavor, which is possibly related to their higher lipid content. The obtained result also suggests that the lipid content can vary considerably among different coffee genotypes and may be influenced by factors such as the specific growing and processing conditions, including the drying time. Benoit *et al.* (2006) reported that the lipid content in coffee beans increases with an increase in the altitude of the growing area.

The total fat content of coffee beans can affect the overall quality and taste of coffee. Lipids contribute to the aroma and flavor of coffee. Thus, a higher lipid content can result in a more intense and complex aroma and flavor. However, a higher lipid content also results in greater susceptibility to rancidity, which can negatively affect the flavor and aroma of coffee.

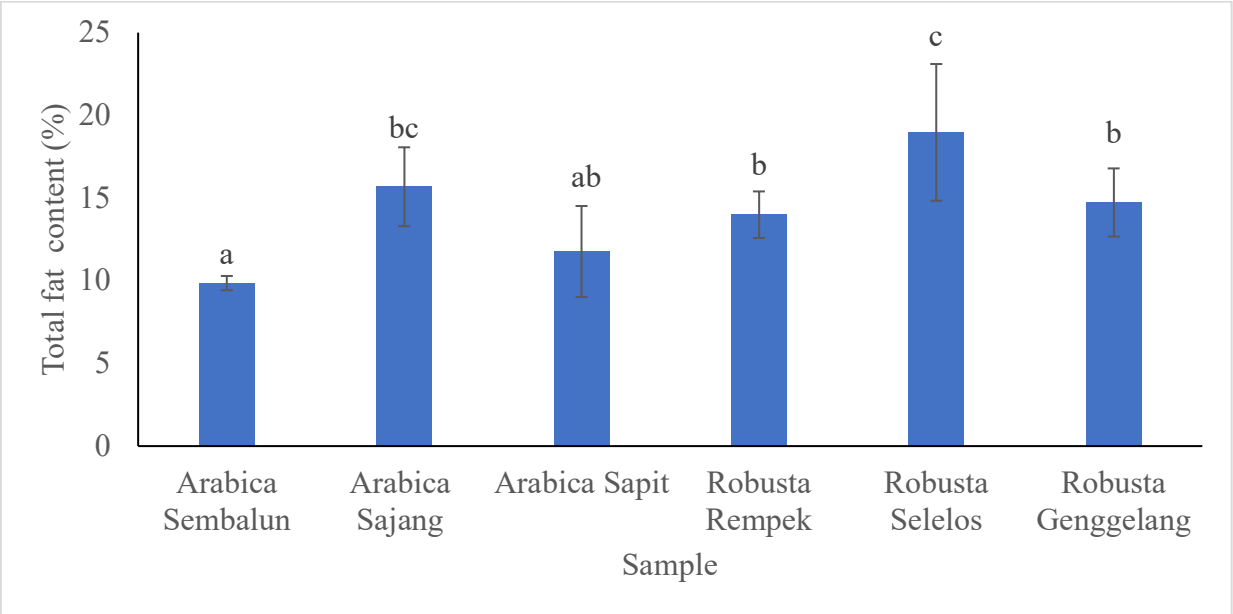

Figure 4 The total fat content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$).

Protein content

As illustrated in Figure 5, the protein contents of the samples showed significant differences. The protein content of the Robusta beans appeared to be higher than that of the Arabica beans. The protein content of Arabica coffee beans was between 11.06 and 12.02%, whereas the Robusta coffee beans contained protein levels ranging from 13.55 to 14.50%. Arabica Sembalun had the lowest protein content among all the samples, whereas Robusta Selelos had the highest protein content. The differences in protein content among the different coffee varieties may be due to various factors, such as the growing conditions, genetics, and post-harvest and processing methods (Wibowo *et al.* 2021). According to Marcone (2004), differences in the protein contents of green coffee beans may be due to variations in the maturity level of the coffee fruit, leading to diverse chemical compositions. The average protein content in dried green coffee beans ranges from 8% to 12%. Arabica Sapit green beans underwent the longest drying process, as it took three weeks, compared to other green beans, which only took two weeks to dry. Prolonged drying or sun-drying can result in protein degradation. In excessive natural fermentation during prolonged drying, microorganisms can use the protein in coffee beans as well as other nutrients such as carbohydrates and fats as energy sources (Wibowo *et al.* 2021).

Total carbohydrate content

Carbohydrates are essential components in coffee. Figure 6 shows the total carbohydrate contents of Arabica and Robusta green coffee beans from different growing areas. The results revealed that the carbohydrate contents of Arabica Sembalun and Arabica Sapit were significantly different than those of Arabica Sajang, Robusta Rempek, Robusta Genggelang, and Robusta Selelos. Among the coffee bean samples, the highest carbohydrate content was found in Arabica Sembalun (65.46%), whereas the lowest carbohydrate content was found in Robusta Selelos (52.95%). The data suggests that Arabica beans tend to have a higher carbohydrate content than Robusta beans. However, there was considerable variation in the two types of coffee, as indicated by the high standard deviation values.

The carbohydrate content can affect the flavor of roasted coffee, as it is responsible for the formation of desirable flavors during the roasting process. Furthermore, the carbohydrate composition can also affect the functional properties of the final product, such as the body and mouthfeel of coffee. Therefore, a higher carbohydrate content may contribute to a more full-bodied and smoother coffee, whereas a lower carbohydrate content may result in a lighter and more acidic coffee. However, the correlation between the carbohydrate content and coffee flavor profile is complex and can be affected by many factors (Arya & Rao 2007).

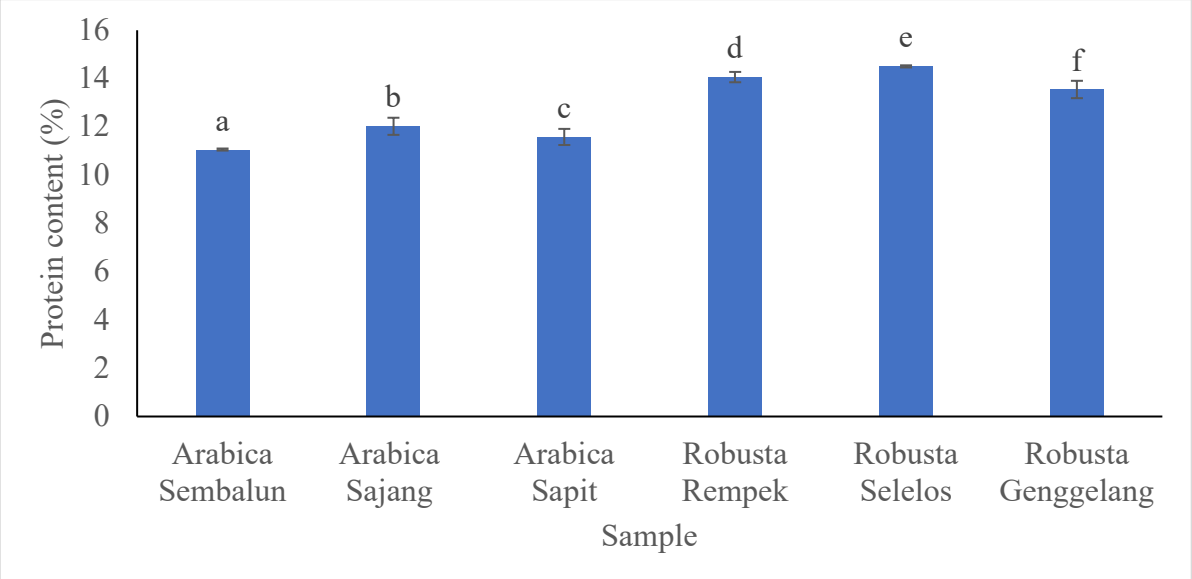

Figure 5 The protein content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$)

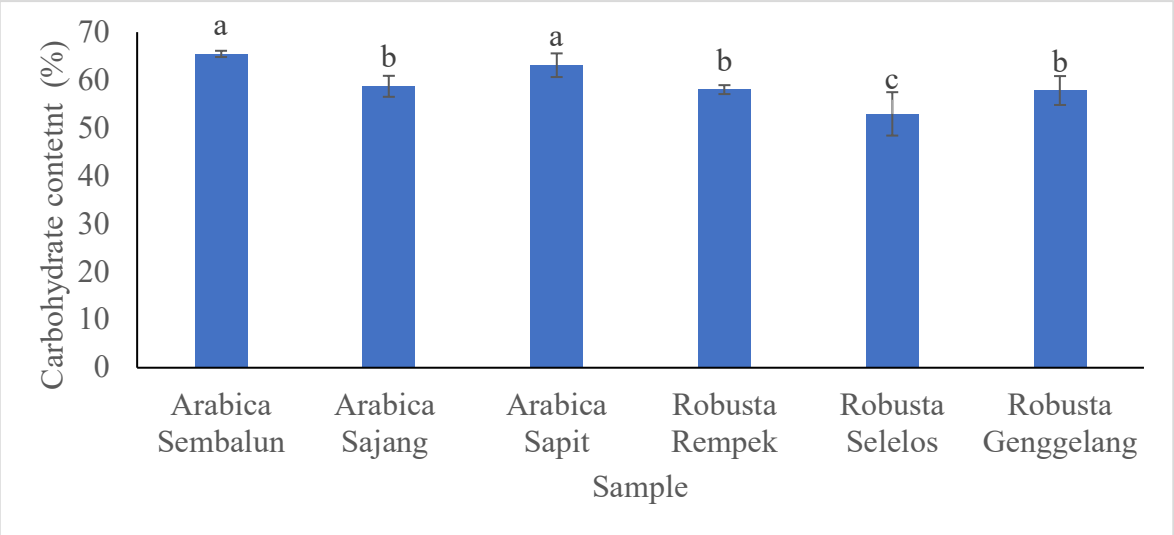

Figure 6 The total carbohydrate content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate statistically significant differences ($P \leq 0.05$)

pH

As shown in Figure 7, Arabica Sembalun has the highest pH (5.81) among the Arabica samples. Arabica Sajang and Arabica Sapit have slightly lower pHs, at 5.55 and 5.33, respectively. The Robusta samples have slightly higher pHs, with Rempek having the lowest (5.4675), followed by Selelos (5.4525), and Genggelang (5.595). These data suggest that the pHs of the Arabica beans from three different locations were more consistent than those of the Robusta beans, as indicated by their lower standard deviations. Moreover, the Robusta coffee bean samples appeared to have a slightly lower pH than the Arabica samples. Saputri et al. (2020) reported that Arabica Gayo coffee beans have an average pH of 5.66, which is slightly higher than that of Robusta Gayo coffee beans (4.89). However, Lee *et al.* (2017) reported that the pH of Arabica coffee beans is lower (4.60–5.60) than that of Robusta coffee beans (5.30–6.10).

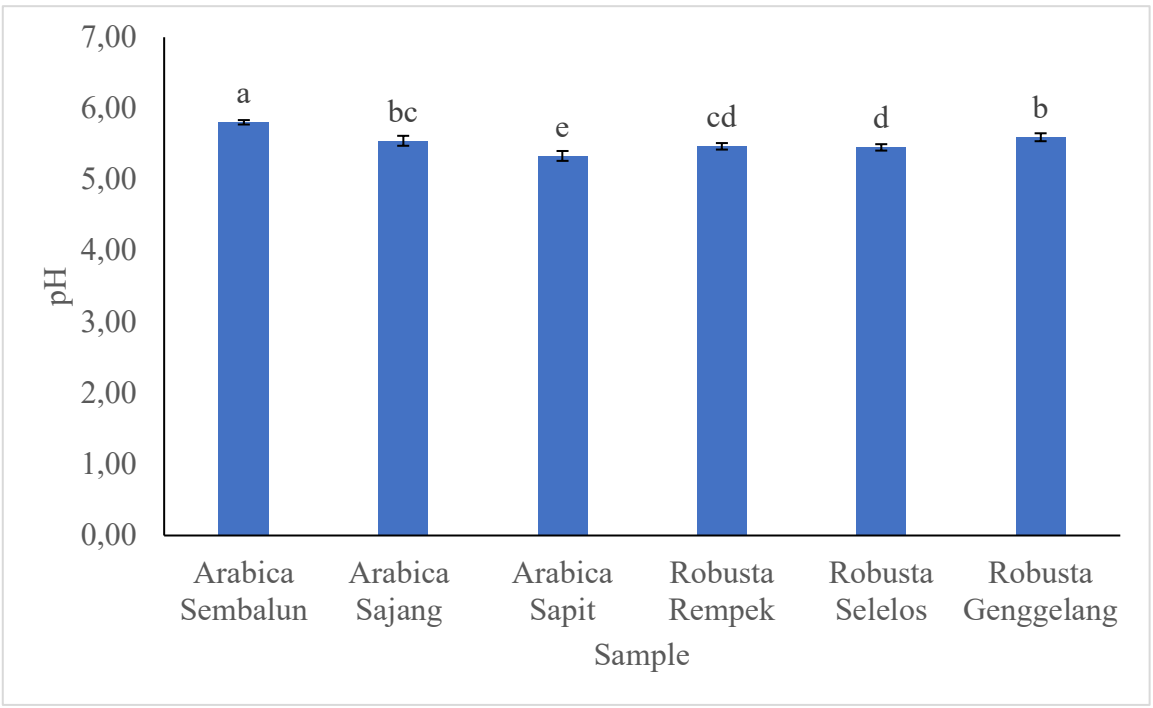

Figure 7 The pH of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$)

The pH of coffee beans is influenced by acidic compounds, specifically carboxylic acids. These acids are present in the free form after their release by the breakage of glycoside bonds. Variations in the pH values of coffee may be influenced by the location where the plants are grown. Sajang Village has andosol soils and is located in Geopark Rinjani at an altitude greater than 800 masl; its soil pH ranges from acidic to neutral (pH 5.0–7.0). Meanwhile, Sapit Village has regosol soils. Based on its geography, Sapit Village has a 25–40% slope and less than 2500 mm/year of rainfall with a soil pH ranging from 6 to 7. On the other hand, Rempek, Selelos, and Genggelang, where the Robusta samples were obtained, have inceptisol soils with a thick layer of approximately 1–2 m and pH in the range of 5.0–7.0. The three soil types in the coffee-growing areas of Geopark Rinjani-Lombok are derived from the volcanic ash of Mount Rinjani, and the pH ranges from 5 to 7.

Total acidity

The acidity of coffee beans is essential as it can affect the flavor profile. Generally, Arabica beans are considered to have a milder and more nuanced flavor, whereas Robusta beans are known for their bold and robust flavor. The difference in acidity levels between these two types of beans may contribute to these distinct flavor profiles.

Figure 8 shows that the Arabica and Robusta coffee samples from different regions in Geopark Rinjani had distinct acidity levels. The Arabica samples had similar acidity levels, ranging from 0.20 to 0.21, whereas the Robusta samples had higher acidity levels, ranging from 0.23 to 0.25. These differences in acidity may be due to variations in the growing conditions of the different samples, such as soil composition and climate, as well as genetic differences between the Arabica and Robusta coffee plants. Towaha *et al.*, (2014a) proposed that altitude affects the acidity level of Arabica coffee beans.

The processing methods and roasting techniques can also influence the acidity of coffee beans. According to Kasim *et al.* (2020), the titrated acidity in the dry-processed Robusta coffee is 3.65%, whereas in the wet-processed Robusta coffee is 3.42%. On the other hand, according to Winarno *et al.* (2021), the free acidity in dry-processed Arabica coffee is approximately 0.05%, and that of wet-processed Arabica coffee is approximately 0.07%.

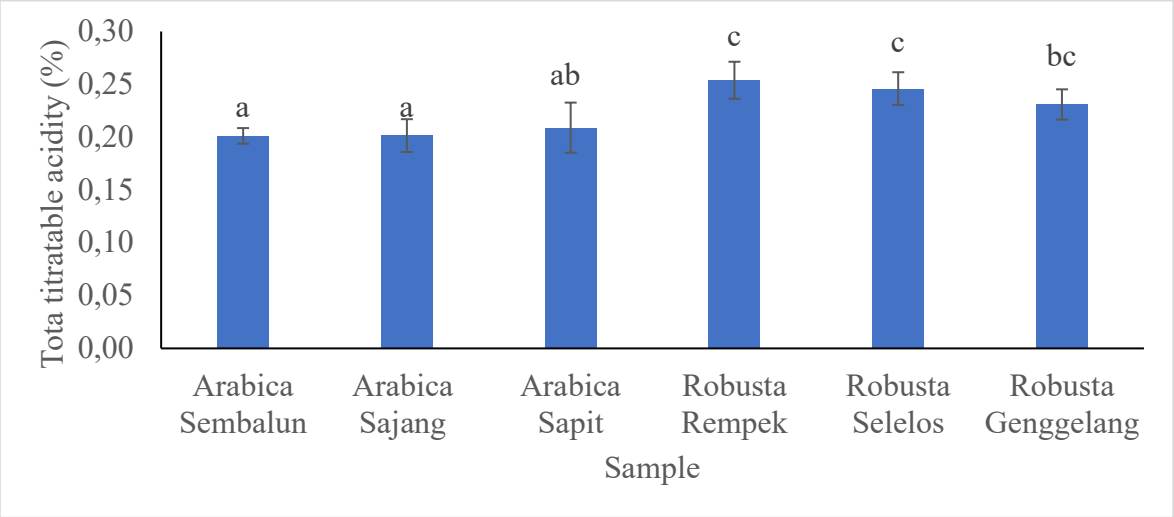

Figure 8 The total acidity of Arabica and Robusta green beans from a different region in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$)

Total solid content

The total solid content refers to the percentage of dry matter in the coffee beans, indicating the quality and taste of coffee. Arabica Sembalun had the lowest total solid content (4.43%), whereas, Arabica Sajang had the highest total solid content (7.45%). Moreover, Robusta Rempek had a total solid content of 7.275% (Figure 9). The total solid content can affect the flavor of coffee. Saputri *et al.* (2020) reported that Robusta coffee beans showed a higher total solid content compared to Arabica coffee beans. This result was consistent with the lower levels of organic acids and amino acids found in Arabica coffee beans compared to Robusta coffee beans. The total solid content can consist of organic acids, amino acids, and simple carbohydrates, which can contribute to the taste and aroma formation of coffee during the roasting process.

Caffeine content

Caffeine, one of the most well-known compounds found in coffee, plays an important role in the strength, body, and bitterness of coffee, and is responsible for many of the physiological effects associated with coffee consumption (Buffo and Cardelli-Freire 2004). Figure 10 shows a significant difference between the caffeine of Arabica and Robusta coffee beans produced in Geopark Rinjani. Arabica beans had a lower (avg. 1.09%) caffeine content than Robusta beans (avg. 2.09%). This result aligns with the general understanding that Arabica beans typically have a lower caffeine content than Robusta beans. Moreover, the data shows that there were variations in the caffeine contents of beans of the same species but from different areas, which can be attributed to factors such as growing conditions, processing methods, and genetics. Towaha *et al.* (2014) also reported that Arabica coffee beans produced at different altitudes exhibited different caffeine levels.

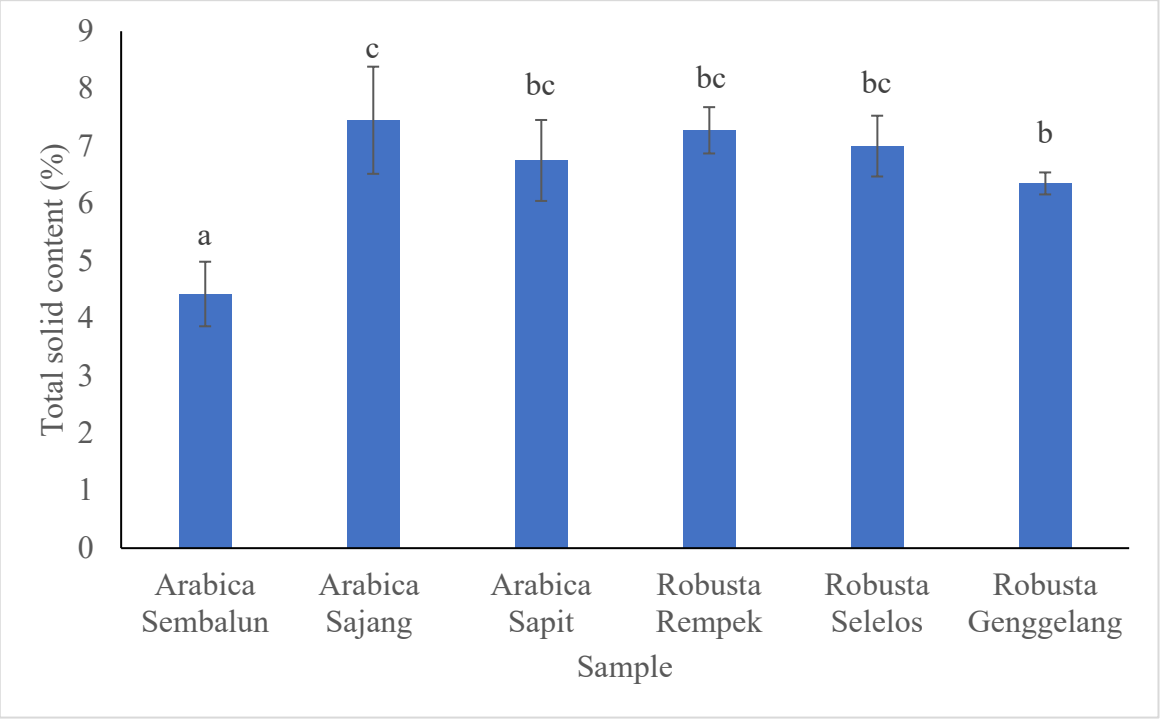

Figure 9 The total solid content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$)

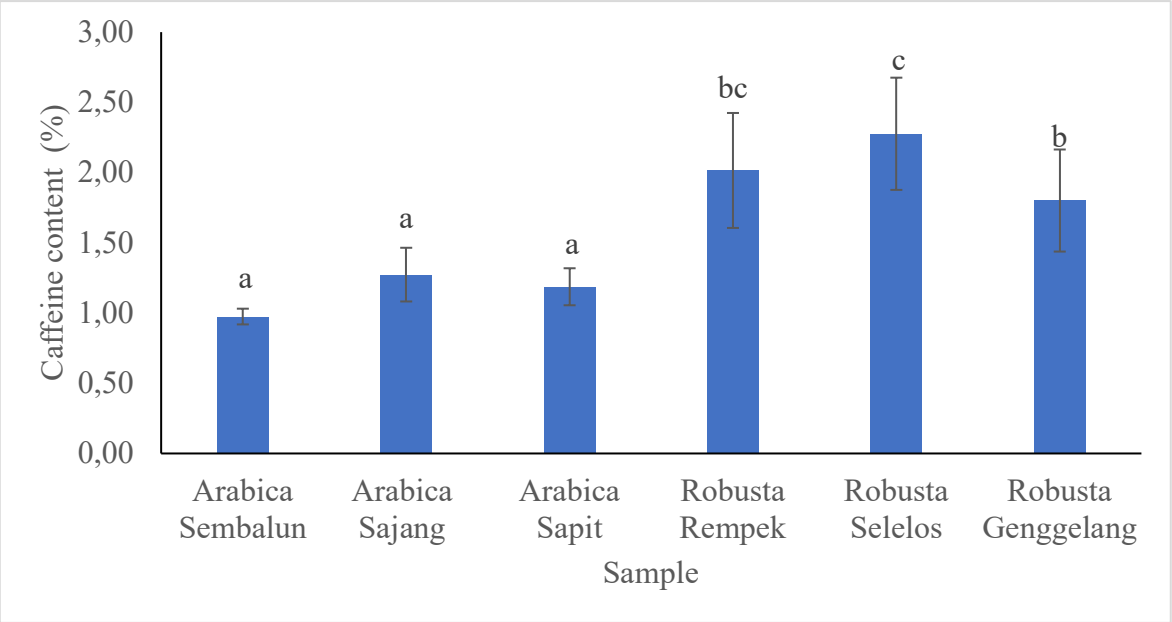

Figure 10 The caffeine content of Arabica and Robusta green beans from different regions in Geopark Rinjani, Lombok Island, Indonesia. Different letters indicate significant differences ($P \leq 0.05$)

According to Clemente *et al.* (2013), the chemical elements in the soil (particularly potassium, K) can affect the caffeine content of coffee beans. K plays an essential role in coffee plant reproduction, especially in the size and yield of coffee beans. In addition, K affects the taste of coffee by activating the polyphenol oxidase enzyme and determining the caffeine and phenol content in coffee beans. Moreover, the caffeine content is not only influenced by the growing location but may also be affected by the processing methods (Zhang *et al.* 2013).

CONCLUSION

The chemical composition of Arabica and Robusta coffee beans was found to be significantly ($P \leq 0.05$) different. Arabica beans have a lower pH, acidity, and caffeine content than Robusta beans. Meanwhile, Robusta beans have a higher total solid content than Arabica beans. These results suggest that the growing conditions, processing methods, and genetics of coffee beans determine their chemical composition, which in turn can contribute to the distinct flavor profiles of Arabica and Robusta coffee beans.

ACKNOWLEDGMENTS

The University of Mataram funded this research through the PNBP Grant in 2022.

REFERENCES

Ahmed S, Brinkley S, Smith E, Sela A, Theisen M, Thibodeau C, Warne T, Anderson E, van Dusen N, Giuliano P, et al. 2021. Climate Change and Coffee Quality: Systematic Review on the Effects of Environmental and Management Variation on Secondary Metabolites and Sensory Attributes of Coffea arabica and Coffea canephora. Front Plant Sci. 12. doi: 10.3389/fpls.2021.708013.

Arya M, Rao LJM. 2007. An impression of coffee carbohydrates. Crit Rev Food Sci Nutr. 47(1): 51-67. doi: 10.1080/10408390600550315.

Benoit B, Vaast P, Alpizar E, Etienne H, Davrieux F, Charmetant P. 2006. Comparison of bean biochemical composition and beverage quality ofArabica hybrids involving Sudanese-Ethiopian origins with traditionalvarieties at various elevations in Central America. Tree Physiiology. 26: 1239-1248.

Buffo RA, Cardelli-Freire C. 2004. Coffee flavour: An overview. Flavour Fragr J. 19(2): 99-104. doi:10.1002/ffj.1325.

Cassamo CT, Mangueze AVJ, Leitão AE, Pais IP, Moreira R, Campa C, Chiulele R, Reis FO, Marques I, Scotti-Campos P, *et al.* 2022. Shade and Altitude Implications on the Physical and Chemical

Attributes of Green Coffee Beans from Gorongosa Mountain, Mozambique. Agronomy. 12(10). doi: 10.3390/agronomy12102540.

Clemente JM, Martinez HEP, Alves LC, Lara MCR. 2013. Effect of N and K doses in nutritive solution on growth, production and coffee bean size. Revista Ceres. 60(2): 279-285. doi: 10.1590/S0034-737X2013000200018.

Collazos-Escobar GA, Gutiérrez-Guzmán N, Váquiro-Herrera HA, Amorocho-Cruz CM. 2020. Moisture dynamic sorption isotherms and thermodynamic properties of parchment specialty coffee (*Coffea arabica* L.). Coffee Sci. 15(1). doi: 10.25186/.v15i.1684.

Dessalegn Y, Labuschagne MT, Osthoff G, Herselman L. 2008. Genetic diversity and correlation of bean caffeine content with cup quality and green bean physical characteristics in coffee (Coffea arabica L.). J Sci Food Agric. 88(10): 1726-1730. doi: 10.1002/jsfa.3271.

Kasim S, Liong S, Lullung A. 2020. Penurunan Kadar Asam dalam Kopi Robusta (*Coffea canephora*) dari Desa Rantebua Kabupaten Toraja Utara dengan Teknik Pemanasan [Reduce Acid Levels in Robusta Coffee (*Coffea canephora*) from Rantebua Village, North Toraja District by Heating Techniques. Jurnal Riset Kimia. 6(2): 118-125.

Lee SJ, Kim MK, Lee KG. 2017. Effect of reversed coffee grinding and roasting process on physicochemical properties including volatile compound profiles. Innovative Food Science and Emerging Technologies. 44: 97-102. doi: 10.1016/j.ifset.2017.07.009. http://dx.doi.org/10.1016/j.ifset.2017.07.009.

Liu C, Yang N, Yang Q, Ayed C, Linforth R, Fisk ID. 2019. Enhancing Robusta coffee aroma by modifying flavour precursors in the green coffee bean. Food Chem. 281: 8-17. doi: 10.1016/j.foodchem.2018.12.080. https://doi.org/10.1016/j.foodchem.2018.12.080.

Marcone MF. 2004. Composition and properties of Indonesian palm civet coffee (Kopi Luwak) and Ethiopian civet coffee. Food Research International. 37(9): 901-912. doi: 10.1016/j.foodres.2004.05.008.

Mendes G de A, de Oliveira MAL, Rodarte MP, de Carvalho dos Anjos V, Bell MJV. 2022. Origin geographical classification of green coffee beans (*Coffea arabica* L.) produced in different regions of the Minas Gerais state by FT-MIR and chemometric. Curr Res Food Sci. 5(November 2021): 298-305. doi: 10.1016/j.crfs.2022.01.017. https://doi.org/10.1016/j.crfs.2022.01.017.

Pizarro C, Esteban-Díez I, Nistal AJ, González-Sáiz JM. 2004. Influence of data pre-processing on the quantitative determination of the ash content and lipids in roasted coffee by near infrared spectroscopy. Anal Chim Acta. 509(2): 217-227. doi: 10.1016/j.aca.2003.11.008.

Reh CT, Gerber A, Prodolliet J, Vuataz G. 2006. Water content determination in green coffee - Method comparison to study specificity and accuracy. Food Chem. 96(3): 423-430. doi: 10.1016/j.foodchem.2005.02.055.

Saputri M, Lioe HN, Wijaya CH. 2020. PEMETAAN KARAKTERISTIK KIMIA BIJI KOPI ARABIKA GAYO DAN ROBUSTA GAYO. Jurnal Teknologi dan Industri Pangan. 31(1): 76-85. doi: 10.6066/jtip.2020.31.1.76. https://journal.ipb.ac.id/index.php/jtip/article/view/26680.

Towaha J, Aunillah A, Heri Purwanto E, Handi Supriadi Balai Penelitian Tanaman Industri dan Penyegar Jalan Raya Pakuwon Km dan. 2014. Pengaruh Elevasi dan Pengolahan Terhadap Kandungan Kimia dan Citarasa Kopi Robusta Lampung. J TIDP. 1(1): 57-62.

Wibowo NA, Mangunwardoyo W, Santoso TJ, Yasman. 2021. Effect of fermentation on sensory quality of liberica coffee beans inoculated with bacteria from saliva arctictis binturong raffles, 1821. Biodiversitas. 22(9): 3922-3928. doi: 10.13057/biodiv/d220938.

Winarno RA, Perangin-angin MIB, Sembiring N v. 2021. Karakterisitik Sifat Kimia Biji Kopi Arabika dengan Beberapa Metoda Pengolahan di Kabupaten Simalungun Provinsi Sumatera Utara. Agrivet : Jurnal Ilmu-Ilmu Pertanian dan Peternakan (Journal of Agricultural Sciences and Veteriner). 9(2): 237-243. doi: 10.31949/agrivet.v9i2.1701.

Zhang X, Li W, Yin B, Chen W, Kelly DP, Wang X, Zheng K, Du Y. 2013. Improvement of near infrared spectroscopic (NIRS) analysis of caffeine in roasted arabica coffee by variable selection method of stability competitive adaptive reweighted sampling (SCARS). Spectrochim Acta A Mol Biomol Spectrosc. 114: 350-356. doi: 10.1016/j.saa.2013.05.053. http://dx.doi.org/10.1016/j.saa.2013.05.053.

FURTHER STUDY ON TWO SPECIES OF LOACH FISHES (Cypriniformes: Nemacheilidae: *Nemacheilus*) BASED ON MORPHOLOGY AND MOLECULAR DATA

DWI ANGGOROWATI RAHAYU[1*], SUNU KUNTJORO[1], WIDOWATI BUDIJASTUTI[1], WINARSIH WINARSIH, RENI AMBARWATI[1], ENDIK DENI NUGROHO[2], ABDUL BASITH[3], NIA KURNIAWAN[4] AND HARYONO[5]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya, Surabaya 60231, Indonesia*
[2]*Department of Biology Education, Faculty of Science Education, ITSNU, Pasuruan 60231, Indonesia*
[3]*Indonesian Genetic and Biodiversity Community, Malang 67118, Indonesia*
[4]*Department of Biology, Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang 65145, Indonesia*
[5]*Research Center for Biosystematics and Evolution, National Reserearch and Innovation Agency (BRIN), Cibinong 16911, Indonesia*

Received 11 March 2023 / Revised 8 May 2023 / Accepted 8 May 2023

ABSTRACT

The identities of two local loaches, *Nemacheilus chrysolaimos* (Valenciennes, 1846) and *N. fasciatus* (Valenciennes, 1846) from six rivers, were obtained through a comprehensive examination of their morphology and molecular characteristics in Biltar Regency, East Java, Indonesia. Therefore, this study identified *Nemacheilus* spp. from Blitar based on morphology and partial sequence of COI. The meristic data obtained for *N. chrysolaimos* included DII. 7–8 (dorsal fin), AI. 3–5 (anal fin), PI. 9 (pectoral fin), VI. 6–7 (ventral fin), and C. 17 (caudal fin). On the other hand, *N. fasciatus* exhibited the following meristic data, namely D II 7–8 (dorsal fin), AI. 6 (anal fin), PI. 9–10 (pectoral fin), VI. 6–7 (ventral fin), and C. 17 (caudal fin). A significant difference was observed in the morphometric characteristics of *N. fasciatus* across various sampling sites, as determined by the Kruskal-Wallis Test. Furthermore, the nucleotide base composition sequences of *Nemacheilus spp.* consisted of Thiamine (T), Cytosine (C), Adenine (A), and Guanine (G) with a mean of 29.565%, 32.023%, 23.88%, and 16.244%. Maximum Likelihood (ML) and Minimum Evolution (ME) phylogenetic analysis was also conducted using the Kimura 2 Parameter model to establish two major clades on *Nemacheilus* spp. and one out-group significantly different from the *Nemacheilus* spp. The results showed that these major clades exhibited a close relationship at 100% bootstrap support and were grouped under the genus *Nemacheilus*. The study on *Nemacheilus* spp. from the Blitar locality differentiated COI sequences between *N. fasciatus* and *N. chrysolaimos*. Additionally, *N. chrysolaimos*, as inferred from reference sequences, was identified as the ancestral species to *N. chrysolaimos* MZB 26540 and MZB 26539. ABGD analyses, employing a prior maximal distance of 0.025, also indicated the separation of these species into distinct partitions. The integration of morphology and genetic data for *Nemacheilus* spp. should provide valuable insights for future genetic population studies and conservation initiatives.

Keywords: DNA barcoding, morphology, *Nemacheilus,* phylogenetic, taxonomy

INTRODUCTION

The family Nemacheilidae comprises one of the most diverse groups of freshwater fish worldwide, encompassing 43 genera and 714 species (Froese & Pauly 2022a). One of the members of this family is the genus *Nemacheilus*, which has been described and validated with a total of 55 species. According to (Kottelat 1984; Kottelat *et al.* 1993; Kottelat 2013a; Froese & Pauly 2022b; Kottela, 2022), the highest diversity of *Nemacheilus* species has been recorded in the Asiatic region. Among the total of 55 species, only two species, namely *Nemacheilus chrysolaimos* and *N. fasciatus*, have been reported to be highly abundant in Java Island (Kottelat 1984; Kottelat

*Corresponding author, email: dwirahayu@unesa.ac.id

et al. 1993; Hadiaty and Yamahira 2014; Hubert *et al.* 2019).

The taxonomic history of the two *Nemacheilus* fishes was initially documented by Cuvier and Valenciennes in 1846. They classified these species under the genus Cobitis, naming them *Cobitis chrysolaimos* and *C. fasciata* (Cuvier & Valenciennes, 1846). Subsequently, the genus was revised by Bleecker in 1853, and the name *Nemacheilus* became a valid designation. These *Nemacheilus* spp. have gained significant attention due to the exclusive distribution in Indonesian waters, as well as their strikingly similar morphology but distinct body colorations. Kottelat (1984) elucidated the differences between these two species and in subsequent publications (Kottelat *et al.* 1993), additional information was provided. Moreover, Hadiaty and Yamahira (2014) presented an updated identification key specifically for the species of *Nemacheilus* spp. found in Asian waters within Indonesia.

In addition to the morphological analysis, the molecular examination of the two *Nemacheilus* fishes showed their dissimilarities. DNA barcoding identified the Most Recent Common Ancestor (MRCA) to have existed approximately 1.5 million years and 0.5 million years ago in *N. fasciatus* and *N. Chrysolaimos,* respectively (Hubert *et al.* 2019). Šlechtová *et al.* (2021) also supported that *N. chrysolaimos* was different from *N. masyae* through genomic DNA isolation. According to Kusuma *et al.* (2021), *Nemacheilus chrysolaimos* from Temanggung and Yogyakarta using the partial sequence of COI gene indicated a haplotype and nucleotide diversity (Hd) of 0.679 and 0.00117, respectively. The differentiation between these two species needs to be reinforced through DNA barcoding in contrast to the study conducted by Ath-thar *et al.* (2018) using PCR-RAPD analysis on *Neimacheilus fasciatus*, which revealed a high level of genetic diversity. This study also augments the understanding of *Nemacheilus* spp. by employing morphological and molecular approaches. A noteworthy addition is the inclusion of the fins formula, which has not been previously described. The outcomes are expected to offer valuable insights and resolve the taxonomy of these two fishes yet to be disclosed.

MATERIALS AND METHODS

Field Sampling

The specimens of *Nemacheilus* spp. from Blitar Regency were collected from three main locations, namely Garum, Ponggok, and Wlingi at six rivers. Specifically, Garum consisted of three rivers (Slorok, Sumber Ronje and Glawah), Ponggok comprised two rivers (Loadeng and Tunjung), and Wlingi included one river (Lekso), as shown in Figs. 1 & 2. To capture the fish samples, gill nets were employed, and the collected fishes were preserved in sample bottles containing a 10% formaldehyde solution. Furthermore, the samples were transported to the laboratory at the State University of Surabaya (Universitas Negeri Surabaya) for subsequent identification, measurement, and analysis.

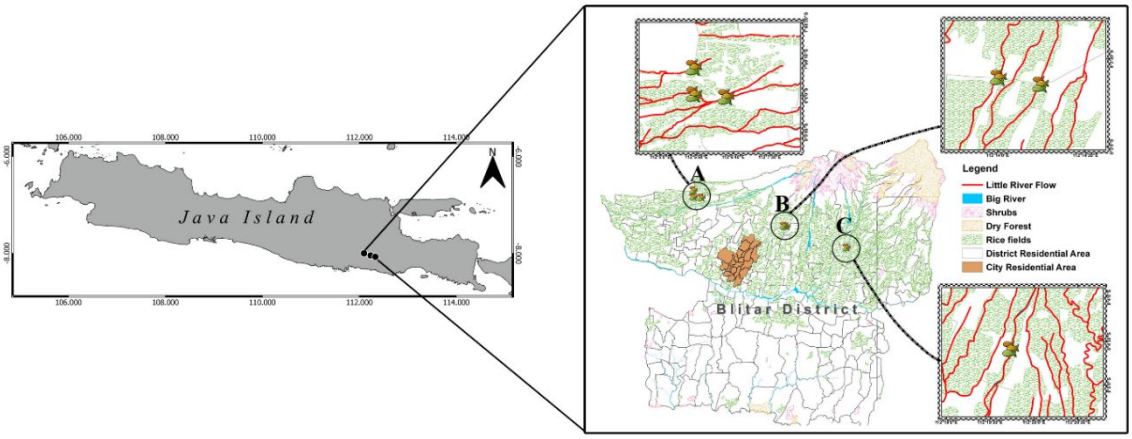

Figure 1 The map showing sampling localities of two Nemacheilus fishes at three rivers of Blitar, East Java, Indonesia. A. Garum (7°59.967'S, 112°5.775'E), B. Ponggok (8°2.604'S, 112°14.004'E) and C. Wlingi (8°4.215'S, 112°19.894'E)

Figure 2 The habitat of two *Nemacheilus* fishes in Blitar, East Java, Indonesia. A. Slorok river, B. Sumber Ronje river, C. Glawah river, D. Loadeng river, E. Tunjung river and F. Lekso river

Morphology Work

In the laboratory, all of the fish were sorted, washed, and cleaned for morphological observation and identification. Measurement of the morphological characteristics was made on 14 characters, as shown in Fig. 3, using modifications of Kottelat (1984) and Kottelat and Freyhof (2007) with a digital caliper and 0.01 mm accuracy. Furthermore, the identification was performed in line with the study of Kottelat (1984) and Kottelat *et al.* (1993). The specimens were then stored in 70% alcohol and deposited in the Museum Zoologicum Bogoriense (MZB), Cibinong, Indonesia. Before molecular work, the specimens were frozen at -20°C for DNA extraction.

DNA Extraction and Sequencing

The isolation of total DNA (whole genome) from stomach tissue samples was carried out using the DNA Isolation Kit (Roche), with several modifications. A DNA fragment of approximately 526 base pairs (bp), corresponding to the COI gene region of the mitochondrial DNA (mtDNA), was successfully amplified using gradient PCR. The universal primers LCO1490 (5' GGT CAA CAA ATC ATA AAG ATA TTG G 3') and HCO2198 (5' TAA ACT TCA GGG TGA CCA AAA AAT CA 3') were used for this purpose (Folmer *et al.* 1994). The hotstart PCR method was employed, using a Kapa master and two Taq master mixes. The PCR process consisted of 35 cycles, each encompassing the following steps, initial double-strand attachment (pre-denaturation) at 95°C for 3 minutes, denaturation at 94°C for 45 seconds, annealing at 45°C for 45 seconds, and extension at 72°C for 2 minutes. A final elongation step was conducted at 72°C for 10 minutes. To visualize the PCR products, gel electrophoresis was performed on a 1% agarose gel prepared with 0.5 g of agarose and 50 mL of TAE buffer. Additionally, 4 µL of Ethidium Bromide (EtBr) was added as a dye to the gel, and the subsequent step was to mix 3 µL of the PCR samples with 1 µL of loading dye before putting the mixture in an agarose well. The electrophoresis was performed using a machine with a voltage of 220 V and a current of 400 mA, for 25 minutes. PCR products were also purified using a Qiagen purification kit according to the manufacturer's instructions and subsequently sequenced at First Base, Malaysia.

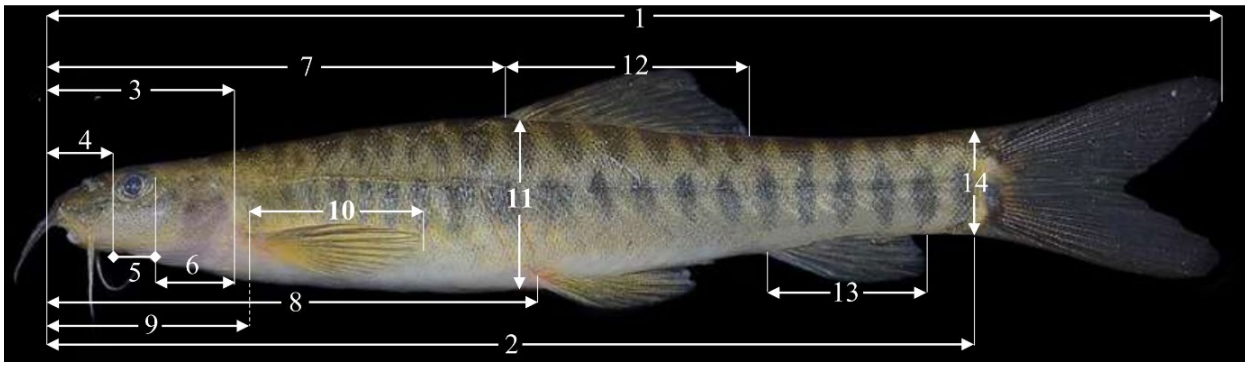

Figure 3 Morphometric of the *Nemacheilus* fish from Blitar, East Java. Abbreviation: **1.** Total length (TL); **2.** Standard length (SL); **3.** Head length; **4.** Snout length; **5.** Eye diameter; **6.** Postorbital length; **7.** Predorsal length; **8.** Prepelvic length; **9.** Prepectoral length; **10.** Pectoral fin length; **11.** Height of the body; **12.** Dorsal fin length; **13.** Anal fin length; **14.** Height of caudal peduncle. The fish image was adopted from Hubert *et al.* (2019); measurement was modified from Kottelat (1984) and also Kottelat & Freyhof (2007)

Data Analysis

A description of the two *Nemacheilus* spp. was presented based on morphological observation. Since the data from morphological measurements were not normally distributed, a non-parametric test (Kruskal-Wallis test) was employed to investigate any significant difference in the morphometric of *N. fasciatus*, from sampling localities. This was because only this species occurred at all six rivers, while *N. chrysolaimos* had a limited number and was only found in one river. The molecular data obtained from the study were subjected to the following analytical procedures:

Sequence Composition and Genetic Diversity

A partial sequence of the COI gene along 503 bp was obtained from 11 *Nemacheilus* spp. from Blitar Regency, East Java, as the final dataset. Each sequence was initially translated into an amino acid to check and remove pseudogene (Song *et al.* 2008; Buhay 2009). Nucleotide sequencing was then continued by carrying out the chromatogram analysis, using Finch TV software and translating into amino acid sequence through the ExPASy website (Duvaud *et al.* 2021). Subsequently, all sequences were checked through the BLAST (*Basic Local Alignment Search Tool*) (Boratyn *et al.* 2013) and the BOLD System (Ratnasingham & Hebert 2007) to be compared with close relatives of *Nemacheilus* spp. A total of 5 accessions from GenBank (NCBI) were selected as in-group and out-group for the phylogenetic tree reconstruction. Multiple sequence alignment was performed by using the Clustal X (Larkin *et al.* 2007) and then checked manually through the BioEdit software (Hall 1999). Furthermore, partial sequences of the COI gene from *Nemacheilus* spp. were submitted to GenBank with referred accession numbers, as shown in Table 2. All the data including taxonomic characteristics and GenBank accession numbers were tagged with the voucher specimens preserved at Zoologicum Bogoriense (MZB) at Juanda Street, Bogor, West Java, Indonesia.

The calculation of similarity values was performed as follows: similarity percentage = (1-Genetic Distance) × 100%. The substitution of transitions and transversion of nucleotide bases was calculated by the K2P (Kimura 2-Parameter) model. Information on the genetic diversity of all sequences used for phylogenetic reconstruction was analyzed through items, such as the value of nucleotide diversity (Pi), the number of polymorphic sites (S), the haplotype analysis (haplotype diversity (Hd), and the number of haplotypes (nHap) (Nei 1972). In addition, the software version of MEGA X was 10.2.6 (Kumar *et al.* 2018) used to calculate nucleotide frequencies, transition/conversion ratio (k), transition/conversion, rate ratio bias (R), and probabilities.

Phylogenetic Reconstruction

Reconstruction of the phylogenetic tree based on the partial sequence of the COI gene was conducted on a total of 17 sequences using MEGA X version 10.2.6. The purpose is to determine the grouping of different species and

the applied methods were Minimum Evolution (ME) and Maximum-Likelihood (ML). The settings used for ME phylogenetic tree reconstruction begins with the setting of a bootstrap consensus tree inferred from 1000 replicates retrieved to represent the evolutionary history of the taxa (Felsenstein 1985). Branching corresponding to partitions reproduced in less than 50% of bootstrap replicates was eliminated. Furthermore, evolutionary distances were calculated using the Kimura 2-parameter (K2P) method and in units of the number of base substitutions per site (Kimura, 1980). The rate variation among sites was modeled with a gamma distribution (shape parameter = 63). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm at search level 2 (Nei & Kumar 2000) and the neighbor-joining algorithm was used to generate the initial tree (Saitou & Nei 1987).

The settings used for ML phylogenetic tree reconstructions were calculated by using the K2P substitution model (Saitou & Nei 1987), and the rate variation among sites was modeled with a Gamma distribution and bootstrap consensus tree inferred from 1000 replicates (Felsenstein 1985). In addition, the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) were shown next to the branches. The barcode gap analysis generated by Automatic Barcode Gap Discovery (ABGD) was used to strengthen the identification of this species, and grouping analysis (Puillandre *et al.* 2012) was conducted through a web interface to check the distribution and size of a potential barcoding gap for the partial sequence of COI gene dataset with the following settings: Pmin: 0.001, Pmax: 0.9, Step: 10, X (relative gap width):1.5, Kimura (K80), number of bins: 20.

RESULTS AND DISCUSSION

Class Actinopteri Cope, 1871
Ordo Cypriniformes Bleeker, 1859
Family Nemacheilidae Regan, 1911
Genus *Nemacheilus* Bleeker, 1863
***Nemacheilus chrysolaimos* (Valenciennes, 1846)** (Fig. 4)

Noemacheilus fasciatus Kuhl & van Hasselt in van Hasselt, 1823: 133 (Buitenzorg) (partim; nomen nudum).
Cobitis chrysolaimos Valenciennes in Cuvier & Valenciennes, 1846: 27, fig. 521.
Noemacheilus chrysolaimos Kottelat, 1984: 241, figs. 14a, 15.
Nemacheilus chrysolaimos Kottelat, 1993: 75, pl. 25.—Roberts, 1993: 25, fig. 29.—Hardiaty & Yamahira, 2014: 84 (list), 87 (list), 90 (list), 92 (key).

Material examined. Slorok river, Garum (MZB 26539, SL. 52.5 mm; MZB 26540, SL. 51.3 mm; 08°02'36.25"S, 112°14'00.23"E), Blitar, East Java, 26 June 2022, Coll. D.A. Rahayu & E.D. Nugroho.

Description. Morphometric data are presented in Table 1. The head is rounded with a pair of eyes, a pair of nares, a short blunt snout, a small subterminal mouth, and a circular lip around the mouth. Furthermore, the eyes are elliptical and nares are located between the snout and the eyes. The mouth contains three pairs of rostral barbels, two pairs on the upper jaw, and one pair on the upper snout maxillary barbels may reach half of the postorbital length of the head. The inner rostral spines are present and reach about half of the eye.

The body is elongated, fusiform, weakly compressed, and laterally flattened at the base of the tail without sharp scales. TL is about 1.18–1.28 (1.24 ± 0.01) times as long as SL and head length is about 2.21–3.09 (2.65 ± 0.09) times as the snout. Meanwhile, the eye diameter is about 0.38–0.57 (0.46 ± 0.02), 0.36–0.50 (0.41 ± 0.01), and 0.02–0.04 (0.03 ± 0.002) times as long as the snout, postorbital length, and SL, respectively. Predorsal length, Prepectoral length, and height of the body are about 0.99–1.10 (1.02 ± 0.01), 1.07 (0.94 ± 0.02), and 1.00–1.50 (1.19 ± 0.05) times as the prepelvic length, length of the head, and height of the caudal peduncle, respectively.

Pectoral fins almost reach more than half of pelvic fin bases. An axillary lobe presents at the pelvic fins bases under the first to third of branched dorsal rays. The anal fin does not reach the caudal fin bases. The dorsal fin is opposite the ventral fin or just behind the vertical fin. The anal fin is short, far behind the ventral fin. The ventral fin does not reach the anal fin; pectoral fins are shorter than the head. The caudal fin is very

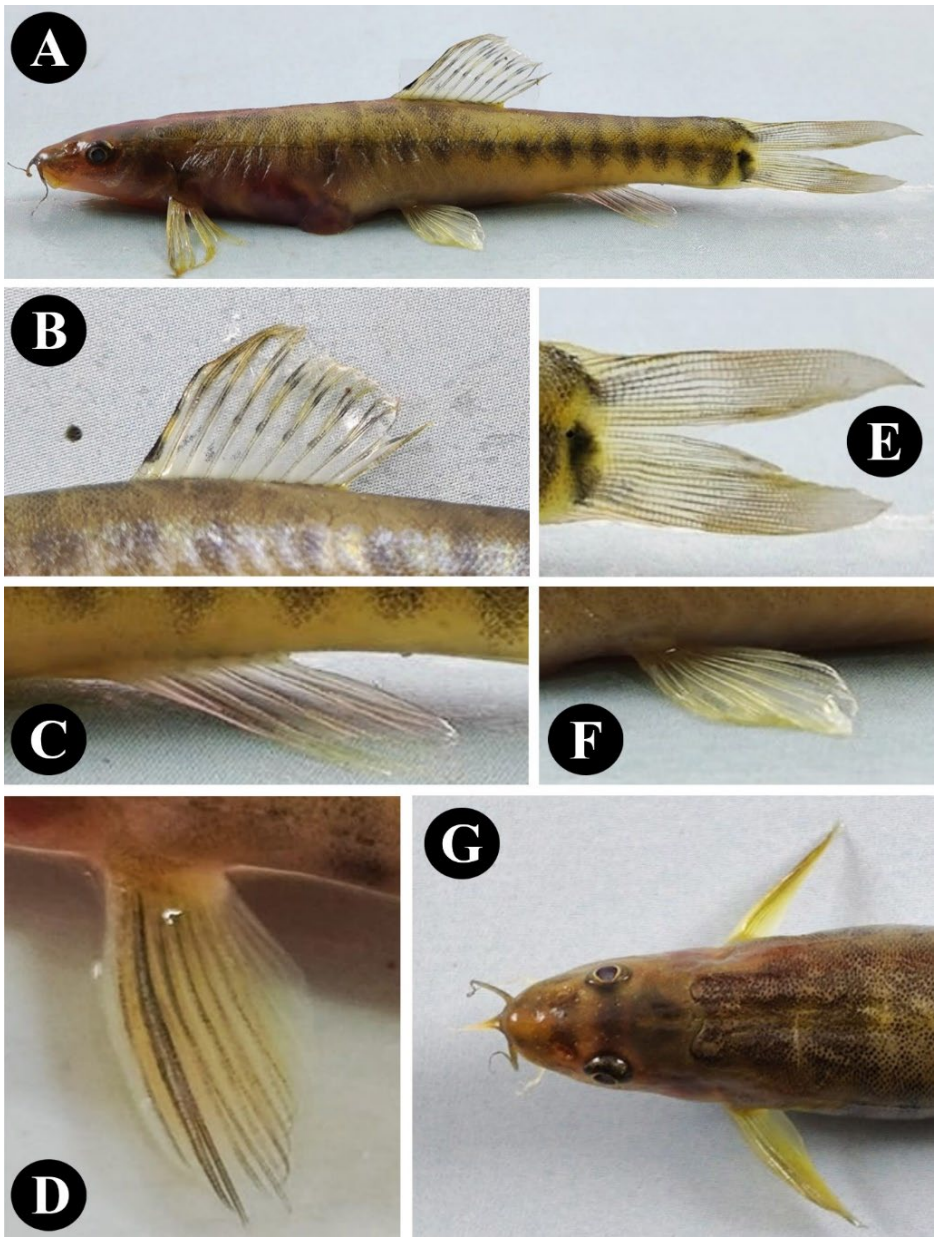

Figure 4 *Nemacheilus chrysolaimos* (Valenciennes, 1846) (Standar Length: 52.5 mm; MZB 26539) from Blitar, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Anal fin, lateral view; D. Pectoral fin, lateral view; E. Caudal fin, lateral view; F. Ventral fin, lateral view; G. Anterior part, dorsal view

emarginate, the lobes are acute, caudal fin is longer than the head with the rays all forked. The initial dorsal fin is in front of the vertical line of the anal fin base, closer to the tip of the snout than the base of the caudal fin; the dorsal fin is medium in size, the base of the posterior tip is the opposite the base of the ventral fin and the tip is not reaching anal fin. The caudal fin is crescent in shape, the pectoral fin is rounded, and the length is almost equal to the head, ending less than the length in front of the ventral fin; Anal fin is shorter than the pectoral fin and the shape is tapered or slightly pointed and bearing hard and soft rays, not or barely lower than the body, higher than the length of the base; the caudal fin is emarginate or crescent-shaped-emarginate. The first anterior dorsal rays are the longest. Dorsal fins DII. 7–8, anal fins AI. 3–5, pectoral fin PI. 9, ventral fins VI. 6–7 and caudal fin C. 17.

Coloration. The body is black-yellowish in color with 12–18 dark bars irregular shape on the lateral part. The two pairs of rostral barbels are black and one pair with yellowish coloration. The base of the caudal fin is red in the anterior part of the caudal peduncle and the initial base of the dorsal fin rays has a black spot. The head is brown with a dark color in the center; a darker pattern is also present on the snout and opercula.

Furthermore, the base and first rays of the pectoral fins are dark in color and the last dorsal fin rays are dark with black spots.

Body length. Standard length (SL) and total length (TL) ranged from 32.1–52.4 mm and 40.60–66.40 mm, with a mean of 43.47 ± 2.05 mm and 55.32 ± 2.93 mm (n = 9).

Distribution. According to Kottelat (1984) and Kottelat *et al.* (1993), the species *N. chrysolaimos* was distributed in Java Island, particularly in West Java. Meanwhile, Hubert *et al.* (2019) reported that species can be found in almost at all provinces in Java Island, from West Java, Central Java to East Java.

Remarks. There is a contradiction information on the distribution of this species between Kottelat's (1984) finding with Hubert *et al.* (2019) information. We believe that so far, the Kottelat's collected materials maybe restricted to the West Java only and have not ever been expanded to another location, meanwhile, Hubert *et al.* (2019) revisited and collected this species at all 3 provinces in Java Island, therefore, the information is totally different.

Nemacheilus fasciatus (Valenciennes, 1846)

(Fig. 5)

Noemacheilus fasciatus Kuhl & van Hasselt in van Hasselt, 1823: 133; 1824: 376; Kottelat, 1984: 247, fig. 18a.

Cobitis fasciata Valenciennes in Cuvier & Valenciennes, 1846: 25.—Bleeker, 1854: 96; 1860: 78.

Cobitis suborbltalis Valenciennes in Cuvier and Valenciennes, 1846: 26.

Cobitis chrysolaimos Valenciennes in Cuvier and Valenciennes, 1846: 27.

Nemacheilus fasciatus Bleeker 1863a: 41, 366 (in part); 1863b: 7 (in part).—Kottelat, 1993: 25.—Roberts, 1993: 26.—Hardiaty & Yamahira, 2014: 84 (list), 87 (list), 90 (list), 92 (key).

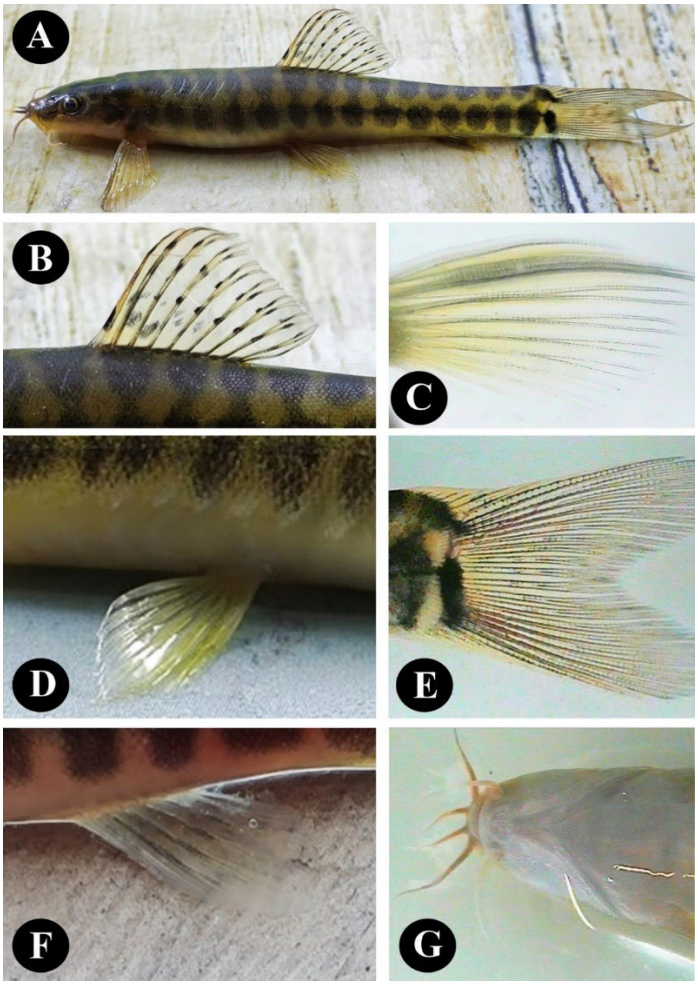

Figure 5 *Nemacheilus fasciatus* (Valenciennes, 1846) (Standar Length: 52.9 mm; MZB 26539) from Blitar, East Java, Indonesia. A. Habitus, lateral view; B. Dorsal fin, lateral view; C. Pectoral fin, lateral view; D. Ventral fin, lateral view; E. Caudal fin, lateral view; F. Anal fin, lateral view; G. Anterior part, ventral view

Material examined. Glawah River (MZB 26552, SL. 45.7 mm; 07°59'58.02"S, 112°05'46.52"E), Loadeng River (MZB 26545, SL. 52.9 mm; 08°00'01.61"S 112°06'30.43"E), Blitar, East Java, 26 June 2022, Coll. D.A. Rahayu & E.D. Nugroho.

Description. Morphometric and statistical data are presented in Table 1. The head is rounded with a pair of eyes, a pair of nares, a short blunt snout, a small subterminal mouth and circular lips around the mouth. The eyes are elliptical and nares are located between the snout and the eyes. Three pairs of rostral barbels are present at the mouth, two pairs on the upper jaw and one pair on the upper snout. The length of the snout is medium, slightly pointed and slightly shorter than the postorbital part of the head. The mouth is arched and the anterior lip is slightly furrowed anteriorly (see Fig. 5). The posterior lip has 4–5 deep grooves on each side of the different median incisions. The posterior part of the lips is smooth. The maxillary and outer rostral barbels reach the mid-length of the postorbital area of the head.

The body is elongated, fusiform, weakly compressed and without sharp scales at the base of the tail. Total length (TL) is about 1.14–1.50 (1.26 ± 0.06) times as long as SL. The head length is about 1.69–3.65 (2.56 ± 0.04) times as long as the snout. Eye diameter is about 0.33–0.69 (0.46 ± 0.005) times as long as the snout; 0.25–0.58 (0.39 ± 0.008) times as long as postorbital length; 0.02–0.06 (0.04 ± 0.0008) times as long as SL. Predorsal length is about 0.72–1.52 (1.01 ± 0.007) times as long as prepelvic length. Prepectoral length is about 0.70–1.22 (0.96 ± 0.008) times as long as the length of the head. The body height is about 1.02–3.22 (1.62 ± 0.04) times as long as the height of the caudal peduncle.

The position of the dorsal fin base is located in front of the vertical line of the pelvic fin. It is closer to the tip of the snout than the base of the caudal fin; the size of the dorsal fin is medium; the base of the posterior tip is opposite to the ventral fin and the tip does not reach the anal fin. The anal fin is short, far behind the ventral fin. The ventral fin does not reach the anal fin; the pectoral fins are shorter than the head. The caudal fin is emarginated, the lobes are pointed and caudal fin is longer than the head. The base of the dorsal fin is almost at the middle of the tip of the snout and the base of the caudal fin. There is no black spot at the base of the anterior dorsal fin. The pectoral fin is rounded and the length is almost equal to the head, ending less than its length in front of the ventral fin, shorter than the pectoral fin, ending less than its length in front of the anal fin; the anal fin is rounded or slightly pointed, not or barely branched lower than body size, higher than base length; the caudal fin is emarginated or crescent-emarginate. The pectoral fin does not reach the base of the ventral fin. A small axillary lobe presents at the base of the ventral fin which is inserted under the dorsal forked finger, the anal fin does not reach the base of the caudal fin and the last fin is branched with a subequal lobe. Dorsal fin D II 7–8; anal fin A I. 6; pectoral fin P I. 9–10; ventral fin V I. 6–7, Caudal fin C. 17.

Coloration. The body is yellowish with 16–18 vertical elongated black spots. The anterior spots are thinner than the posterior ones. There are about 5-6 black saddles on the back in front of the dorsal fin. There is a black spot at the base of the caudal fin. The head is dark. There are about 7 colors of the saddle with positions below and behind the dorsal fin. There is a black spot on the proximal third of the dorsal fin rays. Half of the dorsal fin rays are dark. There are two longitudinal rows of spots on the dorsal rays: in the middle and above all four fin rays. The other fin is hyaline.

Body length. Standard length (SL) ranged from 24.20 to 71.70 mm, with a mean of 42.59 ± 0.87 mm and total length (TL) ranged from 31.30 to 93.50 mm, with a mean of 53.56 ± 1.31 mm (n = 147). The smallest was found from Loadeng river and the largest was found from Lekso river.

Distribution. According to Kottelat (1984) and Kottelat *et al.* (1993), this species was distributed up to southern Sumatra and also Java Island (West Java, Central Java and East Java). On the other hand, Hubert *et al.* (2019) noted that this species was distributed only in West Java. In this study, *N. fasciatus* was found in all six rivers from Blitar, East Java.

Remarks. There is a contradiction information on the distribution of this species between Kottelat (1984), Kottelat *et al.* (1993) with Hubert *et al.* (2019). We believe that Kottelat's information is correct compared to Hubert *et al.* (2019), because Hubert's claimed that this species only distributed in West Java after revisiting the Java Island. Meanwhile, we

found *N. fasciatus* in East Java, different from Hubert's information and similar to Kottelat's information.

Statistical analysis. The Kruskal-Wallis test showed that all of the 14 measured characters were significantly different ($P < 0.001$) (Table 1).

The morphological characteristics of the two *Nemacheilus* fishes can be distinguished from their body colorations (*N. chrysolaimos* with black-yellowish, while *N. fasciatus* with yellowish), pattern (irregular shape/bars or dots), ray ornamentation on the dorsal fin (black dots in *N. chrysolaimos*, while reddish spot in *N. fasciatus*), anterior naris (*N. chrysolaimos* with valve pierced tube-like, while in *N. fasciatus*, anterior margin with a winged flap) and also the fins meristic. The four preceding characters were almost similar and also found from Kottelat's (1984) observations but Kottelat (1984) and Kottelat *et al.* (1993) did not mention the detail on the meristic fins of *N. chrysolaimos* or *N. Fasciatus*. The fins provide valuable insights into the morphological characteristics of the two fish species. *N. chrysolaimos* has shorter proportions of eye diameter, head length, and lateral length of the head concerning SL, as opposed to *N. longipectoralis*. This information contributes to a more comprehensive understanding of the morphological distinctions between the two species. Similarly, it becomes apparent that *N. fasciatus* bears a resemblance to *N. masyae*, with a few notable differences. The upper caudal lobe and the eye diameter of *N. fasciatus* are larger in comparison to *N. Masyae*, and this distinction in size provides an updated perspective on the morphological variations between the two closely related species.

The significant difference in the morphometric of *N. fasciatus* may be related to the condition of the rivers or the habitats. For example, the differences in the river flow have caused a morphological variation in western rainbowfish (*Melanotaenia australis*) (Kelley *et al.* 2017). Flow regime differences in the streams result in morphological variation in *Cyprinella venusta* (Haas *et al.* 2010). The availability and prey type also lead to differences in morphological features (Hendry *et al.* 2002) but there was no evidence to support the differences among *N. fasciatus*. Therefore, further study should be conducted to elucidate the morphological variation among *N. fasciatus* from the six rivers in Blitar, East Java, Indonesia.

Sequence Composition and genetic diversity

A total of 11 partial sequences of the COI gene with a length of 503 base pairs (bp) for *N. fasciatus* and *N. chrysolaimos* were successfully amplified and analyzed to determine genetic variations within the related species based on the database in Table 2. A universal primer for the partial sequence of the COI gene carried out through accurate calculations, was successfully

Table 1 Morphometric data (mm) and Kruskal-Wallis's test (H) on two species of *Nemacheilus* fishes. from Blitar, East Java, Indonesia

Characters	*N. chrysolaimos* (n = 9)						*N. fasciatus* (n = 147)					
	Min	Max	Mean	Std. Error	H	*p*	Min	Max	Mean	Std. Error	H	*p*
Total length (TL)	40.6	66.4	55.3	2.9	N/A	N/A	31.3	93.5	53.6	1.1	92.6	< 0.001
Standard length (SL)	32.1	52.4	44.4	2.4	N/A	N/A	24.2	71.7	42.6	0.9	87.3	< 0.001
Head length	6.1	10.6	8.7	0.5	N/A	N/A	5.2	13.9	8.6	0.1	69.5	< 0.001
Snout length	2.1	4.8	3.4	0.3	N/A	N/A	1.8	6.4	3.4	0.1	16.4	< 0.001
Eye diameter	0.8	2.2	1.5	0.1	N/A	N/A	1	3.6	1.7	0	24.7	< 0.001
Postorbital length	2	5.4	3.8	0.4	N/A	N/A	2	7.9	4.2	0.1	41.2	< 0.001
Predorsal length	14.5	25.9	21	1.3	N/A	N/A	10.5	34.4	21.3	0.4	70.4	< 0.001
Pre-pelvic length	14.2	25.7	20.6	1.4	N/A	N/A	10.8	34.8	21.2	0.4	66.1	< 0.001
Pre-pectoral distance	5.7	10.4	8.2	0.5	N/A	N/A	4.8	80	8.8	0.5	40.7	< 0.001
Pectoral fin length	4.9	8.6	7.1	0.4	N/A	N/A	0.9	12.6	6.1	0.2	85	< 0.001
Body height	2.6	8.1	4.9	0.5	N/A	N/A	3.3	11.3	6.1	0.1	41.8	< 0.001
Dorsal fin length	5.4	10.2	7.9	0.4	N/A	N/A	3.2	17.3	7.2	0.2	77.9	< 0.001
Anal fin length	3.7	6.6	5.5	0.3	N/A	N/A	1.3	9.5	4.9	0.2	84	< 0.001
Height of caudal peduncle	2.6	5.4	4	0.3	N/A	N/A	1.9	6.6	3.9	0.1	36.3	< 0.001

Note: N/A is not available.

Table 2 GenBank accession numbers for partial sequence of COI gene of *Nemachaeilus* spp. with references

No	Species	MZB Code	Locality	GenBank Accession
1	*Nemacheilus chrysolaimos*	MZB 26539	Slorok River, Garum	OP379585
2	*Nemacheilus chrysolaimos*	MZB 26540	Slorok River, Garum	OP381212
3	*Nemacheilus fasciatus*	MZB 26550	Slorok River, Garum	OP412496
4	*Nemacheilus fasciatus*	MZB 26545	Loadeng river, Ponggok	OP412495
5	*Nemacheilus fasciatus*	MZB 26543	Sumber Ronje River, Ponggok	OP379588
6	*Nemacheilus fasciatus*	MZB 26544	Sumber Ronje River, Ponggok	OP412493
7	*Nemacheilus fasciatus*	MZB 26552	Glawah River, Ponggok	OP412492
8	*Nemacheilus fasciatus*	MZB 26551	Glawah River, Ponggok	OP412789
9	*Nemacheilus fasciatus*	MZB 26547	Kali Tanjung River, Garum	OP412790
10	*Nemacheilus fasciatus*	MZB 26541	Lekso River, Wlingi	OP380384
11	*Nemacheilus fasciatus*	MZB 26549	Slorok River, Garum	OP412790
12	*Nemacheilus fasciatus*	BIF3609	Dauwan River, Mojokerto	KU692665.1
13	*Nemacheilus fasciatus*	BIF0832	Pelus River, Purwokerto	KT960792.1
14	*Nemacheilus fasciatus*	BIF3609	Dauwan River, Mojokerto	KU692665.1
15	*Nemacheilus chrysolaimos*	BIF0170	Ci Seupan, Sukabumi	KU692664.1
16	*Rasbora argyrotaenia*	BIF0975	Teluk Peny, Cilacap, West Java	KT960805.1

applied to *Nemacheilus* spp. From Blitar Regency, East Java, Indonesia. The details of the sequence characteristics based on a length of 503 bp are summarized in Table 3. The percentage of base adenine (A), cytosine (C), guanine (G), and thymine (T) in all *Nemacheilus* spp. were 16.244%, 32.023%, 23.88%, and 29.565%, as shown in Table 3. Furthermore, the percentage of G+C content in the partial sequence of the COI gene was 48.26%.

The absence of stop codons in these sequences indicated a successful amplification of functional mitochondrial COI sequences. Therefore, nuclear DNA sequences derived from the mitochondrial DNA (NUMTs) were not sequenced since vertebrates NUMT was less than 600 bp (Wong *et al.* 2009). The characteristics of the partial sequence of the COI gene were analyzed which included haplotype diversity (Hd) 0.978 with nucleotide (π) 0.10532, Frequency of parsimony informative sites 25.646%, Polymorphic sites 165, ts/tv ratio (k) Purines= 7.006, Pyrimidines= 0.042; ts/tv ratio (R) 1.322; and mean of evolutionary rate 0.00, 0.02, 0.05, 0.09, 0.15, 0.22, 0.31, 0.42, 0.56, 0.72, 0.93, 1.19, 1.53, 2.01, 2.79, and 4.99 substitutions per site. The characteristics indicated that the partial sequence of the COI gene was suitable for determining the species of *Nemacheilus* spp. Genetic distance referred to the ratio of genetic differences between species or populations. Based on the genetic distance matrix of 2 species of *Nemacheilus* spp., the highest distance was found between *N. fasciatus* and *N. Chrysolaimos* with a value of 0.22 (Table 4). Therefore, a smaller genetic distance value generated a more similar appearance partial sequence of COI genes compared to related species.

DNA barcoding distinguished freshwater fish species with barcodes in Australia, Canada, India, Thailand, Germany, and Indonesia (Ward *et al.* 2005, Hubert *et al.* 2008, Knebelsberger *et al.* 2014; Lakra *et al.* 2015, Pampromin *et al.* 2019, Rahayu *et al.* 2019). The partial sequences of the COI gene profile for *N. chrysolaimos* and *N. fasciatus*, which were local freshwater fish species in different locations were compiled. Sequence validation was also performed using the online facility provided by BLAST (NCBI) and the BOLD system. The results indicated that the sequence samples matched the available accessions in the database, with query coverage ranging from 98% to 99.8%, and this confirmed the effectiveness of using DNA barcodes for species identification. After analyzing the nucleotide sequences, no insertions, deletions, or codon stops were observed. This supported the notion that all the amplified sequences represented functional mitochondrial COI sequences. Additionally, the average length of the amplified sequences exceeded 503 bp, which was typically the limit observed for nuclear DNA sequences originating from mtDNA (NUMT). These findings strengthened the reliability of the results and underscored the suitability of the COI gene as a marker for distinguishing between *N. chrysolaimos* and *N. fasciatus* in the local freshwater fish populations of Blitar Regency, Indonesia (Buhay, 2009; Gunbin *et al.* 2017).

Table 3 Characteristics of partial sequence of COI gene used for phylogenetic trees reconstruction and genetic distance analysis include sequences from the study sample and the GenBank/BOLD system (in group and out group)

Parameters	Position at codon			
	1st	2nd	3rd	Total
Thyrosine frequency	26.757%	41.737%	20.203%	503 bp
Cytosine frequency	30.382%	31.723%	33.964%	503 bp
Adenine frequency	22,875%	12.5%	31.127%	503 bp
Guanine frequency	19.986%	14.041%	14.706%	503 bp
Frequency of invariable sites	67.196%			
Frequency of parsimony informative sites	25.646%			
Nucleotide diversity (Pi)	0,10532			
Haplotype diversity	0.882			
Number of haplotypes	8			
Total number of mutations	199			
Polymorphic sites	165			
ts/tv ratio (k)	Purines= 7.006, Pyrimidines= 0.042			
ts/tv ratio (R)	1.322			
Gamma discrete distribution	0.5428			
Mean of evolutionary rate	0.00, 0.02, 0.05, 0.09, 0.15, 0.22, 0.31, 0.42, 0.56, 0.72, 0.93, 1.19, 1.53, 2.01, 2.79, and 4.99 substitutions per site			

Note: The COI gene sequence characteristics were based on the 503 bp sequence length.

The partial sequence of the COI gene of *Nemacheilus* spp. showed that the values of the nucleotide base composition of G+C and A+T were between 48.26% and 51.74%, as shown in Table 3. The value of the nucleotide base composition and content of the A+T result was higher than G+C, consistent with the characteristics of the mitochondrial base composition. The analysis of the partial sequence of the COI gene showed that AT content (54.44%) was higher than GC content (48.26%), These data were observed in Australia (Ward *et al.* 2005), Canadian (Steinke *et al.* 2009); Cuban (Lara *et al.* 2010), and Bangladesh (Ahmed *et al.* 2020) fish species. Clusters 1 and 2 were resolved as sister taxa with 99% bootstrap and the genetic diversity was very low or less than 2%. A genetic distance value of more than 2% indicated that there were species different from other group members. Meanwhile, a genetic distance value of less than 3% indicated that the group or cluster was obtained from the same species (Hebert *et al.* 2003; Hebert *et al.* 2004). Based on the standards from Nei (1972), the genetic distance of *Nemacheilus* spp. obtained in Blitar Regency waters was categorized into low (0.01–0.045) and medium (0.17–0.18) similar to *Nemacheilus* spp. genetic distance calculations reported by Hubert *et al.* (2019).

Phylogenetic reconstruction

Phylogenetic relationships were shown in the ME tree (Fig. 6) and ML tree (Fig. 7). Each species was associated with a specific DNA barcode cluster and the relationship among these species was obtained. Closer species in terms of genetic divergence, were clustered at the same nodes to determine the distance between the terminal branches of the ME & ML trees, consisting of two divergent clusters.

Table 4 Pairwise genetic distance of *Nemacheilus fasciatus* dan *N. crysolaimos* compared to all congeners and outgroups

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
NF MZB 26551																
NF MZB 26552	0.000															
NF MZB 26549	0.000	0.000														
NF MZB 26550	0.000	0.000	0.000													
NF MZB 26545	0.004	0.004	0.004	0.004												
NF MZB 26547	0.045	0.045	0.045	0.045	0.045											
NF MZB 26543	0.045	0.045	0.045	0.045	0.045	0.000										
NF MZB 26541	0.045	0.045	0.045	0.045	0.045	0.000	0.000									
KU692665.1_NF	0.045	0.045	0.045	0.045	0.045	0.000	0.000	0.000								
KU692666.1_NF	0.060	0.060	0.060	0.060	0.060	0.014	0.014	0.014	0.014							
NF MZB 26544	0.047	0.047	0.047	0.047	0.047	0.002	0.002	0.002	0.002	0.012						
KT960792.1_NF	0.047	0.047	0.047	0.047	0.047	0.002	0.002	0.002	0.002	0.012	0.000					
NC MZB 26540	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179				
KU692664.1_NC	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179	0.000			
NC MZB 26539	0.229	0.229	0.229	0.229	0.229	0.181	0.181	0.181	0.181	0.170	0.179	0.179	0.000	0.000		
KT960805.1	0.278	0.278	0.278	0.278	0.278	0.229	0.229	0.229	0.229	0.223	0.226	0.226	0.243	0.243	0.243	
LC130692	0.288	0.288	0.288	0.288	0.288	0.241	0.241	0.241	0.241	0.241	0.238	0.238	0.244	0.244	0.244	0.144

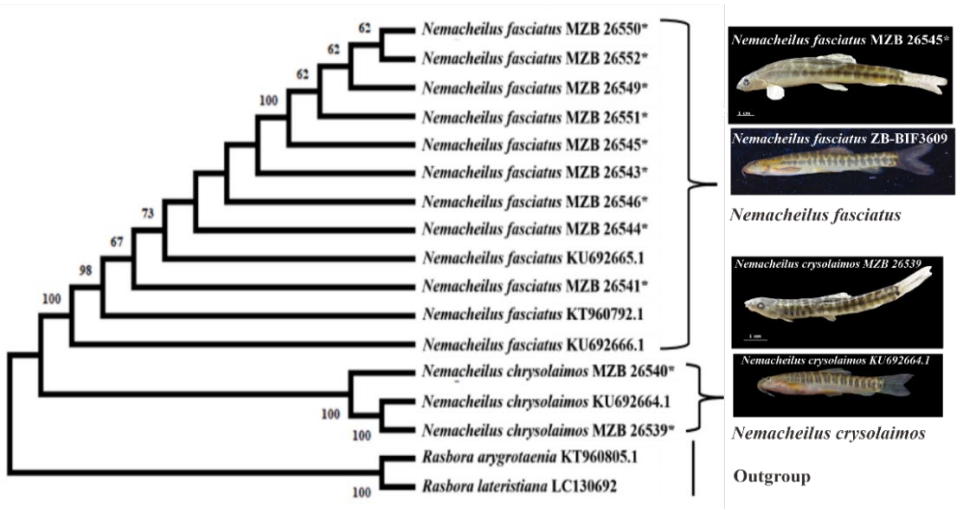

Figure 6 Minimum Evolution (ME) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of COI gene. The asterisk (*) denotes the sequence of *Nemacheilus* spp. obtained from Blitar Regency, East Java, Indonesia and *Rasbora* spp. as the outgroup

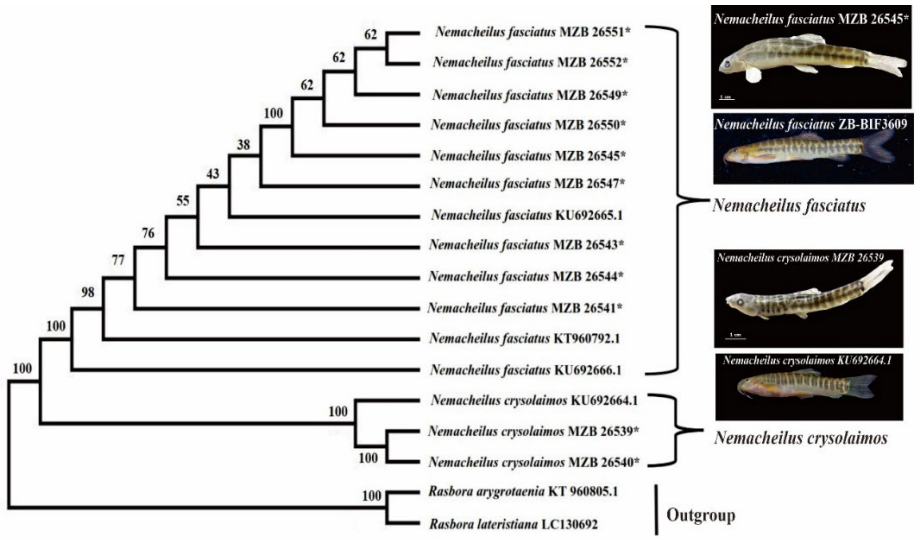

Figure 7 Maximum-Likelihood (ML) phylogenetic tree of *Nemacheilus* spp. based on partial sequence of COI gene. The asterisk (*) denotes the sequence of *Nemacheilus* spp. obtained from Blitar Regency, East Java, Indonesia and *Rasbora* spp. as the outgroup

The phylogenetic analysis of *Nemacheilus* spp. using both ME and ML methods resulted in unambiguous branching patterns, as illustrated in Figs. 6 and 7. The phylogenetic trees showed that *N. fasciatus* and *N. chrysolaimos* species formed distinct monophyletic branches. However, their proximity at the same node indicated genetic relatedness and the positioning of these branches corresponded with a calculated genetic distance of 0.22, signifying the greatest divergence between these two species. The ME, ML, and genetic distance data collectively provided strong evidence that *N. fasciatus* and *N. chrysolaimos* were genetically distant from each other.

In addition, the ABGD method identified 3 groups for *Nemacheilus* spp. specimens in with the initial approach and the barcode gap threshold calculated by the COI dataset as shown in Figs. 8A & 8B). The value of the barcode gap distance was 0.025 in line with the results of the ABGD grouping which divided the species into 3 groups, as shown in Fig 8C. Group [1] (*Nemacheilus fasciatus* MZB 2655, *Nemacheilus fasciatus* MZB 26549, *Nemacheilus fasciatus* MZB 26550, *Nemacheilus fasciatus* MZB 26552, *Nemacheilus fasciatus* MZB 26545, Group [2] (*Nemacheilus fasciatus* MZB 26543, *Nemacheilus fasciatus* MZB 26546, *Nemacheilus fasciatus* MZB 26544, *Nemacheilus fasciatus* MZB 26541), and Group [3] (*Nemacheilus crysolaimos* MZB 26540, and *Nemacheilus crysolaimos* MZB 26539).

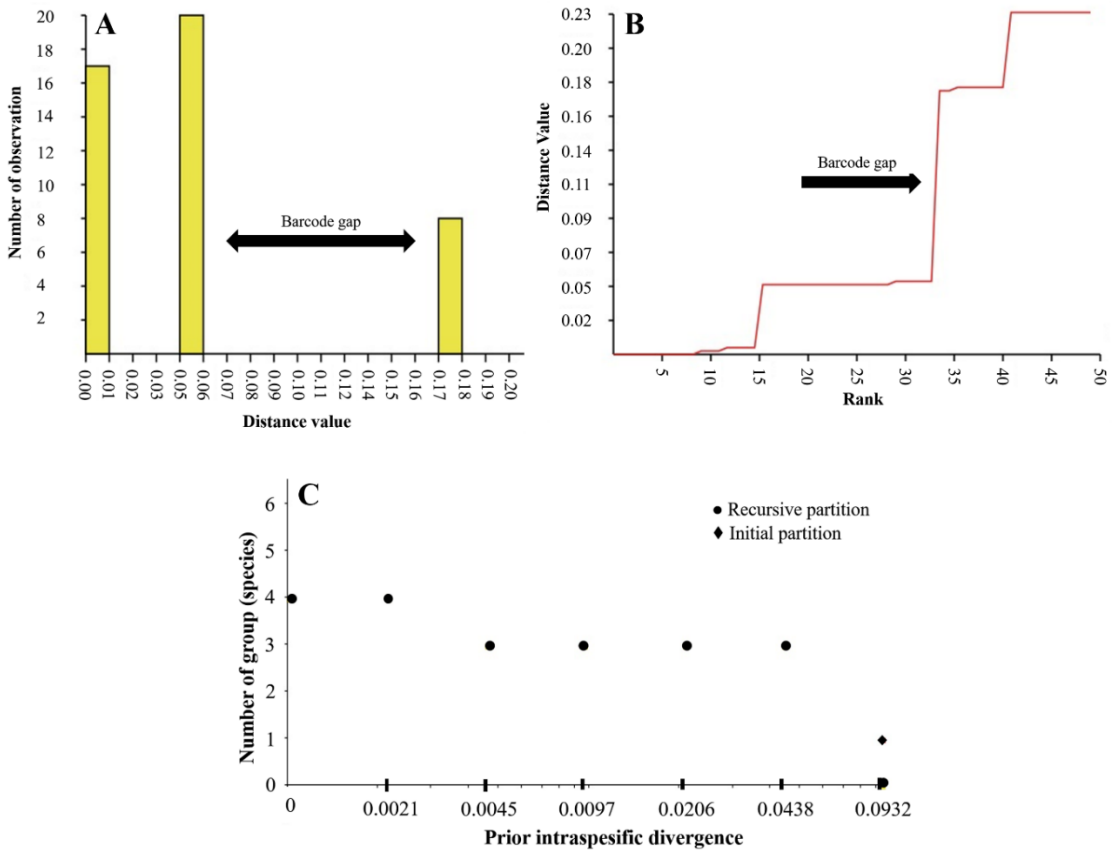

Figure 8 Barcode Gap Analysis of COI sequences performed by ABGD (Puillandre *et al.* 2012). Histograms show the distribution of pairwise genetic distances (uncorrected p-distances) between each pair of specimens. The arrow indicates the gap that allows to distinguish intraspecific (left) and interspecific (right) distances for the COI region. (A) Histogram of distance, (B) Ranked distance, and (C) Number of Primary Species Hypotheses (PSHs) obtained, for each prior intraspecific divergence

The application of ABGD analyses, with a prior maximal distance set at 0.025, further reinforced the separation of *N. chrysolaimos* and *N. fasciatus* into distinct partitions. These additional analyses align with differentiation of these species. Consequently, the combination of genetic distance, phylogenetic analysis, and ABGD analyses collectively confirmed the successful identification of *Nemacheilus spp.* from Blitar Regency. Based on the comprehensive evidence derived from DNA barcoding, with morphological characteristics, it can be concluded that the targeted utilization of these tools offered an efficient and reliable means of identifying *Nemacheilus* spp. at the species level. Therefore, this study was the first to report on the morphology in accordance with Kottelat (1984); Kottelat *et al* (1993); Hardiaty *et al.* (2014)] genetic identification and phylogenetic reconstruction of *Nemacheilus* spp. using the partial sequence of the COI gene. Conservation management of *N. chrysolaimos* and *N. fasciatus* in grouping animal units should be conducted according to species and genetic entity, as well as the potential of developing cryopreservation for sustainability. A molecular approach using the partial sequence supported the identification results based on a morphological approach in *Nemacheilus* spp. and obtained an accession number from GenBank (NCBI) database. This study indicated that improved morphology and molecular characteristics of local loaches (*Nemachelius* spp.) were obtained from Blitar Regency. Therefore, a reliable DNA barcode reference library for East Java, Indonesia, freshwater fish was established to assign fish species by screening sequences. This initiative aimed to enhance the achievement of better monitoring, conservation, and management of fisheries in this overexploited region.

CONCLUSION

In conclusion, this study has successfully identified analysis for loach fishes such as *N. chrysolaimos* and *N. fasciatus* from six rivers at Biltar, East Java, Indonesia, based on morphology and molecular data. The main characteristic used to distinguish these species is the color pattern on their lateral bodies, such as dark bars or spots, along with the morphological variations of anal fins. Moreover, through the utilization of genetic approaches, including phylogenetic reconstruction, sequence composition, genetic diversity analysis, and ABGD analysis, it was determined that *N. chrysolaimos* and *N. fasciatus* are distinct species.

ACKNOWLEDGEMENTS

The authors are grateful to the people around the river of Blitar Regency for their assistance during sampling session. Mr. Didik for his great help at the molecular laboratory and Museum Zoological Bogor, Directorate of Scientific Collection Management-BRIN for great help to saving this specimen. Rofiza Yolanda for his valuable time for checking the preparation of this manuscript. M. Kottelat and Prof Joerg Bohlen for checking morphological and molecular analysis. We gratefully acknowledge to the reviewers and the Editor for the thorough and constructive reviews of this manuscript. This works was supported by the Research Grant from Faculty of Mathematics and Natural Sciences, Universitas Negeri Surabaya for the fiscal year 2022, (Grant No. 659/UN38/HK/PP/2022, "Policy Research Theme").

REFERENCES

Ahmed MD, Datta SK, Zhilik AA. 2020. Molecular diversity of freshwater fishes of Bangladesh assessed by DNA barcoding. Bangladesh J Zool 48(1): 1-19.

Ath-thar MHF, Ambarwati A, Soelistyowati DT, Kristanto AH. 2018. Keragaman genotipe dan fenotipe ikan uceng *Nemacheilus Fasciatus* (Valenciennes, 1846) asal Bogor, Temanggung, dan Blitar. J Ris Akuakultur 13(1): 1-10.

Bleeker P. 1853. Diagnostische beschrijvingen van nieuwe of weinig bekende vischsoorten van Sumatra. [Diagnostic descriptions of new or little-known fish species from Sumatra]. Tiental V–X. Natuurk Tijdschr Ned.-Indië 4: 243-302.

Bleeker P. 1854. Overzigt der ichthyologische fauna van Sumatra, met beschrijving van eenige nieuwe soorten. [Overview of the ichthyological fauna of Sumatra, with descriptions of some new species]. Natuurk Tijdschr Ned.-Indië 7: 49-108.

Bleeker P. 1859. Negende bijdrage tot de kennis der vischfauna van Banka. [Ninth contribution to the

knowledge of the fish fauna of Bangka]. Natuurk Tijdschr Ned.-Indië 18: 359-78.

Bleeker P. 1862–63. Atlas ichthyologique des Indes Orientales Néêrlandaises, publié sous les auspices du Gouvernement colonial néêrlandais. Tome II. Siluroïdes, Chacoïdes et Hétérobranchoïdes. [Fish Atlas of the Dutch East Indies, published under the auspices of the Dutch Colonial Government. Volume II. Siluroids, Chacoids and Heterobranchoids]. Amsterdam: F. Muller, 112 pp., Pls. 49–101. [pp. 1–32 (November 1862), pp. 33–64 (January 1863), pp. 65–96 (April 1863), pp. 97–112 (September 1863), plates published 1862–1863, see Kottelat (2013b), p. 283].

Bleeker P. 1863a. Sur les genres de la famille des Cobitioïdes. [On the genera of the Cobitioid family]. Verslagen en Mededeelingen der Koninklijke Akademie van Wetenschappen, Afdeeling Natuurkunde 15: 32-44.

Bleeker P. 1863b. Atlas ichthyologique des Indes Orientales Néêrlandaises. Tome III. Cyprins. [Fish Atlas of the Dutch East Indies. Volume III. Cyprines]. Amsterdam: Müller.

Bleeker, P. 1860. De visschen van den Indischen Archipel beschreven en toegelicht. Deel 2. Ordo Cyprini, karpers. [The fish of the Indian Archipelago described and explained. Part 2. Ordo Cyprini, Carp.]. Acta Soc Regiae Sci Indo-Neêrl 7(2): 1-492.

Boratyn GM, Camacho C, Cooper PS, Coulouris G, Fong A, Ma N, Madden TL, Matten WT, McGinnis SD, Merezhuk Y, Raytselis Y, Sayers EW, Tao T, Ye J, Zaretskaya I. 2013. BLAST: a more efficient report with usability improvements. Nucleic Acids Res 41: 29-33.

Buhay JE. 2009. "COI-like" sequences are becoming problematic in molecular systematic and DNA barcoding studies. J Crustac Biol 29(1): 96-110.

Cope ED. 1871. Contribution to the ichthyology of the Lesser Antilles. Trans Am Philos Soc, New Series, 14(3): 445-83.

Cuvier G, Valenciennes A. 1846. Histoire naturelle des poissons. Paris: Tome dix-huitième. Bertrand.

Duvaud S, Gabella C, Lisacek F, Stockinger H, Ioannidis V, Durinx C. 2021. Expasy, the Swiss Bioinformatics Resource Portal, as designed by its users. Nucleic Acids Res. 49(W1): W216-W227.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-91.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial Cytochrome C Oxidase Subunit I from diverse metazoan invertebrates. Mol Marine Biol Biotechnol 3(5): 294-99.

Froese R, Pauly D. (Eds.) 2022a. FishBase. Family Nemacheilidae - Brook loaches. [cited 2022 October 01]. Available from: https://www.fishbase.se/summary/FamilySummary.php?ID=692#famList_tab

Froese R, Pauly D. (Eds.). 2022b. FishBase. *Nemacheilus* Bleeker, 1863. [cited 2022 October 22]. Available from https://www.marinespecies.org/aphia.php?p=taxdetails&id=154189

Gunbin K, Peshkin L, Popadin K, Annis S, Ackermann RR, Khrapko K. 2017. Data on the time of integration of the human mitochondrial pseudogenes (NUMTs) into the nuclear genome. Data Br 13: 536-44.

Haas TC, Blumand MJ, Hein DC. 2010. Morphological responses of a stream fish to water impoundment. Biol Lett 6: 803-06.

Hadiaty RK, Yamahira K. 2014. The loaches of the genus *Nemacheilus* (Teleostei: Nemacheilidae) in Sunda Islands, with an identification key. Indones J Ichthyol 14: 1-18.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for windows 95/98/NT. Nucleic Acids Symp Ser 41: 95-98.

Hebert PDN, Cywinska A, Ball SL, deWaard JR. 2003. Biological identifications through DNA barcodes. Proc R Soc B: Biol Sci 270(1512): 313-321.

Hebert PDN, Stoeckle MY, Zemlak TS, Francis CM. 2004. Identification of Birds through DNA Barcodes. PLoS Biol 2(10): e312.

Hendry AP, Taylor EB, McPhail JD. 2002. Adaptive divergence and the balance between selection and gene flow: lake and stream stickleback in the misty system. Evolution 56: 1199-1216.

Hubert N, Hanner R, Holm E, Mandrak NE, Taylor E, Burridge M, Zhang J. 2008. Identifying Canadian freshwater fishes through DNA barcodes. PLoS ONE 3(6): 19.e2490.

Hubert N, Lumbantobing D, Sholihah A, Dahrudin H, Busson F, Sauri S, Keith, P. 2019. Revisiting species boundaries and distribution ranges of *Nemacheilus* spp. (Cypriniformes: Nemacheilidae) and *Rasbora* spp. (Cypriniformes: Cyprinidae) in Java, Bali and Lombok through DNA barcodes: implications for conservation in a biodiversity hotspot. Conserv Genet 20: 517-29.

Kelley JL, Davies PM, Collin SP, Grierson PF. 2017. Morphological plasticity in a native freshwater fish from semiarid Australia in response to variable water flows. Ecol Evo 7(16): 6595-6605.

Kimura M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. J Mol Evol 16: 111-120.

Knebelsberger T, Dunz AR, Neumann D, Geiger MF. 2015. Molecular diversity of Germany's freshwater fishes and lampreys assessed by DNA barcoding. Mol Ecol Resour 15(3): 562-72.

Kottelat M, Freyhof, J. 2007. *Handbook of European freshwater fishes*. Berlin: Kottelat, Cornol & Freyhof.

Kottelat M, Whitten AJ, Kartikasari SN, Wirjoatmodjo S. 1993. Freshwater fishes of Western Indonesia and Sulawesi. Hong Kong: Periplus.

Kottelat M. 1984. Revision of the Indonesian and Malaysian loaches of the subfamily Nemacheilinae. Japanese J Ichthyol 31: 225-60.

Kottelat M. 2013a. The fishes of the inland waters of Southeast Asia: a catalog and core bibliography of the fishes known to occur in freshwaters, mangroves and estuaries. Raffles Bull Zool Suppl 27: 1-663.

Kottelat M. 2013b. Dates of publication of Bleeker's Atlas ichthyologique and Poissons de Madagascar. Zootaxa 3681(3): 281-85.

Kottelat M. 2022. *Nemacheilus pezidion*, a new species of loach from southern Laos (Teleostei: Nemacheilidae). Zootaxa 5129(1): 92-104.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Bio Evol 35: 1547-49.

Kusuma RO, Dadlono MS, Kusum B, Syakuri H. 2021. Genetic Diversity of Stone loaches (Nemacheilus) in River of Banyumas Area based on Cytochrome Oxidase Subunit I (COI). Jurnal Perikanan 23(2): 89-94.

Lakra WS, Singh M, Goswami M, Gopalakrishnan A, Lal KK, Mohindra V, Sarkar UK, Punia PP, Singh KV, Bhatt JP, Ayyappan S. 2015. DNA Barcoding Indian Freshwater Fishes. Mitochondrial DNA A: DNA Mapp Seq Anal 27(6): 4510-17.

Lara A, Ponce De León JL, Rodriguez R, Casane D, Cote G, Bernatchez L, García-Machado ERIK. 2010. DNA barcoding of Cuban freshwater fishes: evidence for cryptic species and taxonomic conflicts. Mol Eco Resour 10(3): 421-30.

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. 2007. Clustal W and Clustal X version 2.0. Bioinformatics (Oxford, England), 23(21): 2947-48.

Nei M, Kumar S. 2000. Molecular evolution and phylogenetics. Oxford University Press, New York.

Nei M. 1972. Genetic distance between populations. Am Nat 106: 283-92.

Panprommin D, Soontornprasit K, Tuncharoen S, Pithakpol S, Keereelang J. 2019. DNA barcodes for the identification of species diversity in fish from Kwan Phayao, Thailand. J Asia-Pac Biodivers 12(3): 382-89.

Puillandre N, Lambert A, Brouillet S, Achaz G. 2012. ABGD, Automatic Barcode Gap Discovery for primary species delimitation. Mol Ecol 21(8): 1864-77.

Rahayu DA, Nugroho ED, Listyorini D. 2019. DNA Barcoding ikan introduksi khas telaga sari, Kabupaten Pasuruan. [DNA Barcoding of Introduced Fish Typical of Telaga Sari, Pasuruan Regency]. Biotropika 7(2): 51-62.

Ratnasingham S, Hebert PD. 2007. BOLD: the Barcode of Life Data System (http://www.barcodinglife.org). Mol Ecol Notes 7(3): 355-64.

Regan CT. 1911. The classification of the teleostean fishes of the order Ostariophysii. – 1. Cyprinoidea. Annals and Magazine of Natural History, Series 8, 8(43): 13-32.

Roberts TR. 1993. The freshwater fishes of Java, as observed by Kuhl and van Hasselt in 1820–23. Zoologische Verhandelingen 285: 1-94.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Bio Evol 4: 406-25.

Sayers EW, Cavanaugh M, Clark K, Pruitt KD, Schoch CL, Sherry ST, Karsch-Mizrachi I. 2022. GenBank. *Nucleic Acids Res* 50(D1): D161-64.

Šlechtová V, Musilova Z, Tan HK, Kottelat M, Bohlen, J. 2021. One northward, one southward: Contrasting biogeographical history in two benthic freshwater fish genera across Southeast Asia (Teleostei: Cobitoidea: Nemacheilus, Pangio). Mol Phylogenet Evol 161: 107139.

Song H, Buhay JE, Whiting MF, Crandall KA. 2008. Many species in one: DNA barcoding overestimates the number of species when nuclear mitochondrial pseudogenes are coamplified. PNAS 105(36): 13486-91.

Steinke D, Zemlak TS, Boutillier JA, Hebert PD. 2009. DNA barcoding of Pacific Canada's fishes. Mar Biol 156(12): 2641-47.

van Hasselt, J.C. 1823. Uittreksel uit enn' brief van den Heer J. C. van Hasselt, aan den Heer C. J. Temminck, geschreven uit Tjecande, Residentie Bantam, den 29sten December 1822. [Extract from another letter from Mr. J. C. van Hasselt, to Mr. C. J. Temminck, written from Tjecande, Residentie Bantam, on 29 December 1822]. Algemeene Konst-en Letter-Bode voor het Jaar 1823, 2: 130-33.

van Hasselt, J.C. 1824. Extrait d'une seconde lettre sur les poissons de Java, écrite par M. van Hasselt à M. C.-J. Temminck, datée de Tjecande, résidence de Bantam, 29 décembre 1822 [Extract from a second letter on the fishes of Java, written by M. van Hasselt to M. C. -J. Temminck, date of Tjecande, residence of Bantam, December 29, 1822]. Bulletin Universel des Sciences et de l'Industrie, Section 2, Bulletin des Sciences Naturelles et de Géologie 2: 374-77.

Ward RD, Zemlak TS, Innes BH, Last PR, Hebert PD. 2005. DNA barcoding Australia's fish species. Philos Trans R Soc Lond B Biol Sci 360(1462): 1847-57.

Wong EHK, Shivji MS, Hanner RH. 2009. Identifying sharks with DNA barcodes: Assessing the utility of a nucleotide diagnostic approach. Mol Eco.

BIOTROPIA Vol. 30 No. 3, 2023: 346 - 354 DOI: 10.11598/btb.2023.30.3.1956

POTENTIAL OF CARBON SINK IN MANGROVE SUBSTRATES IN LEMBAR BAY, WEST LOMBOK, INDONESIA

FIRMAN ALI RAHMAN[1*], DEWI PUTRI LESTARI[2], ALFIAN PUJIAN HADI[3&5], ANIS SYAKIRATUR RIZKI[4], AISHA ZEA ALMAHYRA[5], ARSYA ZAFRAN ALVARENDRA[5], NUZULY ILMIA CERMINAND[5], NENING LISTARI[6], R. DIDI KUSWARA[6], ZULKARNAIN GAZALI[6], MAYA EKANINGTIAS[6], SITI WARDATUL JANNAH[6] AND BAIQ YULIA HASNI PRATIWI[7]

[1]*Department of Biology Education, Faculty of Education and Teacher Training, Universitas Islam Negeri Mataram, Mataram 83127,West Nusa Tenggara, Indonesia*
[2]*Departemen of Aquaculture, Faculty of Agriculture, Universitas Mataram, Mataram 83125, West Nusa Tenggara, Indonesia.*
[3]*Departemen of Geography Education, Universitas Muhammadiyah Mataram, Mataram 83127, West Nusa Tenggara, Indonesia.*
[4]*Department of Health Analyst, Faculty of Nursing and Health, Universitas Muhammadiyah Semarang, Semarang 50273, Indonesia.*
[5]*Indonesian Tajuk Foundation, Mataram 83116, West Nusa Tenggara, Indonesia.*
[6]*Departemen of Biology Education, Faculty of Education and Teacher Training, Universitas Nahdlatul Wathan Mataram, Mataram 83126, West Nusa Tenggara, Indonesia*
[7]*Departement of Pharmacy, Faculty of Public Health, Universitas Bumigora, Mataram 83127, Nusa Tenggara Barat, Indonesia.*

Received 20 March 2023 / Revised 25 July 2023 / Accepted 7 September 2023

ABSTRACT

Mangroves are one of the coastal vegetation that can mitigate carbon (carbon sink and carbon storage). This study aimed to determine the potential for soil carbon stock found under stands of mangroves in Lembar Bay, West Lombok, and West Nusa Tenggara. The research began with the identification of the species and then proceeded toa sampling of the soil, which was then analyzed using the Walkley and Black method. The results showed that there were ten species of mangroves, namely, *Rhizophora stylosa, Avicennia lanata, Avicennia marina, Bruguiera gymnorrhiza, Ceriops decandra, Excoecaria agallocha, Lumnitzera racemosa, Scyphiphora hydrophyllacea, Thespesia populnea,* and *Xylocarpus maluccensis*. The highest soil carbon content percentage was found in the lower soil of the *A. lanata* (1.43 %C) mangrove, and the lowest was found in the lower-stand soil of *E. agallocha* (0.21 %C). Meanwhile, the carbon sinks per meter were 0.002-0.066 gC/m^2, with an average of 0.020±0.020 gC/m^2. The estimated total soil carbon sink in 10 mangrove stands was 0.20-6.60 tons C/ha, with an average of 2.18±2.010 tons C/ha. The average total estimated soil carbon stock found in 20.49 ha of the mangrove area studied was 44.67 tonsC, which is equivalent to 263.69 tonsC in a mangrove area of 120.96 ha in Lembar Bay.

Keywords: carbon stock, c-organic, mangroves, soil

INTRODUCTION

Mangroves are one of the plants in coastal areas that play a role in disaster mitigation (abrasion, breakwater, sea wind barrier, and tsunami), biota habitats, and germplasms. The environmental benefits of mangrove ecosystems that have not been widely studied include their potential as carbon sinks and carbon storage, especially in mangrove ecosystem soil (Brath *et al.* 2015; Lovelock & Duarte 2019; Macreadie *et al.* 2019). Based on Murray *et al.*(2011), the average annual carbon sequestration potential of mangrove ecosystems is between 6 and 8 Mg CO_2 e/ha (tonnes CO_2 equivalent per hectare) and is two to four times greater than the carbon sink potential of tropical forests (Nellemann *et al.* 2009).

One of the coasts in the Mangrove Corridor Essential Ecosystem Area is Lembar Bay, West Lombok, which is directly affected by the activities of the Lembar harbor. Lembar harbor is an inter-island sea and goods transportation route that continues to be developed as a port area (reclamation) covering 22 ha. It has a direct impact on the degradation of mangrove

*Corresponding author, email:

ecosystems, resulting in a decrease in the mangrove ecosystem area, which currently only has an area of ±120.96 ha (Saraswati 2019).

The ecological potential of mangrove ecosystems as carbon sinks and carbon storage has been widely studied. The following data are some of those previous findings. The soil carbon stock of the Tanjung Lesung Banten mangrove ecosystem of 27.92 tons C/ha; the mangrove carbon stock in Dukuh Tapak, Tugurejo Village, Semarang amounted to 708.2 tons C/ha; mangroves in Timbulsloko Village, Demak, Central Java had carbon stock of 1,307.77 tons/ha; Perancak mangroves in Jembrana, Baliretain carbon stock of 119.75 tons C/ha; mangroves of the Batang Apar Estuary of West Sumatra had carbon stock of 2,561.90 tons C/ha (Handoyo *et al.* 2020); mangroves in Sungai Sembilan, Dumai had carbon stock of 1,819.31 tons C/ha (Handoyo *et al.* 2020); mangroves in Tambakbulusan Village, Demak, Central Java carbon stock of 57.74 tons C/ha; and Gili Meno mangroves, North Lombok had carbon stock of 154.62±99.78 tons C/ha, equivalent to a total soil carbon stock of 1,020.50 tonsC in a total 6.6 ha of the mangrove ecosystem area (Hilyana & Rahman 2022).

In general, research on carbon sinks in mangrove ecosystems is still related to the potential for carbon sinks in certain locations, and not specifically related to the potential for soil carbon stock under mangroves. This is in line with the opinion of Mcleod *et al.* (2011) and Howard *et al.*(2017) that in-depth analysis related to the potential of mangroves as carbon sinks and carbon storage in different species and habitats is very important. Due to this, this study can be a source of information on the potential for soil carbon storage found under ten types of mangroves in the harbor area of Lembar Bay, West Lombok, Indonesia.

MATERIALS AND METHODS

The study was carried out in the mangrove ecosystem located in Lembar Bay, Lembar, West Lombok, in February-March 2023 with a research site encompassing an area of 20.49 ha (located at 116°3'-116°4' E and 8°43'-8°44' S) (Fig 1). This was a quantitative descriptive study that began with the identification of mangroves and sampling of soil found under mangrove stands. Soil samples were collected fromunder ten mangrove standsat the research site. These stands represented various species, including *Avicennia lanata, Avicennia marina, Bruguiera gymnorrhiza, Ceriops decandra, Excoecaria agallocha, Lumnitzera racemosa, Rhizophora stylosa, Scyphiphora hydrophyllacea, Thespesia populcarapus,* and *Xylocarpus maluccensis*.

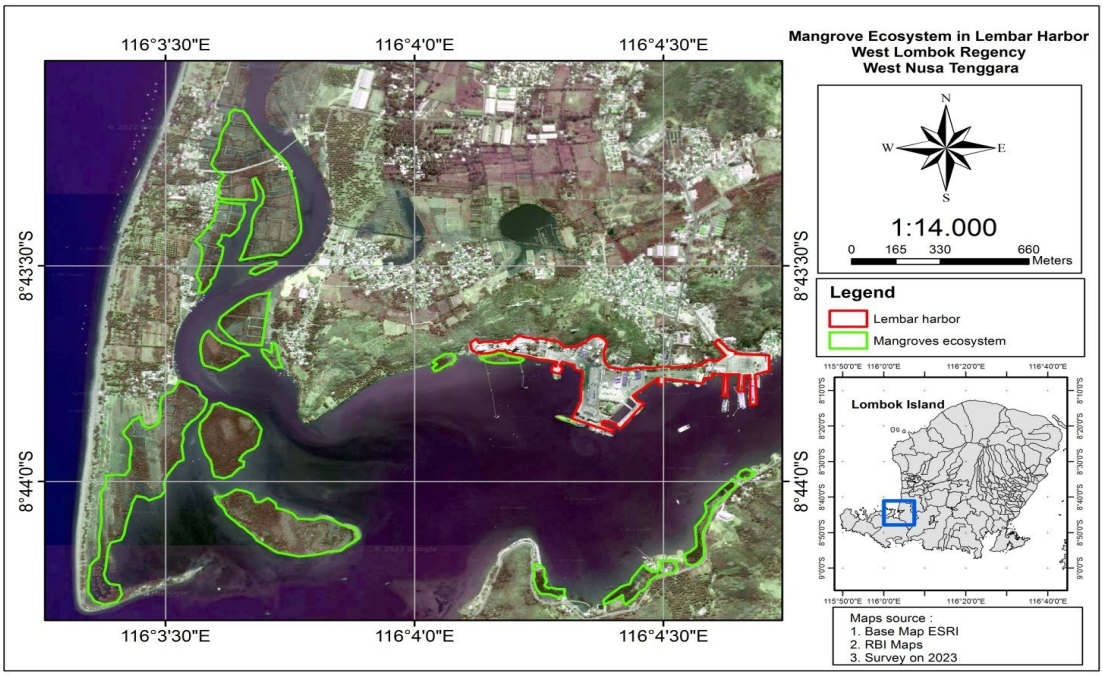

Figure 1 Mangrove ecosystem of Lembar Bay, West Lombok, Indonesia

Mangrove Identification

Mangroves were identified in situ based on morphological characteristics by referring to the introductory guide to Mangroves in Indonesia (Noor *et al.* 2006).

Analysis of Soil Organic Carbon Content

Soil sampling was carried out to a depth of 30 cm around the roots and a slope of 30° using pipes with a diameter of 5 cm and a length of 35 cm. The soil's carbon organic content was analyzed using the Walkley and Black method (Walkley & Black 1934). A soil sample weighing 0.5 g with a size of less than 0.5 mm was placed in a 100 ml volumetric flask. Then, 5 ml of 1 N $K_2Cr_2O_7$ was added, and the mixture was shaken. Following that, 7.5 ml of concentrated H_2SO_4 was added, and the mixture was shaken and left to stand for 30 minutes. It was then diluted with ion-free waterand the clear solution sample's absorbance was measured using a spectrophotometer at a wavelength of 561 nm. As a comparison, 0 and 250 ppm standards were made by pipetting 0 and 5 ml of the 5.000 ppm standard solution into a 100 ml volumetric flask with the same treatment as the sample procedure.

Data Analysis

Soil Carbon Content

The soil's carbon content was calculated using the following formula (Sulaeman *et al.* 2005):

$$\text{Soil c – organic content} = \frac{\text{Ppm curve}}{500 \times \text{correction factor}}$$

where:

ppm curve = The sample content obtained from the curve of therelationship between the standard series contentand its reading after corrected for blanks

Correction factor = 100/(100 - % water content)

Soil Carbon Stock

The soil's carbon stockwas calculated using the following formula (Badan Standarisasi Nasional, 2011):

$$Ct = Kd \times \rho \times \% \text{ c-organic}$$

where:

Ct = Soil carbon stock (g/cm^2)

Kd = Soil sample depth or soil depth (cm)

ρ = Bulk density is the ratio of the soil's dry weight to its volume (g/cm^3)

% c-organic = Value of carbon content percentage (0.47)

Soil Carbon Stock in Hectare Area

The soil's organic carbon content in hectare area was calculated using the following formula (Badan Standarisasi Nasional 2011):

$$\text{C soil (ton C/ha)} = Ct \times 100$$

where:

C_{soil} = Soil carbon stock (tons C/ha)

Ct = Soil organic carbon (g/cm^2)

100 = Conversion factor from g/cm^2 to tons C/ha

Total Carbon Stock Area

The total carbon stock area was calculated using the following formula (Lugina *et al.* 2017):

$$C_{totals} = C_n + C_{soil}$$

Description:

C_{totals} = Total carbon stock (tons C/ha)

C_n = Carbon stocks per hectare in each carbon pool in each plot (tons C)

C_{soil} = Soil carbon stock (tons/ha)

RESULTS AND DISCUSSION

Mangrove Species

A total of 10 mangrove stands were identified in Lembar Bay, namely, *Avicennia lanata, Avicennia marina, Bruguiera gymnorrhiza, Ceriops decandra, Excoecaria agallocha*, *Lumnitzera racemosa, Rhizophora stylosa, Scyphiphora hydrophyllacea, Thespesia populnea,* and *Xylocarpus maluccensis*. There were more strands discovered in this study than that of Syarifuddin & Zulhamran (2012), which found five species, namely, *Avicennia marina, Rhizophora stylosa, Rhizophora mucronata, Rhizophora apiculata,* and *Sonneratia alba.* On the other hand, Sukuryadi *et al.*(2021) found 12 species in a Lembar Bay mangrove area of 168.9 ha, those species being *Avicennia alba, Avicennia marina, Bruguiera cylindrica, Ceriop decandra, Ceriop tagal, Lumnitzera littorea, Lumnitzera racemosa, Phemphis acidula, Rhizhopora*

stylosa, Rhizophora apiculata, Rhizophora mucronata, and *Sonneratia alba*. These different findings may have occurred due to differences in research areas in the port. This has the potential to disrupt the mangroves' growth and development due to potential contamination from port activities and the loading and unloading of goods. Besides that, the research methodology utilized quadrant points, which limited the collection area of species composition data.

Soil of C-Organic Content

The largest percentage of soil carbon content was found in the bottom soil of *Avicennia lanata* (1.43% C) when compared to nine other species (Table 1). Meanwhile, the lowest percentage soil carbon content was found under *Excoecaria agallocha*st ands at 0.21% C. The soil carbon content percentage found in Lembar Bay waslower than that of the soil carbon in the Gili Meno at the range of 4.85-20.00 %C (Rahman & Hadi 2021; Hilyana & Rahman 2022). The high and low soil carbon content found under the Lembar Bay mangrove stands could generally be caused by the soil fraction size. This is in line with the research results of Lee *et al.* 2014, Ati *et al.*(2015); Sidik *et al.*(2016); and Lestariningsih *et al.*(2018). Another supporting factor is the large amount of organic matter sourced from litter weathering mixed with the soil (Rahman *et al.* 2023). In addition, it could be influenced by species density, species age, soil fraction, and each mangrove's growing zoning position (Schwarzer *et al.* 2016; Hilmi 2018; Bomer *et al.* 2020; Wang *et al.* 2020; Jannah *et al.* 2021). This is confirmed by the results reported by (Susilowati *et al.* 2020) that species density can affect litter production, which is one of the main sources of organic material for soil mangrove ecosystems.

Another factor is the water's condition, one of which is its pH, which can cause low weathering activity for organic matter by organisms (Abdelhakeem *et al.* 2016; Barreto *et al.* 2016; Hilmi *et al.* 2017; Hilmi *et al.* 2019). The physical factors that affect the waters of Lembar Bay are wind speed, temperature, and humidity. This is related to the amount of litter production in each mangrove species in Lembar Bay. Another factor is mangrove vegetation zoning, which is always flooded. This causes litter, fruit, and flowers, as the main sources of organic matter, to be affected by currents and carried to the open sea. This contributes to the organic sinking process in Lembar Bay. Greater attention should be directed towards the activities at the Lembar portas they have the potential to exacerbate environmental pollution through the changes in the water conditions. This is in line with several other studies on factors that affect carbon conservation in mangrove ecosystems, such as Matsui *et al.* (2015), Jones *et al.* (2016), Weiss *et al.* (2016), Martuti *et al.* (2017), Suhendra *et al.* (2018), Asadi *et al.* (2018), Pérez *et al.* (2018), Gao *et al.* (2019), and Kida & Fujitake (2020).

Table 1 Soil carbon content under mangrove stands in Lembar Bay

No.	Mangroves	Soil Carbon Content Under Mangrove Stands						
		Gross Weight	Dry Weight	Moisture Level	Correction Factor	Absorbance	Ppm curve	% C
1	*Avicennia lanata*	8.00	6.91	15.77	1.16	0.10	61.63	1.43
2	*Avicennia marina*	22.30	21.08	5.78	1.06	0.05	26.91	0.57
3	*Bruguiera gymnorrhiza*	20.00	19.35	3.36	1.03	0.04	20.71	0.43
4	*Ceriops decandra*	17.74	17.09	3.84	1.04	0.02	10.17	0.21
5	*Excoecaria agallocha*	15.57	15.21	2.30	1.02	0.01	5.83	0.12
6	*Lumnitzera racemosa*	17.31	16.53	4.51	1.05	0.03	16.06	0.34
7	*Rhizophora stylosa*	26.14	24.74	5.67	1.06	0.04	23.81	0.50
8	*Scyphiphora hydrophyllacea*	11.34	10.38	9.30	1.09	0.08	47.37	1.04
9	*Thespesia populnea*	16.70	16.15	3.43	1.03	0.02	9.55	0.20
10	*Xylocarpus maluccensis*	14.03	13.15	6.67	1.07	0.04	20.71	0.44
	Average	16.91	16.06	6.06	1.06	0.04	24.28	0.53
	Standard Deviation	5.220	5.141	3.959	0.041	0.028	17.591	0.409

The soil carbon content percentage (%C) can affect the total accumulation of potential carbon sinks in the research area. This study's results indicate that the soil carbon content stored 0.002-0.066 gC/m^2 with an average of 0.020±0.020 gC/m^2 (Table 2). The soil carbon content in each area is determined by its bulk density and percentage value. The soil carbon content under the *Avicennia lanata* mangrove stands had the largest amount of storage compared to the other nine species. However, it was lower than the results from another study which observed five mangrove stands (*Avecennia marina, Bruguera cylindrica, Rhizophora apiculata, Lumnitzera racemosa*, and *Excoecaria agallocha*) in Gili Meno, North Lombok. That study found an average of 0.57-3.08 gC/m^2 with an average of 1.55±1.000 gC/m^2 (Hilyana & Rahman 2022).

Several factors can determine the level of total accumulated soil carbon storage. These factors can be influenced by the percentage of soil carbon content, soil specific gravity, sampling depth, bulk density, litter, and topography of the area (Mahasani *et al.* 2015; Stringer *et al.* 2016; Rahman *et al.* 2019; Gao *et al.* 2019; Susilowati *et al.* 2020; Dencer-Brown *et al.* 2020). In addition, it has been reinforced by Leopold *et al.* (2013) and Pham *et al.*(2019) stating that species dominance correlates with an uptake of carbon, oxygen, and nutrients from soil and air, and species relationships develop patterns of grouping and species association.

It is estimated that the total soil carbon stock of Lembar Bay found in 10 mangrove stands was 0.20-6.60 tons C/ha with an average of 2.18±2.010 tons C/ha (Table 3). This value is smaller than that in some previous research results. The soil carbon stock of the Tanjung Lesung Banten mangrove was discovered to be 27.92 tons C/ha (Ati *et al.* 2015); the mangrove soil carbon stock in Dusun Pandan Sari Brebes, Central Java amounted to 326.46 tons C/ha;

Table 2 Carbon content under each mangrove stand in Lembar Bay

No.	Mangroves	Soil Carbon Content Under Mangrove Stands					
		Gross Weight (g)	Dry Weight (g)	Biomass	Bulk Density	Soil Carbon Content (% C)	Soil Carbon (gC/m^2)
1	*Avicennia lanata*	8.00	6.91	1.09	0.002	1.43	0.066
2	*Avicennia marina*	22.30	21.08	1.22	0.002	0.57	0.029
3	*Bruguiera gymnorrhiza*	20.00	19.35	0.65	0.001	0.43	0.012
4	*Ceriops decandra*	17.74	17.09	0.65	0.001	0.21	0.006
5	*Excoecaria agallocha*	15.57	15.21	0.36	0.001	0.12	0.002
6	*Lumnitzera racemosa*	17.31	16.53	0.78	0.001	0.34	0.011
7	*Rhizophora stylosa*	26.14	24.74	1.40	0.002	0.50	0.029
8	*Scyphiphora hydrophyllacea*	11.34	10.38	0.96	0.001	1.04	0.042
9	*Thespesia populnea*	16.70	16.15	0.55	0.001	0.20	0.005
10	*Xylocarpus maluccensis*	14.03	13.15	0.88	0.001	0.44	0.016
Average		16.91	16.06	0.85	0.001	0.53	0.02
Standard Deviation		5.220	5.141	0.321	0.000	0.409	0.020

Table 3 Soil carbon stock of mangrove ecosystem in Lembar Bay

No.	Mangroves	SoilOrganic Carbon StockUnder Mangrove Stands		
		Soil Carbon (% C)	Soil Carbon (g C/m^2)	Soil Carbon (tons C/ha)
1	*Avicennia lanata*	1.43	0.066	6.60
2	*Avicennia marina*	0.57	0.029	2.90
3	*Bruguiera gymnorrhiza*	0.43	0.012	1.20
4	*Ceriops decandra*	0.21	0.006	0.60
5	*Excoecaria agallocha*	0.12	0.002	0.20
6	*Lumnitzera racemosa*	0.34	0.011	1.10
7	*Rhizophora stylosa*	0.50	0.029	2.90
8	*Scyphiphora hydrophyllacea*	1.04	0.042	4.20
9	*Thespesia populnea*	0.20	0.005	0.50
10	*Xylocarpus maluccensis*	0.44	0.016	1.60
Average		0.53	0.02	2.18
Standard Deviation		0.409	0.020	2.010

the mangrove soil carbon stock of the Perancak mangrove forest, Jembrana, Bali totaled 119.75 tons C/ha; the mangrove soil carbon stock in Sungai Sembilan, Dumai was calculated to be 1,819.31 tons C/ha (Handoyo *et al.* 2020); the mangrove soil carbon stock in Tambakbulusan Village, Demak, Central Javawas 57.74 tons C/ha (Susilowati *et al.* 2020); and the soil carbon stock in the mangrove ecosystem of Gili Meno, North Lombok was found to be 154.62±99.78 tons C/ha (Hilyana & Rahman 2022).

Based on the potential value of carbon sinks and storage in mangrove ecosystems calculated in several places in Indonesia, there is a greater potential for mangrove ecosystems to store carbon than tropical forests. This can be seen in the results of studies by Daud *et al.* (2015), Raynaldo *et al.* (2022), and Yaqin *et al.* (2022). A report by Alongi (2020) also supports this finding, stating that, globally, mangrove ecosystems have a total carbon stock of 738 ± 27.9 MgC/ha. It has also been reported that the largest potential carbon sink is in the soil, and it is equivalent to 77% of the total carbon stock although mangrove forests only make up 0.2% of this stock compared to forestson land (Hamilton & Casey 2016).

The soil carbon content found under 10 mangrove stands in Lembar Bay was lower than the soil carbon stock under five mangrove stands on Gili Meno. The mangroves include *Rizophora apiculata* (307.96 tons C/ha), *Avicennia marina* (197.16 tons C/ha), *Excoecaria agallocha* (114.31 tons C/ha), *Lumnitzera racemosa* (59.90 tons C/ha), and *Bruguiera cylindrica* (57.17 tons C/ha). The average estimated total of soil carbon stock found in 20.49 ha of the mangrove area studied was 44.67 tonsC, which is equivalent to 263.69 tonsC in a 120.96 ha area of mangroves in Lembar Bay (Table 4). If the entire 20.49 ha area wascovered by *Avicennia lanata*, it could contribute 135.23 tonsC of carbon storage. The lowest soil carbon stock capacity was found from *Excoecaria agallocha* at 4.10 tonsC in a mangrove area of 20.49 ha.

Table 4 Carbon pool in mangrove soil in Lembar Bay

No.	Mangroves	Carbon Pool in Mangrove Soil in Lembar Bay		
		Soil Carbon (tons C/ha)	ResearchArea (ha)	Carbon Pool (tonsC)
1	*Avicennia lanata*	6.60	20.49	135.23
2	*Avicennia marina*	2.90	20.49	59.42
3	*Bruguiera gymnorrhiza*	1.20	20.49	24.59
4	*Ceriops decandra*	0.60	20.49	12.29
5	*Excoecaria agallocha*	0.20	20.49	4.10
6	*Lumnitzera racemosa*	1.10	20.49	22.54
7	*Rhizophora stylosa*	2.90	20.49	59.42
8	*Scyphiphora hydrophyllacea*	4.20	20.49	86.06
9	*Thespesia populnea*	0.50	20.49	10.25
10	*Xylocarpus maluccensis*	1.60	20.49	32.78
	Average	0.53	20.49	44.67
	Standard Deviation	0.409	0.000	41.182

CONCLUSION

The highest soil carbon stock in the mangrove ecosystem of the Lembar harbor was found in the subsoil of *Avicennia lanata* stands, while the lowest was found in the subsoil of *Excoecaria agallocha*. The total carbon absorption potential of the mangrove ecosystem soil in the study area was 44.67 tonsC. This is equivalent to 263.69 tonsC in the mangrove forest in Lembar Bay, covering an area of 120.96 ha.

REFERENCES

Abdelhakeem SG, Aboulroos SA, Kamel MM. 2016. Performance of a vertical subsurface flow constructed wetland under different operational conditions. Journal of Advanced Research. 7(5):803–814. https://doi.org/10.1016/j.jare.2015.12.002

Alongi DM. 2020. Carbon Balance in Salt Marsh and Mangrove Ecosystems: A Global Synthesis. JMSE. 8(10):767. https://doi.org/10.3390/jmse8100767

Asadi MA, Yona D, Saputro SE. 2018. Species Diversity, Biomass, and Carbon Stock Assessments of Mangrove Forest in Labuhan, Indonesia. IOP Conf Ser: Earth Environ Sci. 151:012009. https://doi.org/10.1088/1755-1315/151/1/012009

Ati RNA, Rustam A, Kepel TL, Sudirman N, Astrid MA, Daulat A, Mangindaan P, Salim HL, Hutahaean AA. 2015. Stok Karbon Dan Struktur Komunitas Mangrove Sebagai Blue Carbon Di Tanjung Lesung, Banten [Carbon Stock and Mangrove Community Structure as Blue Carbon in Tanjung Lesung, Banten]. Jurnal Segara. 10(2). https://doi.org/10.15578/segara.v10i2.21

Badan Standarisasi Nasional. 2011. Pengukuran dan penghitungan cadangan karbon –Pengukuran lapangan untuk penaksiran cadangan karbon hutan [Measuring and calculating carbon stocks – Field measurements for estimating forest carbon stocks].

Barreto MB, Lo Mónaco S, Díaz R, Barreto-Pittol E, López L, Peralba M do CR. 2016. Soil organic carbon of mangrove forests (Rhizophora and Avicennia) of the Venezuelan Caribbean coast. Organic Geochemistry. 100: 51-61. https://doi.org/10.1016/j.orggeochem.2016.08.002

Bomer EJ, Wilson CA, Hale RP, Hossain ANM, Rahman FMA. 2020. Surface elevation and sedimentation dynamics in the Ganges-Brahmaputra tidal delta plain, Bangladesh: Evidence for mangrove adaptation to human-induced tidal amplification. CATENA. 187: 104312. https://doi.org/10.1016/j.catena.2019.104312

Brath B, Friesen T, Guérard Y, Jacques-Brissette Cg, Lindman C, Lockridge K, Mulgund S, Walke B-J. 2015. Research Paper: Climate Change and Resource Sustainability - An Overview for Actuaries. Canadian Institute of Actuaries: 57.

Daud M, Latifah H, Hikmah, Imran J. 2015. Potensi Cadangan dan Serapan Karbon Dioksida di Hutan Pendidikan Universitas Muhammadiyah Makassar Desa Bissoloro Kabupaten Gowa [Potential for Carbon Dioxide Reserves and Absorption in the Educational Forest of Makassar Muhammadiyah University, Bissoloro Village, Gowa Regency]. Eucalyptus : Jurnal Ilmu Kehutanan. 3(5): 13.

Dencer-Brown AM, Alfaro AC, Bourgeois C, Sharma S, Milne S. 2020. The secret lives of mangroves: Exploring New Zealand's urban mangroves with integrated biodiversity assessments. Ocean & Coastal Management. 191:105185. https://doi.org/10.1016/j.ocecoaman.2020.105185

Gao Y, Zhou J, Wang L, Guo J, Feng J, Wu H, Lin G. 2019. Distribution patterns and controlling factors for the soil organic carbon in four mangrove forests of China. Global Ecology and Conservation. 17: e00575. https://doi.org/10.1016/j.gecco.2019.e00575

Hamilton SE, Casey D. 2016. Creation of a high spatio-temporal resolution global database of continuous mangrove forest cover for the 21st century (CGMFC-21): CGMFC-21. Global Ecol Biogeogr. 25(6): 729-738. https://doi.org/10.1111/geb.12449

Handoyo E, Amin B, Elizal E. 2020. Estimation of Carbon Reserved In Mangrove Forest Of Sungai Sembilan Sub-District, Dumai City, Riau Province. ajoas. 3(2): 123-134. https://doi.org/10.31258/ajoas.3.2.123-134

Hilmi E. 2018. Mangrove landscaping using the modulus of elasticity and rupture properties to reduce coastal disaster risk. Ocean & Coastal Management. 165: 71-79. https://doi.org/10.1016/j.ocecoaman.2018.08.002

Hilmi E, Kusmana C, Suhendang E, Iskandar. 2017. Correlation Analysis Between Seawater Intrusion and Mangrove Greenbelt. InaJForRes. 4(2): 151-168. https://doi.org/10.20886/ijfr.2017.4.2.151-168

Hilmi E, Sari L, Setijanto, Kusmana C, Suhendang E. 2019. The Carbon Conservation of Mangrove Ecosystem in Indonesia. BIOTROPIA. 26(3). https://doi.org/10.11598/btb.2019.26.3.1099

Hilyana S, Rahman FA. 2022. Variabilities of the carbon storage of mangroves in Gili Meno Lake, North Lombok District, Indonesia. Biodiversitas. 23(11). https://doi.org/10.13057/biodiv/d231140

Howard J, McLeod E, Thomas S, Eastwood E, Fox M, Wenzel L, Pidgeon E. 2017. The potential to integrate blue carbon into MPA design and management. Aquatic Conserv: Mar Freshw

Ecosyst. 27:100–115. https://doi.org/10.1002/aqc.2809

Jannah SW, Rahman FA, Hadi AP. 2021. Analisis Kandungan Karbon Pada Vegetasi Mangrove Di Desa Lembar Kabupaten Lombok Barat [Analysis of Carbon Content in Mangrove Vegetation in Sheet Village, West Lombok Regency]. Bioscientist : Jurnal Ilmiah Biologi. 9(2): 11.

Jones R, Bessell-Browne P, Fisher R, Klonowski W, Slivkoff M. 2016. Assessing the impacts of sediments from dredging on corals. Marine Pollution Bulletin. 102(1): 9-29. https://doi.org/10.1016/j.marpolbul.2015.10.049

Kida M, Fujitake N. 2020. Organic Carbon Stabilization Mechanisms in Mangrove Soils: A Review. Forests. 11(9): 981. https://doi.org/10.3390/f11090981

Lee SY, Primavera JH, Dahdouh-Guebas F, McKee K, Bosire JO, Cannicci S, Diele K, Fromard F, Koedam N, Marchand C, et al. 2014. Ecological role and services of tropical mangrove ecosystems: a reassessment: Reassessment of mangrove ecosystem services. Global Ecology and Biogeography. 23(7): 726-743. https://doi.org/10.1111/geb.12155

Leopold A, Marchand C, Deborde J, Chaduteau C, Allenbach M. 2013. Influence of mangrove zonation on CO2 fluxes at the sediment–air interface (New Caledonia). Geoderma. 202-203: 62-70. https://doi.org/10.1016/j.geoderma.2013.03.008

Lestariningsih WA, Soenardjo N, Pribadi R. 2018. Estimasi Cadangan Karbon pada Kawasan Mangrove di Desa Timbulsloko, Demak, Jawa Tengah [Estimated Carbon Stocks in Mangrove Areas in Timbulsloko Village, Demak, Central Java]. Bul Oseano Mar. 7(2): 121. https://doi.org/10.14710/buloma.v7i2.19574

Lovelock CE, Duarte CM. 2019. Dimensions of Blue Carbon and emerging perspectives. Biol Lett. 15(3): 20180781. https://doi.org/10.1098/rsbl.2018.0781

Lugina M, Alviya I, Indartik I, Aulia Pribadi M. 2017. Strategi Keberlanjutan Pengelolaan Hutan Mangrove Di Tahura Ngurah Rai Bali [Strategy of Mangrove Management in Ngurah Rai Grand Forest Park]. j.analisis.kebijak.kehutan. 14(1): 61-77. https://doi.org/10.20886/jakk.2017.14.1.61-77

Macreadie PI, Anton A, Raven JA, Beaumont N, Connolly RM, Friess DA, Kelleway JJ, Kennedy H, Kuwae T, Lavery PS, et al. 2019. The future of Blue Carbon science. Nat Commun. 10(1): 3998. https://doi.org/10.1038/s41467-019-11693-w

Mahasani IGAI, Widagti N, Karang IWGA. 2015. Estimasi Persentase Karbon Organik Di Hutan Mangrove Bekas Tambak, Perancak, Jembrana, Bali [Estimation of the Percentage of Organic Carbon in the Former Tambak Mangrove Forest, Perancak, Jembrana, Bali]. Journal of Marine and Aquatic Sciences. 1(1): 14-18. https://doi.org/10.24843/jmas.2015.v1.i01.14-18

Martuti NKT, Setyowati DL, Nugraha SB, Mutiatari DP. 2017. Carbon stock potency of mangrove ecosystem at Tapak Sub-village, Semarang, Indonesia. AACL Bioflux. 10(6): 10.

Matsui N, Meepol W, Chukwamdee J. 2015. Soil Organic Carbon in Mangrove Ecosystems with Different Vegetation and Sedimentological Conditions. JMSE. 3(4): 1404-1424. https://doi.org/10.3390/jmse3041404

Mcleod E, Chmura GL, Bouillon S, Salm R, Björk M, Duarte CM, Lovelock CE, Schlesinger WH, Silliman BR. 2011. A blueprint for blue carbon: toward an improved understanding of the role of vegetated coastal habitats in sequestering CO_2. Frontiers in Ecology and the Environment. 9(10): 552-560. https://doi.org/10.1890/110004

Murray BC, Pendleton L, Jenkins WA, Sifleet S. 2011. Green Payments for Blue Carbon: 52.

Nellemann C, Corcoran E, Duarte C, Valdes Y, De Young C, Fonseca L, Grimsditch G. 2009. Blue carbon: The role of oceans as carbon sinks. In: Blue carbon: The role of healthy oceans in binding carbon: A rapid response assessment. Arendal, Norway: GRID-Arendal: UNEP.

Noor YR, Khazali M, Suryadiputra INN. 2006. Panduan pengenalan mangrove di Indonesia [Guide to Introduction to Mangroves in Indonesia]. Bogor: Ditjen PHKA: Wetlands International, Indonesia Programme.

Pérez A, Libardoni BG, Sanders CJ. 2018. Factors influencing organic carbon accumulation in mangrove ecosystems. Biol Lett. 14(10): 20180237. https://doi.org/10.1098/rsbl.2018.0237

Pham LTH, Vo TQ, Dang TD, Nguyen UTN. 2019. Monitoring mangrove association changes in the Can Gio biosphere reserve and implications for management. Remote Sensing Applications: Society and Environment. 13: 298-305. https://doi.org/10.1016/j.rsase.2018.11.009

Rahman FA, Hadi AP. 2021. Kandungan C-Organik Substrat Ekosistem Mangrove di Danau Air Asin Gili Meno Kabupaten Lombok Utara [C-Organic Content of Mangrove Ecosystem Substrates in Gili Meno Saltwater Lake, North Lombok Regency]. Bioscientist : Jurnal Ilmiah Biologi. 9(2): 516-526.

Rahman FA, Qayim I, Wardiatno Y. 2023. Carbon Stored on Seagrass Beds in Gili Maringkik, Lombok, Indonesia. BIOTROPIA - The Southeast Asian Journal of Tropical Biology. 30(1): 63-73. https://doi.org/10.11598/btb.2023.30.1.1776

Rahman FA, Rohyani IS, Hadi AP, Lestari DP. 2019. Komposisi Vegetasi Mangrove Berdasarkan Strata Pertumbuhan di Teluk Sereweh, Kabupaten Lombok Timur, Nusa Tenggara Barat

[Composition of Mangrove Vegetation Based on Growth Strata in Sereweh Bay, East Lombok Regency, West Nusa Tenggara]. Jurnal Pendidikan Biologi Dan Sains (PENBIOS). 4(2): 53-61.

Raynaldo A, Marista E, Shofiyah SS, Linda R, Rafdinal R. 2022. Estimasi Cadangan Karbon Kawasan Taman Wisata Hutan Mangrove Kecamatan Sukadana, Kabupaten Kayong Utara, Kalimantan Barat [Estimation Of Carbon Stock In Mangrove Forest Tourism Park, Sukadana District, Kayong Utara Regency, West Kalimantan]. JK. 15(1): 23-30. https://doi.org/10.21107/jk.v15i1.11170

Saraswati NA. 2019. Pemantauan Mangrove di Teluk Lembar, Lombok Barat Menggunakan Landsat Tahun 1995 hingga 2019 [Monitoring Mangrove in Lembar Bay, West Lombok Using Landsat from 1995 to 2019]. Seminar Nasional Penginderaan Jauh. 6: 404-408.

Schwarzer K, Thanh NC, Ricklefs K. 2016. Sediment re-deposition in the mangrove environment of Can Gio, Saigon River estuary (Vietnam). Journal of Coastal Research. 75(sp1): 138-142. https://doi.org/10.2112/SI75-028.1

Sidik F, Neil D, Lovelock CE. 2016. Effect of high sedimentation rates on surface sediment dynamics and mangrove growth in the Porong River, Indonesia. Marine Pollution Bulletin. 107(1): 355-363. https://doi.org/10.1016/j.marpolbul.2016.02.048

Stringer CE, Trettin CC, Zarnoch SJ. 2016. Soil properties of mangroves in contrasting geomorphic settings within the Zambezi River Delta, Mozambique. Wetlands Ecol Manage. 24(2): 139-152. https://doi.org/10.1007/s11273-015-9478-3

Suhendra, Amron Amron, Hilmi E. 2018. The pattern of coastline change based on the characteristics of sediment and coastal slope in Pangenan coast of Cirebon, West Java.Prayogo NA, Amron A., editors. E3S Web Conf. 47: 06001. https://doi.org/10.1051/e3sconf/20184706001

Sukuryadi, Harahab N, Primyastanto M, Hadi AP. 2021. Short Communication: Structure and composition of mangrove vegetation in Lembar bay area, West Lombok District, Indonesia. Biodiversitas. 22(12). https://doi.org/10.13057/biodiv/d221243

Sulaeman, Suparto, Eviati. 2005. Petunjuk Teknis Analisis Tanah, Tanaman, Air dan Pupuk [Technical Instructions for Soil, Plant, Water and Fertilizer Analysis].

Susilowati MW, Purnomo PW, Solichin A. 2020. Estimation of CO_2 Absorption Based on Carbon Storage in the Mangrove Forest of Tambakbulusan Village, Demak Jawa Tengah. 4(2): 11.

Syarifuddin A, Zulhamran. 2012. Analisa Vegetasi Hutan Mangrove Pelabuhan Lembar Kabupaten Lombok Barat [Analysis Of Mangrove Forest Mangrove Port Sheet West Lombok Regency West Nusa Tenggara]. Jurnal Gamma. 7(2): 1-13.

Walkley A, Black IA. 1934. An Examination Of The Degtjareff Method For Determining Soil Organic Matter, And A Proposed Modification Of The Chromic Acid Titration Method. Soil Science. 37(1): 29-38. https://doi.org/10.1097/00010694-193401000-00003

Wang H, Liu G, Li Z, Zhang L, Wang Z. 2020. Processes and driving forces for changing vegetation ecosystem services: Insights from the Shaanxi Province of China. Ecological Indicators. 112: 106105. https://doi.org/10.1016/j.ecolind.2020.106105

Weiss C, Weiss J, Boy J, Iskandar I, Mikutta R, Guggenberger G. 2016. Soil organic carbon stocks in estuarine and marine mangrove ecosystems are driven by nutrient colimitation of P and N. Ecol Evol. 6(14): 5043-5056. https://doi.org/10.1002/ece3.2258

Yaqin N, Rizkiyah M, Putra EA, Suryanti S, Febrianto S. 2022. Estimasi Serapan Karbon pada Kawasan Mangrove Tapak di Desa Tugurejo Semarang [Estimated Carbon Uptake in the Tapak Mangrove Area in Tugurejo Village, Semarang]. Bul Oseano Mar. 11(1): 19-29. https://doi.org/10.14710/buloma.v11i1.38256

BRYOPHYTE DIVERSITY AND ATMOSPHERIC POLLUTION IN A RESIDENTIAL AREA AND AN INDUSTRIAL URBAN FOREST IN JAKARTA, INDONESIA

AFIATRY PUTRIKA[12], ANDI SALAMAH[*1], MEGA ATRIA[12], DIAN HENDRAYANTI[1], NURUL LAKSMI WINARNI[1,2], ELIZA PRICILIA PURBA[1], SARAH TSABITUDINILLAH[1] AND NUNIK SRI ARIYANTI[3]

[1]*Department of Biology, Faculty of Mathematics and Natural Science, Universitas Indonesia, Depok 16424, Indonesia*
[2]*Research Group of Wildlife Biology and Sustainable Landscape, Universitas Indonesia, Depok 16424, Indonesia*
[3]*Department of Biology, Faculty of Mathematics and Natural Science, IPB University, Bogor 16680, Indonesia*

Received 25 March 2023 / Revised 15 August 2023 / Accepted 11 September 2023

ABSTRACT

Bryophytes are nonvascular plants that have simple structures that are sensitive to environmental changes, and they can, therefore be used as indicators of air quality. The presence of bryophytes in disturbed urban ecosystems, such as residential and industrial areas, indicates that their structures have adapted to survive in such areas. The objective of this study was to compare the bryophyte diversity and air quality indices between a residential area and an industrial area in Jakarta. The research was conducted in the Bona Indah residential area in South Jakarta and the Jakarta Industrial Estate Pulogadung (JEIP) urban forest. Sampling was carried out using the transect method in the residential area and the quadratic method in the urban forest on three different substrates, namely rocks or concrete, soil and tree trunks. The percentage of the epiphytic bryophyte cover was measured using a 10 × 10 cm subplot. Voucher specimens were stored at the Herbarium UI DEP and Herbarium IPB. Twenty-one species of moss and three species of liverwort were found in the two locations. Bryophytes were found on all the substrates in the residential area, but in the urban forest, they were found only on tree trunks and rock/cement substrates. Based on the Shannon–Wiener Index, although both locations had moderate bryophyte diversity, the residential area's bryophyte diversity was higher than that of the urban forest. The index of atmospheric purity in the residential area was 4.3, indicating a high level of pollution, and it was 0.3 in the urban forest, showing that it was also very polluted.

Keywords: atmospheric purity, bryophyte, diversity index, urban area

INTRODUCTION

An urban area is a region with high population density dominated by human activities such as trade, industry and education (Freedman 2018). Transportation and industry produce emissions such as carbon dioxide and nitrogen oxides that contribute to air pollution in urban areas. Pollution causes ecological effects such as the greenhouse effect, harm to plant and animal health and temperature stress (Kanawade *et al.* 2010). It is damaging to urban biodiversity because several species have become more sensitive to environmental stress from pollution and habitat fragmentation (Uttara *et al.* 2012; Szlavecs *et al.* 2011).

Jakarta is one of Indonesia's largest and most populous cities (Permatasari *et al.* 2019). According to worldpopulationreview.com, in 2023, its population was estimated at 11 million. The city's open green spaces have been reduced in area since 2008, leading to the urban heat island phenomenon (Putra *et al.* 2021). An industrial estate is a built-up area with relatively high pollution levels due to transportation and industrial combustion (Putra *et al.* 2021; Lestari *et al.* 2022), while residential areas consist of buildings and vegetation and have less air pollution than industrial areas (Putra *et al.* 2021). The air quality problem in Jakarta causes

*Corresponding author, email: salamah@sci.ui.ac.id

environmental stress, harming species sensitive to high temperatures, drought and air pollution, such as bryophytes (Oishi & Hiura 2017; Oishi 2019).

Bryophytes are non-vascular plants that are sensitive to environmental changes. The structure of bryophytes is simple; they have a thin cuticle layer so that water and minerals can be absorbed directly through the surface through diffusion. This causes the water content of bryophytes to depend on ambient humidity, making them poikilohydric. To survive in a dry environment, bryophytes develop some morphological adaptations for water retention and transportation (Vanderpoorten & Goffinet 2009; Proctor & Tuba 2002; Bahuguna *et al.* 2013; He *et al.* 2016). Based on these characteristics, bryophytes are suitable as air quality indicators, particularly in urban environments. Epiphytic species such as bryophytes and lichens are known to be drought-sensitive organisms. They have been reported to be absent in urban ecosystems, although certain species are known to be drought tolerant (Pescott *et al.* 2015; Bahuguna *et al.* 2013). This suggests that epiphytic bryophytes can be indicators of air quality through the index of atmospheric purity (IAP). Correlations between species distribution, IAP and pollutant concentrations can reflect the level of air pollution in a region (Jiang *et al.* 2020; Bahuguna *et al.* 2013).

Since the middle of the twentieth century, urban bryophyte-related air quality has been studied in subtropical regions, such as in European and Asian (Japan, China, India) countries and America and Australia. The study of air quality monitoring is carried out using a survey and a phytosociological approach, namely IAP, which was first used in 1970 by Le Blanc and Rao (Govindapyari *et al.* 2010). To show air quality, IAP is identified by the frequency and coverage of epiphytic bryophytes and the species resistance factor (Dymitrova 2009; Vanderporrten & Goffinet 2009; Govindapyari *et al.* 2010). Research on urban bryophytes in tropical regions, including in Indonesia, is mostly based on inventories and community structures. Studies of urban bryophytes related to air quality have never been conducted in Indonesia. This study aims to compare bryophyte diversity and determine air quality in a residential area and an industrial urban forest area of Jakarta using the IAP.

MATERIALS AND METHODS

Study site

The research was conducted from July to December 2020 in Komplek Taman Bona Indah, a residential area in South Jakarta, and the JIEP urban forest, an industrial area in East Jakarta (Figure 1). Species identification was carried out at the Taxonomy Laboratory of the Biology Department in the Faculty of Mathematics and Natural Sciences, Universitas Indonesia. The voucher specimen was deposited in the Herbarium UI-DEP.

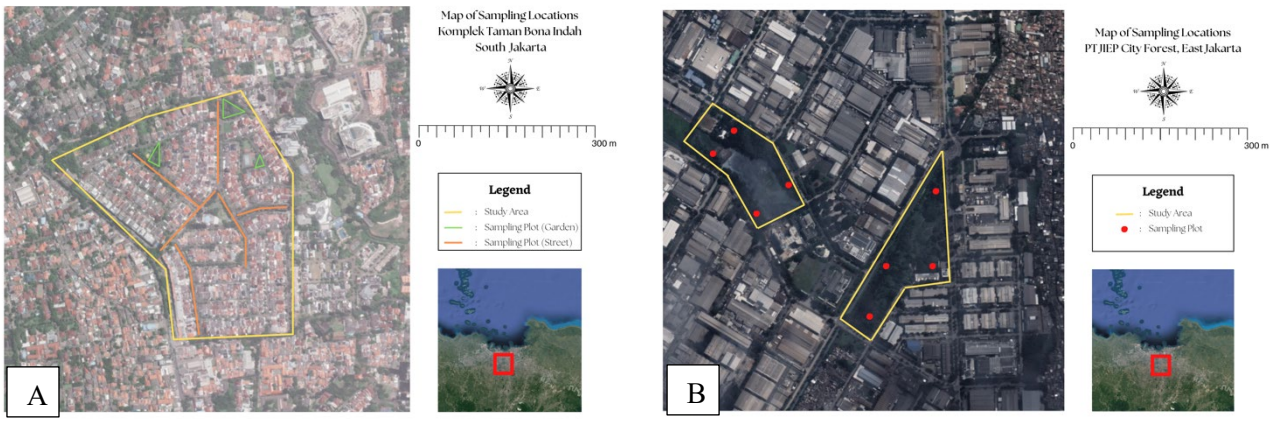

Figure 1 Sampling locations. (A) Taman Bona Indah Residence, (B) JIEP urban forest

Methods

Bryophytes were collected from three substrate types, namely tree trunks, soil and rock or cement in both the Taman Bona Indah residential area and the JIEP urban forest. Samples from the residential area were collected from six sites on a 50 m linear transect at the main roadside and from three sites on a 50 m linear transect in a park. Each transect was divided into 30 × 2 m plots. The bryophytes in the urban forest were collected from eight 25 × 25 m plots. In each plot, five trees with a minimum of 10 cm breast-high diameter were selected. Epiphytic bryophytes were sampled from four cardinal directions with an area of 10 × 10 cm^2 from 0–2 m above ground level. The host tree species and bark type were recorded in a datasheet. In each plot, terrestrial bryophytes were collected from quadrats of 10 × 10 cm^2, and their coverage was estimated using the same quadrats of 10 × 10 cm^2. Voucher bryophyte samples were identified using a stereo and light microscope at the Herbarium UI-DEP, where the voucher specimens were stored.

The bryophyte diversity was analysed using the Shannon–Wiener index and the important value index. The Shannon–Wiener diversity index is calculated using the following formula:

$$H' = -\sum_{i=1}^{S} (pi \ln pi),$$

where:
H' = the Shannon–Wiener diversity index,
S = number of species,
$pi = \frac{Ni}{N}$,
Ni = relative cover species I,
N = relative cover number of S species.

The important value index was measured to analyse species' dominance in each site, as follows (Jiang *et al.* 2020):

$$\text{Important value index} = \frac{\text{Relative cover + relative frequency}}{2}$$

Atmospheric pollution was analysed using IAP according to the following formula (Le Blanc 1971; Jiang *et al.* 2020):

$$\text{IAP} = \sum_{i}^{n} \frac{Q \; x \; f}{10}$$

where:
IAP = index atmospheric purity,
Q = ecological index of species (average number of species that coexist with other species),
f = frequency-coverage scale.

RESULTS AND DISCUSSION

Species composition

A total of 24 bryophyte species were recorded in the residential area and the urban forest (three species of liverwort and 21 species of moss). Based on the locations, the species richness in the residential area was higher than in the urban forest. There were three species of liverworts and 15 species of mosses in the residential area, while there were 11 species of mosses and no liverworts in the urban forest (Table 1).

The number of moss species in the urban areas was higher than liverworts (Giordano *et al.* 2004; Szűcs *et al.* 2017; Godovićová *et al.* 2020; Fastanti & Wulansari 2021). Most moss species are known to be drought tolerant, so they are commonly found in open areas (Vitt *et al.* 2014). According to Mukhia *et al.* (2019), a high abundance of epiphytic liverworts is related to low light intensity, ranging from 460–1900 lux. Outside this light intensity range, liverwort abundance is significantly reduced. Consequently, the abundance and species richness of liverwort in urban areas is lower than in natural habitats because of the difference in canopy coverage density and relative humidity.

The families Pottiaceae, Calymperaceae and Bryaceae were found in both sampling sites, with Pottiaceae having the highest number of species (Figure 2). There were eight species of Pottiaceae in the urban forest (*Barbula consanguinea, Barbula pseudo-ehrenbergii*, *Hyophila apiculata, Hyophila beruensis, Hyophila involuta, Hyophila javanica, Trichostomum brachydontium* and *Weissia edentula*). In the residential area, there were five species of Pottiaceae (*Barbula javanica, Hyophila apiculata, Hyophila beruensis, Hyophila involuta* and *Trichosteleum singapurense*) (Table 1). On the other hand, Calymperaceae was represented by more species in the residential area than in the urban forest. Bryaceae was only represented by one species in each of the sampling sites (Figure 2).

Table 1 Bryophyte species composition in the residential area and the urban forest

No.	Species	Family	Location	
			Residential area	Urban forest
	A. Marchantiophyta			
1	*Haplomitrium blumei*	Haplomitriaceae	√	
2	*Lejeunea cocoes*	Lejeuneaceae	√	
3	*Lejeunea eifrigii*	Lejeuneaceae	√	
	B. Bryophyta			
1	*Bryum apiculatum*	Bryaceae	√	
2	*Calymperes crassinerve*	Calymperaceae	√	
3	*Calymperes motleyi*	Calymperaceae	√	√
4	*Calymperes tenerum*	Calymperaceae	√	√
5	*Leucophanes octoblepharioides*	Calymperaceae	√	
6	*Fissidens atroviridis*	Fissidentaceae	√	
7	*Fissidens biformis*	Fissidentaceae	√	
8	*Isopterygium bancanum*	Hypnaceae	√	
9	*Isopterygium minutirameum*	Hypnaceae	√	
10	*Barbula consanguinea*	Pottiaceae		√
11	*Barbula javanica*	Pottiaceae	√	
12	*Barbula pseudo-ehrenbergii*	Pottiaceae		√
13	*Hyophila apiculata*	Pottiaceae	√	√
14	*Hyophila beruensis*	Pottiaceae	√	√
15	*Hyophila involuta*	Pottiaceae	√	√
16	*Hyophila javanica*	Pottiaceae		√
17	*Trichosteleum singapurense*	Sematophyllaceae	√	
18	*Trichostomum brachydontium*	Sematophyllaceae		√
19	*Weissia edentula*	Pottiaceae		√
20	*Taxithelium nepalense*	Sematophyllaceae	√	
21	*Splachnobryum indicum*	Splachnobryaceae		√
	Number of species		**18**	**11**
	Total number of species		**5**	

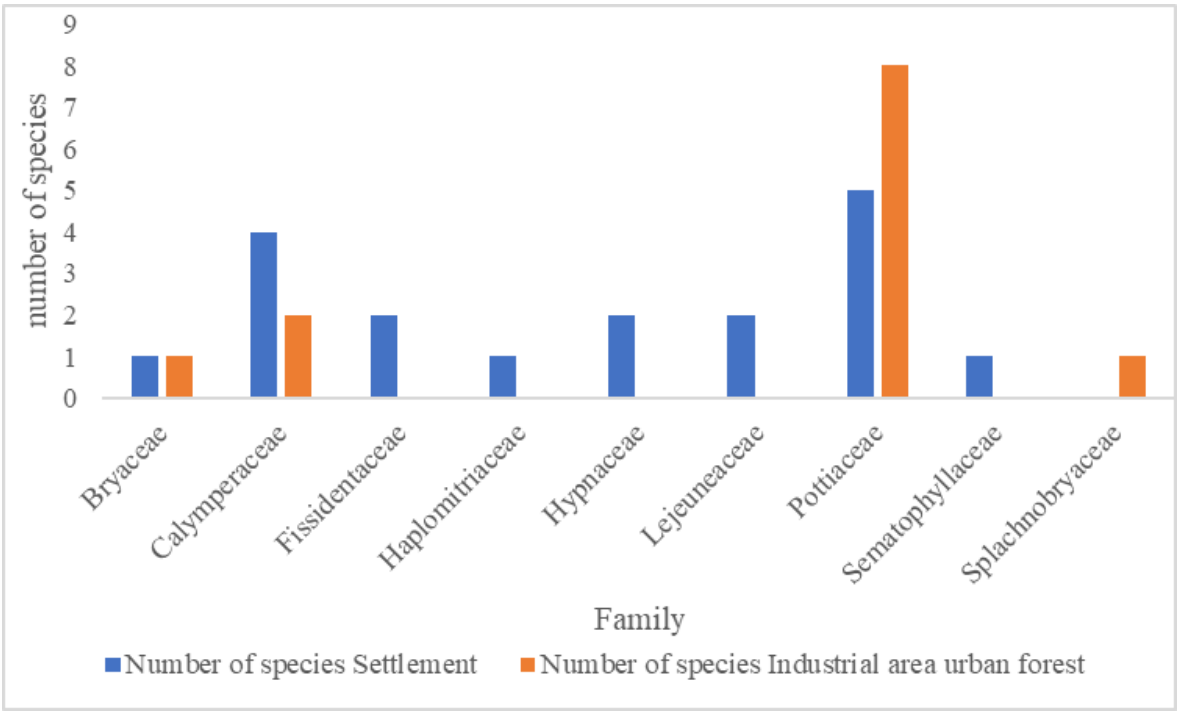

Figure 2 Family composition of bryophytes in the residential area and the urban forest

Pottiaceae is a family of mosses that is present in open and dry environments, particularly in urban areas (Da Costa 2015; Jiang *et al.* 2020). They have a small size, erect stem or acrocarpous with a short turf life-form and dark green gametophore (Figure 3). The members of this family have lanceolate leaves, which become crisped when dry to reduce transpiration due to environmental heat stress. In addition, some species of Pottiaceae have ornamentation on the leaf cell surface, such as papillae. The interstices between papillae are necessary to increase water conduction on the leaf surface (Zander 1993; Kou *et al.* 2014; Glime 2017). Furthermore, they can prevent water loss, making them more successful in a drought.

Calymperaceae is an epiphytic moss that was often found in both sampling locations. Some species of this family have gemmae at the apex of the leaves as asexual reproductive organs, a strategy that helps them survive in unfavorable urban environments. The presence of cancellinae regions (hyaline basal leaf cells) in Calymperaceae is another strategy of this family to retain more water in dry environments (Figure 4) (Glime 2017).

Lejeuneaceae, a cosmopolite family, were the only liverworts in the sampling sites found on the exposed roots of large palm trees 10–20 cm above the ground. This tree root substrate had a low light intensity at around 250 lux, causing a more humid microhabitat. Liverworts generally grow in moist and shady habitats, while epiphytic liverworts grow on large or old trees (Vanderpoorten & Goffinet 2009; Mukhia *et al.* 2019).

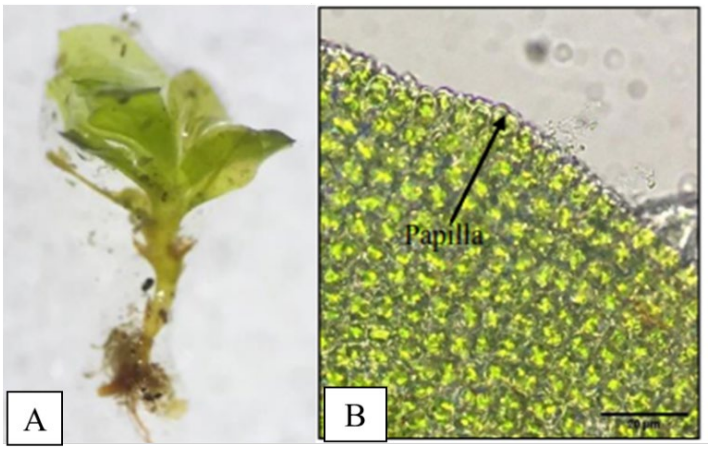

Figure 3 Pottiaceae. (A) Acrocarpous and short turf life-form. (B) Papillae on the leaf surface

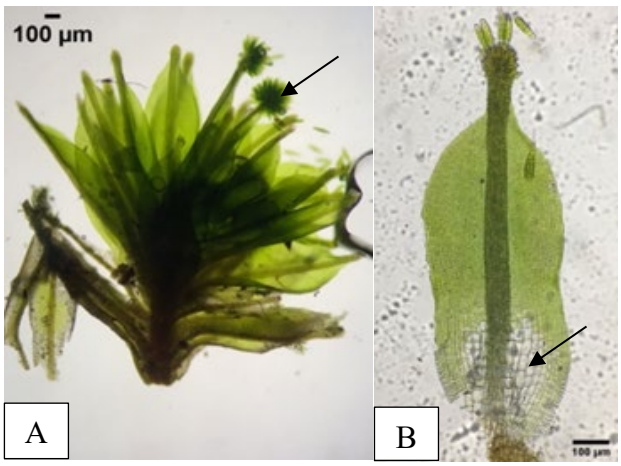

Figure 4 Calymperaceae. A. The gemmae on the leaf apex. B. Cancelinae

The type of substrate with the highest species richness was rock/cement (Figure 5). Cement paths and paving blocks were common kinds of rock and cement substrate in both sampling locations. According to Floyed and Gibson (2012), cement pavement always has crevices and cracked structures that provide a sheltered area for bryophyte colonisation. These surfaces trap soil, water and dust from the atmosphere to support bryophyte growth, provide shelter from mechanical disturbance and retain moisture for a longer period than surrounding surfaces. This substrate was dominated by Pottiaceae, including *Hyophila* and *Barbula*. Those genera are common in disturbed areas, such as urban ecosystems (Zander 1993).

Terricolous bryophytes were found only in the residential area and absent in the urban forest (Figure 5). The soil type in the latter may not have supported bryophyte growth. According to Lubis *et al.* 2013, the JIEP urban forest has a podosol soil type with low moisture and nutrient content. In addition, piles of plastic waste were found in some of the sampling plots. Andayaningsih *et al.* (2013) also reported finding terrestrial pteridophytes in this location, indicating that JIEP's environmental conditions are unsuitable for plants sensitive to dry conditions, such as terrestrial bryophytes and pteridophytes.

The number of epiphytic bryophytes in the residential area was higher than in the urban forest (Figure 5). Air pollution, humidity, temperature and phorophyte variation were limiting factors. The minimum frequency of epiphytic moss in urban areas can be determined from the percentage of host trees covered with epiphytic moss. Twenty-four percent of phorophytes (trees used as hosts of bryophytes) were used as hosts in the residential area. However, only 8% of the urban forest was used by bryophytes as hosts (Figure 6), which shows that industrial areas are more vulnerable to the growth of sensitive bryophyte species than residential areas. Urban forests are surrounded by dense industrial activity and have low vegetation density, which affects microclimate conditions, causing the comfort index in the area to be lower than usual (Syihabuddin *et al.* 2020).

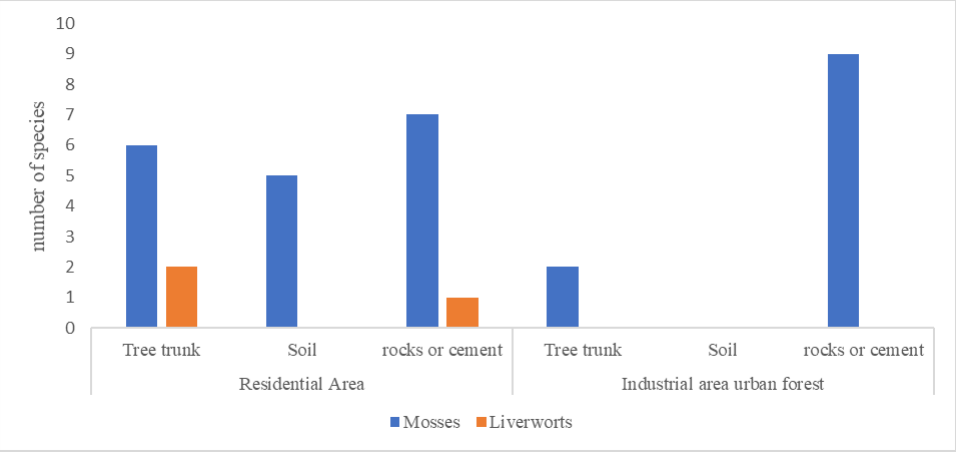

Figure 5 Number of bryophyte species on three substrates in the residential area and the urban forest

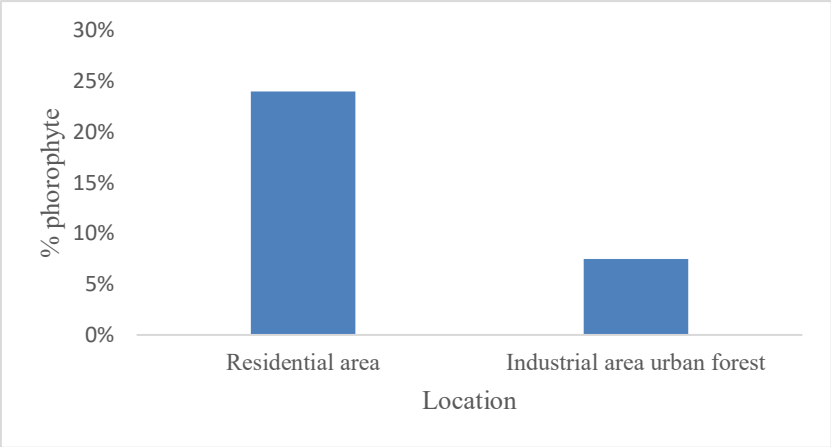

Figure 6 Presentation of phorophytes with epiphyte bryophytes

The characteristics of the host trees also influenced the presence of epiphytic mosses. *Eusideroxylon zwageri* was the only tree species colonized by bryophytes in the urban forest. In the residential area, there were three tree species hosting bryophytes, *Mangifera indica*, *Swietenia mahagoni* and *Roystonia regia*. These trees had rounded canopies and a fissured bark texture, and the pH of the bark varied between 6 and 6.8. The fissured texture had hollow cracks, resulting in a more stable surface for epiphytic bryophytes. It provided a more humid and shaded habitat by retaining water and dust from the atmosphere (Vanderpoorten & Goffinet 2009). Meanwhile, because of the palm trees' smooth bark, there were no epiphytic bryophytes on their bark surface, although they were found on the exposed palm root surfaces near the soil, since these were more humid and shaded than the trunk.

Community structure and index of atmospheric purity

Based on the type of substrate, the bryophyte diversity index in both the residential area and the urban forest ranged from 0 to 2.06. This shows that the bryophyte diversity was very low. No bryophytes were found on the soil substrate in the urban forest, and the lowest diversity index was found in the epiphytic bryophyte community in that location (Table 2). Saxicolous bryophytes on rock/cement substrate in both the residential area and the urban forest had a higher diversity index than those growing on other substrates. This shows that in urban environments, saxicolous bryophytes are more tolerant than the bryophytes on other substrates.

The IAP in the residential area was 4.3, and in the JIEP urban forest, it was 0.3 (Table 2). According to the IAP criteria of Le Blanc and Slover, the air pollution in both locations was found to be highly polluted, in the range of 1–5. Although the level of specific pollutants was unknown, it can be estimated by IAP that the urban forest was more polluted than the residential area in South Jakarta.

Air pollution and microclimate have a negative impact on sensitive species that can reduce IAP values (Jiang *et al.* 2020), which was shown by the lower number of epiphytic bryophytes in the urban forest. Epiphytic bryophytes are not found in urban areas with high pollution levels (Fudali & Żołnierz; 2019: 73; Jiang *et al.* 2020). One of the factors affecting epiphytic bryophyte diversity is traffic density. The absence of liverworts in the urban forest also indicates their sensitivity to industrial environments.

The results showed that the epiphytic mosses with the highest important value index were *Calymperes motley* and *C. tenerum*, while the saxicolous bryophytes with the highest important value index were *Hyophylla javanica* and *H. involuta* in both locations. In addition, the terricolous bryophyte with the highest important value index was *Fissidens biformis* (Table 3).

Table 2 Diversity index and index of atmospheric purity in the residential area and the urban forest

	Location					
	Residential area			Urban forest		
	Tree trunk	Soil	Rock or cement	Tree trunk	Soil	Rock or cement
H'	1.73	1.19	2.06	0.52	–	1.59
IAP	4.3			0.3		

Table 3 The highest important value index of bryophytes

Substrate	Residential area		Urban forest	
	Species	Important value	Species	Important value
Tree	*Calymperes motleyi*	22.02	*Calymperes motleyi*	31.25
	Calymperes tenerum	10.71	*Calymperes tenerum*	26.69
	Calymperes crassinerve	7.74		
Rock or cement substrate	*Hyophila javanica*	37.67	*Hyophila involuta*	15.54
	Hyophila involuta	21.06	*Hyophila javanica*	12.50
	Hyophila apiculata	19.22	*Trichostomum brachydontium*	5.26
Soil	*Fissidens biformis*	35.39		
	Fissidens atroviridis	14.97		
	Isopterygium bancanum	10.10		

Some species of *Calymperes* have also been reported as dominant bryophytes in other urban areas, such as at the Universitas Indonesia campus (Putrika *et al.* 2017) and an urban agroforest in Nigeria (Ezukanma *et al.* 2019). This genus is also commonly found in home yards and oil palm plantations in the Giam Siak Biosphere Reserve (Fastanti *et al.* 2013). From this information, we can assume that some species of *Calymperes* are sun epiphyte bryophytes, since they always grow in open areas and belong to drought-tolerant species.

The highest important value index in both locations was dominated by acrocarpous species, turf and cushion life-forms, which were small, at about 0.4–7 mm. Drought-tolerant moss species are commonly small and have erect stems (Printanakul & Jampetong 2020). In addition, turf and cushion life-forms tend to have dense leaf arrangements, which allows them to optimize water movement and storage as well as prevent excessive transpiration through a dry environment (Spitale *et al.* 2020). Turf and cushion life-forms are commonly found growing terrestrially in open habitats with high light intensity (Kürschner 2004: 105; Glime 2017: 4, 5, 7).

According to the abiotic data, in the residential area and urban forest, the daily temperature and relative humidity ranges were 29–32°C and 50%–61%, respectively. This condition may explain why the diversity of bryophytes in both sampling sites was low. The optimum temperature for bryophyte growth is about 15–25°C (Frahm 2003; He *et al.* 2016). Some bryophyte species in tropical lowland forests cannot survive at 27–30°C. Temperatures above 30°C can inhibit bryophyte photosynthesis (He *et al.* 2016). Furthermore, only specific bryophyte species can survive in urban areas.

CONCLUSION

Bryophyte diversity in both the residential area and the JIEP urban forest was dominated by acrocarpous mosses such as *Calymperes* and *Hyophila*. The residential area had higher bryophyte diversity than the urban forest. The IAP in the urban forest was lower than that in the residential area. However, both locations fell into the category of very heavily polluted areas. Further research is needed to compare the IAP values to the air pollutant quantity to confirm the pollution levels in both study sites.

ACKNOWLEDGMENT

We thank to Research and Community Service (RPM) of Faculty Mathematics and Natural Science Universitas Indonesia for financial

support through Riset Penugasan FMIPA grants under contract number 025/UN2.F3.D/DPPM.00.02/2022.

REFERENCES

Andayaningsih D, Chikmawati T, Sulistijorini. 2013. Keanekaragaman tumbuhan paku terestrial di hutan kota DKI Jakarta. [Terestrial ferns diversity in urban forest DKI Jakarta]. Ber Biol 12(3): 297-365.

Bahuguna YM, Gairola SU, Semwal DP, Uniyal PL, Bhat AB. 2013. Bryophyte and ecosystem. In: Gupta, RK, Kumar K, editors. Biodiversity of lower plants. International Publishing House, New Delhi, India. P. 279-96.

Da Costa DP. 2015. Diversity and conservation of Pottiaceae (Pottiales) in the Atlantic Rainforest. Acta Bot Brasilica 29(3): 354-74.

Dymitrova L. 2009. Epiphytic lichens and bryophytes as indicators of air pollution in Kyiv City (Ukraine). Foloa Crypotog Estonica Fasc 46: 33-44.

Ezukanma IO, Tessler M, Salaam AM, Kanayo S, Chukwuka & Ogunniran AJ. 2019. Epiphytic bryophytes of urban agroforests in Ibadan, Southwest Nigeria. J Bryo [Internet]. [cited 2023 Jan 1]; 41(4): 341-9. Available from: https://www.tandfonline.com/loi/yjbr20, doi: 10.1080/03736687.2019.1611250.

Fastanti FS, Wulansari TRI. 2021. The dynamics of bryophytes species diversity in the Lowland ecosystems, Cibinong Science Center Botanical Garden. Jurnal Ilmiah Biologi Eksperimen dan Keanekaragaman Hayati [Internet]. [cited 2022 Dec 20]; 8(2): 8-17. Available from http://jurnalbiologi.fmipa.unila.ac.id/index.php/jbekh

Fastanti FSF, Fitmawati F, Sofiyanti N. 2013. Mosses in several type of vegetation in n the Giam Siak Kecil-Bukit Batu Biosphere Reserve Area, Riau Province. [Undergraduate Thesis]. Faculty of Mathematics and Natural Science, Universitas Riau Pekanbaru.

Floyed A, Gibson M. 2012. Bryophytes of urban industrial streetscapes in Victoria, Austria. Vic Nat 129(6): 203-14.

Frahm J-P. 2003. Manual of tropical bryology. Trop Bryol 23: 39-48.

Freedman B. 2018. Environmental sciences. Dalhousie University.

Fudali E, Żoknierz, L. 2019. Epiphytic bryophytes in urban forest of Wroclaw (SW Poland). Biodiv Res Conserv [Internet]. [cited 2022 Nov 11]; 53: 73-83. Available from https://sciendo.com/article/10.2478/biorc-2019-0007 doi: 10.2478/biorc-2019-0007

Giordano S, Sorbo S, Adamo P, Basile A, Spagnuolo V, Cobianchi RC. 2004. Biodiversity and trace element content of epiphytic bryophytes in urbanand extraurban sites of southern Italy. Plant Ecol 170: 1-14.

Godovićová K, Mišíková K, Hrabová D. 2020. Bryophyte flora of selected historical parks and gardens of Slovakia. Biol [Internet]. [cited 2022 Nov 11]; 75:1127-34. Available from https://link.springer.com/article/10.2478/s11756-020-00462-6 https://doi.org/10.2478/s11756-020-00462-6

Govindapyari H, Leleeka M, Nivedita M, Uniyal PL. 2010. Bryophytes: Indicators and monitoring agents of pollution. NeBio 1: 35-41.

He X, He KS, Hyvönen J. 2016. Will bryophyte survive in the warm world? Perspect Plant Ecol Evol Syst [Internet]. [cited 2022 Nov 11]; 19:49-60. Available from https://www.sciencedirect.com/science/article/abs/pii/S1433831916300117?via%3Dihub doi: 10.1016/j.ppees.2016.02.005

Kanawade SM, Hamigi AD, Gaikwad RW. 2010. Ecological effect of pollution. Int J Chem Eng Appl 1(4): 332-5.

Kou, J, Feng C, Bai XL, Chen H. 2014. Morphology and taxonomy of leaf papillae and mammilae in Pottiaceae of China. J Syst Evol 52(4): 521-32.

Kürschner, H. 2004. Life strategies and adaptation in bryophytes from the Near and Middle East. Turk J Bot 28: 73-84.

Le Blanc F. 1971. Possibilities and methods for mapping air pollution on the basis of lichen sensitivity. Mitteilungen der forstlichen Bundes-Versuchsanstalt Wien 92: 103-26

Lestari P, Arrohman MK, Damayanti S, Klimont Z. 2022. Emissions and spatial distribution of air pollutants from anthropogenic sources in Jakarta. Atmos Pollut Res [Internet]. [cited 2022 October 10]; 13: 101521. Available from: doi: 10.1016/j.apr.2022.101521

Jiang Y, Shaaban M, Zhang X, Hu R, Zhao J, Fan M, Wu Y. 2020. Urban atmospheric environment quality assessment by naturally growing bryophytes in Central China. Int J Environ Res Public Health [Internet]. [cited 2022 Nov 11]. 17(4537): 1-14. Available from https://www.mdpi.com/1660-4601/17/12/4537 doi: 10.3390/ijerph17124537

Lubis SH, Arifin HS, Samsoedin I. 2013. Analisis cadangan karbon pohon pada lanskap hutan kota di DKI Jakarta [Tree carbon stock analysis of urban forest landscape in DKI Jakarta]. J Penelit Sos Ekon Kehutan 10(1): 1-20.

Mukhia S, Mandal P, Singh DK, Singh D. 2019. The abundance of epiphytic liverworts on the bark of Cryptomeria japonica in relation to different physical and biochemical attributes, found in Senchal Wildlife Sanctuary, Darjeeling, Eastern

Himalaya. BMC Ecol [Internet]. [cited 2023 Jan 2]; 19:37. Available from https://bmcecol.biomedcentral.com/articles/10.1186/s12898-019-0253-9 doi: 10.1186/s12898-019-0253-9

Oishi Y, Hiura T. 2017. Bryophyte as bioindicators of the atmospheric environment in urban-forest landscape. Landsc Urban Plan [Internet]. [cited 2022 Nov 11]; 167: 348-55. Available from https://www.sciencedirect.com/science/article/abs/pii/S016920461730172X doi: 10.1016/j.landurbplan.2017.07.010

Oishi Y. 2019. Urban heat island effects on moss gardens in Kyoto, Japan. Landsc Ecol Eng [Internet]. [cited 2022 Nov 10]; 15:177–184. Available from https://link.springer.com/article/10.1007/s11355-018-0356-z doi: 10.1007/s11355-018-0356-z

Permatasari PA, Amalo LF, Wijayanto AK. 2019. Comparison of urban heat island effect in Jakarta and Surabaya, Indonesia. In Proc of SPIE 2019. Sixth International Symposium on LAPAN-IPB Satellite. P. 1137209-6.

Pescott OL, Simkin JM, August TA, Randle Z, Dore AJD, Botham MSB. 2015. Air pollution and its effects on lichens, bryophytes, and lichen-feeding Lepidoptera: review and evidence from biological records. Biol J Linn Soc 115: 611-35.

Printanakul N, Jampeetong A. 2020. A preliminary study on morphological variations from wet and dry microhabitats of hyophila involuta (Pottiaceae, Bryophyta): A case study from Chiang Mai Province, Northern Thailand. Chiang Mai Univ J Nat Sci [Internet]. [cited 2022 Dec 1]; 20(1): 1-16. Available from https://cmuj.cmu.ac.th/cmu_journal/cmu_journal.php

Proctol, MCF, Tuba Z. 2002. Poikilohydry and homoihydry: Antithesis or spectrum of possibilities? New Phytol 156(3): 327-49.

Putra CD, Ramadhani A, Fatimah E. 2021. Increasing urban heat island area in Jakarta and its relation to land use changes. In IOP Conf Series: Earth and Environmental Science. The 5th International Seminar on Sustainable Urban Development. P. 012002.

Putrika A, Nisyawati, Ariyanti NS. 2017. Keanekaragaman lumut epifit di hutan kota dan jalan utama kampus Universitas Indonesia. [Diversity of ephifit bryophytes in the urban forest and main street margin of Indonesia University campus]. Biosite 3(3): 25-38.

Uttara S, Bhuvandas N, Aggarwal V. 2012. Impacts of urbanization on environment. IJREAS 2(2): 1637-45.

Spitale D, Mair P, Nascimbene J. 2020. Patterns of bryophyte life-forms are predictable across land cover types. Ecol Indic [Internet]. [cited 2022 Nov 11]; 109:1-7. Available from https://www.sciencedirect.com/science/article/pii/S1470160X19307939?via%3Dihub doi: 10.1016/j.ecolind.2019.105799

Syihabuddin A, Darmadi A, Waryono T. 2020. Analisis fungsi jasa bio-ekologis hutan kota kawasan industri. [Analysis of the function of bio-ecological services for industrial area urban forest]. Jurnal Bumi Lestari [Internet]. [cited 2023 Jan 2]; 20(1):1-7. Available from https://ojs.unud.ac.id/index.php/blje/article/view/71759 doi: 10.24843/blje.2020.v20.i01.p01

Szlavecs K, Warren P, Pickett P. 2011. Biodiversity on the urban landscape. In: Cincotta RP, LJ Gorenflo, editors. Human population: Its influences on biological diversity. Berlin: Springer-Verlag. P. 75-101.

Szűcs P, Pénzes-Kónya E, Hofmann T. 2017. The bryophyte flora of the village of Almásfüzitő, a former industrial settlement in NW-Hungary. Cryptogam Bryol [Internet]. [cited 2022 Dec 1]; 38(2): 153-70. Available from https://doi.org/10.7872/cryb/v38.iss2.2017.153

Vanderpoorten A, Goffinet B. 2009. Introduction to bryophytes. Cambridge: Cambridge University Press.

Vitt DH, Crandall-Stotler B, Wood A. 2014. Bryophyte: Survival in a dry world through tolerance and avoidance. In: Rajakaruna N, Boyd R, Harris T, editors. Plant ecology and evolution in harsh environments. Nova Publishers. P. 267-95.

Zander, H. 1993. Genera of the pottiaceae: Mosses of harsh environments. Bull Buffalo Soc Nat Sci.

Zhao D, Zhang C, Sun Z, Wang C, Hao Z, Sun B, Zuo Q, Sun R, Jin J, Wang H. 2021. Urban orchards provide a suitable habitat for epiphytic bryophytes. For Ecol Manag [Internet]. [cited 2022 October 10]; 483:11. Available from: https://doi.org/10.1016/j.foreco.2020.118767

DIVERSITY OF ECTOPARASITES ON BATS IN DRAMAGA, BOGOR, INDONESIA

VIRGILIUS MARTIN KELAKE KEDANG[1], YANRI RIZKY NATANAEL SIMANGUNSONG[1], SUSI SOVIANA[2], UPIK KESUMAWATI HADI[2] AND SUPRIYONO[2]*

[1]*School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, Indonesia*
[2]*Division of Parasitology and Medical Entomology, School of Veterinary Medicine and Biomedical Sciences, IPB University, Bogor 16680, Indonesia*

Received 26 March 2023 / Revised 1 July 2023 / Accepted 2 July 2023

ABSTRACT

Ectoparasites infestation is one of the major health problems affecting animals, including bats, which are known as reservoir hosts for various pathogens. Several reports have shown that a comprehensive understanding of ectoparasites on these animals is crucial from a public health perspective. Therefore, this study aims to identify the diversity of ectoparasites on bats in Dramaga, Bogor, Indonesia. The samples were captured at night with a mist net and then identified using the species identification key. Ectoparasites were collected from the body of the samples and identified using a microscope. A total of 56 bats from 9 species, namely *Cynopterus brachyotis*, *Cynopterus sphinx*, *Cynopterus titthaecheilus*, *Macroglossus sobrinus*, *Rousettus leschenaultii*, *Myotis muricola*, *Nycteris javanica*, *Pipistrellus javanicus*, and *Scotophilus kuhlii* were obtained in this study. The results of ectoparasites identification showed the presence of *Basilia* spp., *Eucampsipoda* sp., *Leptocyclopodia ferrarii* (Nycteribiidae), *Raymondia* sp. (Streblidae), *Meristaspis* spp., *Spinturnix* spp. (Spinturnicidae), and ticks (Ixodidae). The total prevalence of infested bats was 51.7%, with females tending to have a higher intensity compared to males. Bats species with the highest prevalence of infestation were *Rousettus leschenaultii* and *Myotis muricola*. Meanwhile, this study found no ectoparasites on *Macroglossus sobrinus* and *Scotophilus kuhlii*.

Keywords: bats, bats flies, Bogor, ectoparasite, Indonesia

INTRODUCTION

Bats are known to play an essential role in maintaining the ecological balance of ecosystems, serving as seed transmitters, pollinators, and natural insect control (Suyanto 2001). Despite their significant ecological functions, these animals also serve as the natural reservoir of various pathogens. Previous studies showed that bats had unique immune systems, thereby enabling them to carry various zoonotic viruses per host species compared to rodents (Irving *et al.* 2021; Luis *et al.* 2013). Several emerging infectious diseases have also been linked to these animals bats as a source of zoonotic pathogens, including Ebola virus, SARS coronavirus, Nipah virus, and Hendra virus (Brook & Dobson 2015; Calisher *et al.* 2006). Furthermore, studies conducted in Indonesia showed their association with potential zoonotic diseases (Diptyanusa *et al.* 2021; Tsang *et al.* 2021). This indicates that the study of bats and their potential to transmit diseases to humans or other animals demands thorough investigation.

One of the health issues affecting bats is ectoparasites infestation, which warrants further investigation to comprehend the ecology and health of these creatures. The commonly associated ectoparasites belong to the class Insecta (bats flies and bats flea) and Arachnida (ticks and mites). The species within the class Insecta are known to belong to the family Nycteribiidae, Streblidae, Hippoboscidae, and Ischnopsyllidae. Meanwhile, those from class Arachnida are often members of the family Argasidae, Ixodidae, and Spinturnicidae (Fajri *et al.* 2018; Azhar *et al.* 2015). Previous studies showed that these ectoparasites in bats are vectors of pathogens, such as *Bartonella* spp., *Rickettsia* spp., and certain viruses with the ability

*Corresponding author, email: supriyono84@apps.ipb.ac.id

to cause diseases in humans or animals (Szentiványi *et al.* 2019; Reeves *et al.* 2016).

Several studies have been carried out on bat ectoparasites in several Southeast Asia countries, including Malaysia (Azhar *et al.* 2015), Singapore (Lim *et al.* 2020), and the Philippines (Alvarez *et al.* 2015). Based on previous findings, studies related to these ectoparasites in Indonesia remains significantly limited, despite the presence of over 230 distinct bat species in this region (Maryanto *et al.* 2020). Existing reports in Indonesia primarily focused on recording ectoparasite infestation in different locations and species, especially megabats (Nangoy *et al.* 2021; Sauqi *et al.* 2021; Fajri *et al.* 2018). Based on these findings, there is a need to conduct further studies to understand the characteristics of ectoparasites, including their specific host and potential role in transmitting diseases.

Dramaga is a subdistrict located in Bogor, Indonesia, which has been extensively explored due to bats species diversity in Dramaga. Several studies have also been carried out in and around the IPB Dramaga Campus area and its surrounding villages. According to Mustari *et al.* (2014) and Mustari (2020), at least 10 species of bats were found in IPB Dramaga Campus. Another report showed the presence of 11 species in villages around the area (Sumirto 2013). Due to the role of these animals in maintaining ecosystem balance, there is a need to carry out further investigations on their ecology and health problems, such as ectoparasites infestation. Therefore, this study aims to identify the diversity of ectoparasites on bats in Dramaga, Bogor, Indonesia.

MATERIALS AND METHODS

Study Sites and Sampling Techniques

Bats used in this study were obtained from the seven locations in Dramaga, Bogor, Indonesia from November 2021 to February 2022. The habitats selected as sampling locations included arboretums, agricultural land areas, and rural areas. Furthermore, reconnaissance surveys were conducted in the area before the study began to identify the location where bats were likely to congregate. The survey targeted the regions where bats were expected to fly, such as fruiting trees or congregation sites of insects, to determine the appropriate sampling locations. The procedures for sample capturing in this study referred to the Guidelines for Bats Reservoir Data Collection published by the National Institute of Health Research and Development, Ministry of Health, Indonesia. The samples were captured at night using mist-net techniques. The mist nets were installed in determined locations and placed approximately 5 meters above the ground. The installation was carried out for four hours from 18.00 to 22.00 and monitored every hour. Bats were collected as detected trapped in the mist net, placed into a cotton bag, and transported to a nearby station for further identification (Balitbangkes 2015).

Bats Identification and Ectoparasites Collection

Captured bats were identified based on morphological features and morphometries using the species identification key from the Field Guide of Bats in Indonesia (Suyanto 2001). The identified samples were then observed to determine the presence of ectoparasites infestation. The collection procedure for ectoparasites was carried out using small tweezers. Subsequently, the parasites obtained were placed in a labeled vial containing 70% alcohol (Balitbangkes 2015), and bats were released back to the initial location.

Ectoparasites Identification

The initial grouping of ectoparasites was carried out based on their morphological characteristics. Bats flies were distinguished by their large size resembling a fly with or without wings, while mites and ticks were distinguished by their relatively small size compared to bat flies. Subsequently, the collected ectoparasites were identified using a microscope. Bats flies were identified using the publications of Maa (1971, 1968, 1962) and Theodor (1967, 1959), while mites were identified using the publication of Baker & Delfinado (1964) and Delfinado & Baker (1963). Ticks ectoparasites were identified using the publication of Hoogstraal (1955).

Data Analysis

Data on captured bats were analyzed descriptively by making a table that included the species, the number of captured bats, the number

of bats infested with ectoparasites, the prevalence of ectoparasites infestation, and the intensity of ectoparasites. Data on the collected ectoparasites were analyzed descriptively by making a table containing the types of ectoparasites, hosts, sex, and the number of collected samples per host.

RESULTS AND DISCUSSION

Bats Diversity

A total of 56 bats were obtained in this study, belonging to the suborders Megachiroptera and Microchiroptera. The samples represented nine species, namely *Cynopterus brachyotis*, *Cynopterus sphinx*, *Cynopterus titthaecheilus*, *Macroglossus sobrinus*, *Rousettus leschenaultii*, *Myotis muricola*, *Nycteris javanica*, *Pipistrellus javanicus*, and *Scotophilus kuhlii*. The results showed that the most captured species was *Cynopterus brachyotis*, with a total of 20 samples (35.7%). The least caught species was *Scotophilus kuhlii*, with only one sample (1.8%), as shown in Table 1. All bats species found in this study had a conservation status of least concern, except *Nycteris javanica*, which was considered vulnerable. *Nycteris javanica* was an endemic bats and was only found in Java and Timor (Waldien & Wiantoro 2021).

This study found a total of 29 samples that were infested with ectoparasites, accounting for 51.7% of the total population. The species with the highest prevalence (80%) of infestation were *Rousettus leschenaultia* and *Myotis muricola*. Meanwhile, there was no infestation on *Macroglossus sobrinus* and *Scotophilus kuhlii*, as shown in Table 1.

The prevalence and intensity of ectoparasites in each bat sex are presented in Table 2. The results showed that the number and intensity of ectoparasites varied widely among the samples' sex and species. Female *Rousettus lechenaultii* species had the highest intensity of infestation. Female bats tended to have a higher intensity of ectoparasites compared to males, but their population was smaller.

Table 1 Bats species and prevalence of ectoparasites infestation in bats

Bats Species	Number of Captured Bats	Number of Infested Bats	Prevalence (%)
Megachiroptera (Megabat)			
Cynopterus brachyotis	20	11	55
Cynopterus sphinx	5	2	40
Cynopterus titthaecheilus	11	6	54.5
Macroglossus sobrinus	4	0	0
Rousettus leschenaultii	5	4	80
Subtotal	45	23	51.1
Microchiroptera (Microbat)			
Myotis muricola	5	4	80
Nycteris javanica	2	1	50
Pipistrellus javanicus	3	1	33.3
Scotophilus kuhlii	1	0	0
Subtotal	11	6	54.5
Total	**56**	**29**	**51.7**

Table 2. Intensity of ectoparasites based on bats species and sex

Bats Species	Infested Bats/Captured Bats		Total Ectoparasites		Intensity of Ectoparasites	
	Male	Female	Male	Female	Male	Female
Cynopterus brachyotis	10/19	1/1	20	2	2.0	2.0
Cynopterus sphinx	1/3	1/2	3	1	3.0	1.0
Cynopterus titthaecheilus	4/6	2/5	5	2	1.25	1.0
Rousettus leschenaultii	2/3	2/2	8	24	4.0	12.0
Myotis muricola	4/5	0/0	27	0	6.75	0
Nycteris javanica	1/2	0/0	1	0	1.0	0
Pipistrellus javanicus	1/3	0/0	2	0	2.0	0
Macroglossus sobrinus	0/4	0/0	0	0	0	0
Scotophilus kuhlii	0/1	0/0	0	0	0	0
Total	**23/46**	**6/10**	**66**	**29**	**2.87**	**4.83**

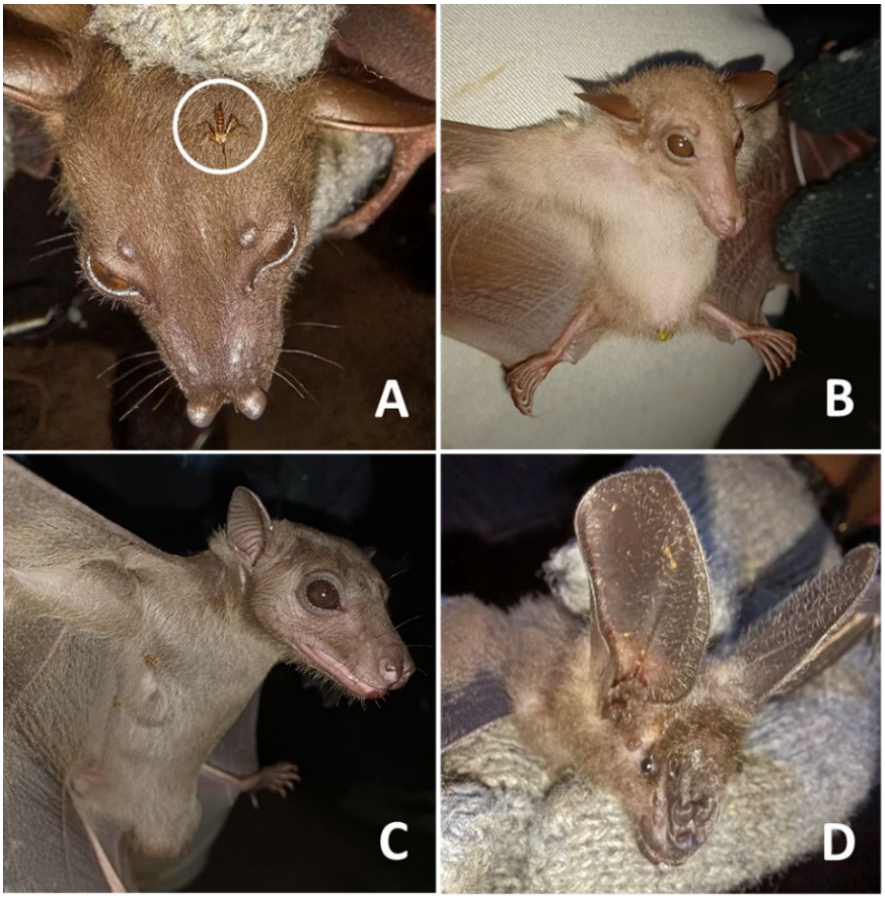

Figure 1 Bats species captured in Dramaga, Bogor: (A) *Cynopterus titthaecheilus* with ectoparasites (white circle), (B) *Macroglossus sobrinus*, (C) *Rousettus leschenaultii*, (D) *Nycteris javanica*

This was the first study to record the diversity of ectoparasites on bats in Dramaga, Bogor. The results could be used to monitor the status of bats species in Dramaga, Bogor, in line with conservation efforts. This study also presented the first record of ectoparasites infestation on vulnerable endemic bats species, namely *Nycteris javanica*. The species was endemic to Java and Timor Islands and not commonly found in nature due to its declining population and conservation status being vulnerable (Waldien & Wiantoro 2021).

Due to the absence of ectoparasites on some species, further studies must be carried out to determine the possibility of infestation on bats. This study found no infestation in *Macroglossus sobrinus* and *Scotophilus kuhlii*, possibly due to the lack of representative samples. Therefore, future reports must explore ectoparasites that could affect these species. Further studies were also needed due to the inability of this current study to cover all bat species reported by Mustari (2020), such as *Kerivoula hardwickii*, *Rhinolophus affinis,* and *Hipposideros diadema*.

Ectoparasites Diversity

Ectoparasites found in this study were from Insecta and Acarina classes, as shown in Table 3. A total of 95 samples were collected representing three groups, namely bats flies (family Nycteribiidae and Streblidae), mites (family Spinturnicidae), and ticks (family Ixodidae). Nycteribiidae bats flies were found on five microbats and megabats, namely *Myotis muricola*, *Cynopterus brachyotis*, *Cynopterus sphinx*, *Cynopterus titthaecheilus*, and *Rousettus leschenaultii*. Streblidae species was only discovered on *Nycteris javanica*. Mites were discovered on *Rousettus leschenaultii*, *Myotis muricola*, and *Pipistrellus javanicus,* while ticks were only found on *Myotis*. Furthermore, *Rousettus leschenaultia* had the highest ectoparasites infestation compared to others in Dramaga. The differences in the number of infestation were due to the variations in number of captured individual bats in each species.

Table 3 Collected ectoparasites found on bats in Dramaga, Bogor

No	Ectoparasites Species	Number of Ectoparasites									Total
		C.b	C.s	C.t	M.sb	R.l	M.m	N.j	P.j	S.k	
	Bats flies										
1	*Basilia* spp.	0	0	0	0	0	10	0	0	0	**10**
2	*Eucampsipoda* sp.	0	0	0	0	21	0	0	0	0	**21**
3	*Leptocyclopodia ferrarii*	22	4	7	0	0	0	0	0	0	**33**
4	*Raymondia* sp.	0	0	0	0	0	0	1	0	0	**1**
	Mites										
1	*Meristaspis* spp.	0	0	0	0	11	0	0	0	0	**11**
2	*Spinturnix* spp.	0	0	0	0	0	16	0	2	0	**18**
	Ticks										
1	Ixodidae	0	0	0	0	0	1	0	0	0	**1**
	Total	**22**	**4**	**7**	**0**	**32**	**27**	**1**	**2**	**0**	**95**

Notes: C.b: *Cynopterus brachyotis*, C.s: *Cynopterus sphinx*, C.t: *Cynopterus titthaecheilus*, M.sb: *Macroglossus sobrinus*, R.l: *Rousettus leschenaultii*, M.m: *Myotis muricola*, N.j: *Nycteris javanica*, P.j: *Pipistrellus javanicus*, S.k: *Scotophilus kuhlii*.

Ectoparasites from the family Nycteribiidae were the most collected ectoparasites in this study. Furthermore, Nycteribiid bats flies were ectoparasites commonly found in megabats from the genus *Cynopterus* and *Rousettus* (Nangoy *et al.* 2021). Species from the genus *Cynopterus* were reported to be the primary hosts of *Leptocyclopodia* flies (Maa 1975). Several studies collected *Leptocyclopodia ferrarii* from *Cynopterus brachyotis* in Indonesia (Nangoy *et al.* 2021; Sauqi *et al.* 2021). *Eucampsipoda* bats flies were common on megabats, and they had infested *Eonycteris spelaea* and *Rousettus leschenaultii* in Southeast Asia countries (Lim *et al.* 2020; Fajri *et al.* 2018; Azhar *et al.* 2015). *Basilia* ectoparasites were commonly obtained in megabats and microbats, and Poerwanto *et al.* (2020) reported their presence on *Miniopterus schreibersii* in Yogyakarta, Indonesia.

Ectoparasites from the genus *Raymondia* included Streblidae bats flies found in microbats (Azhar *et al.* 2015; Maa 1962). These species could be distinguished from Nycteribiidae samples by the presence of the wings (Azhar *et al.* 2015). This study only found *Raymondia* sp. on *Nycteris javanica*. This result was the first record of ectoparasites infestation by the genus *Raymondia* on *Nycteris javanica*.

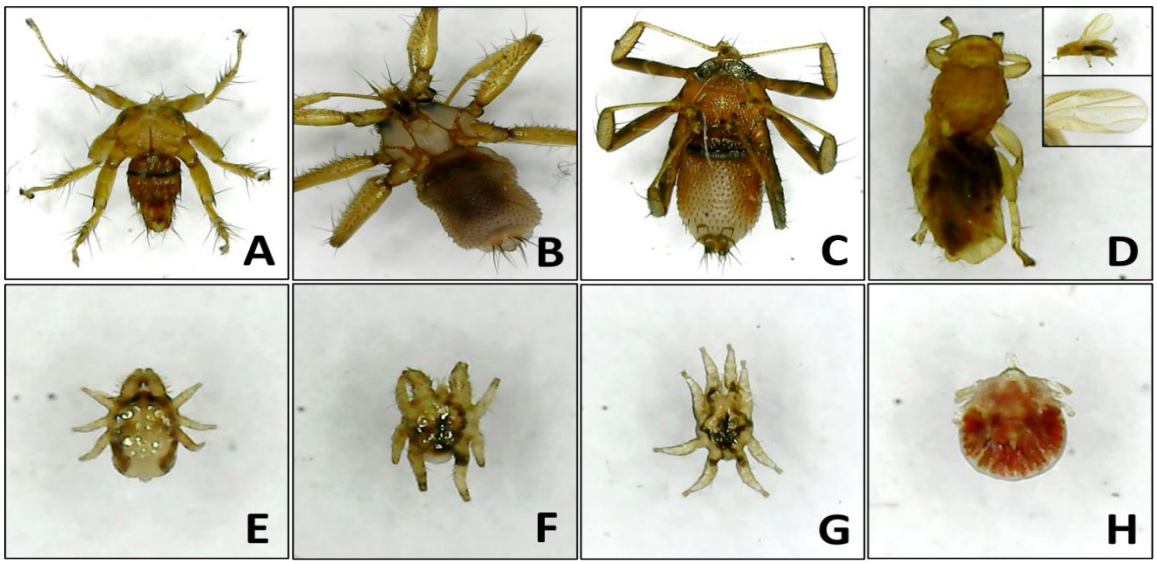

Figure 2 Ectoparasites collected from bats in Dramaga, Bogor: (A) *Basilia* sp. collected from *Myotis muricola*, (B) *Eucampsipoda* sp. collected from *Rousettus leschenaultii*, (C) *Leptocyclopodia ferrarii* collected from *Cynopterus* spp., (D) *Raymondia* sp. collected from *Nycteris javanica*, (E) Mite *Meristaspis* sp. ♀ and (F) *Meristaspis* sp. ♂ collected from *Rousettus leschenaultii*, (G) *Spinturnix* sp. collected from *Myotis muricola* and *Pipistrellus javanicus*, and (H) Ixodid tick (Ixodidae) collected from *Myotis muricola*

Mites and ticks were found in the genus *Rousettus*, *Myotis*, and *Pipistrellus*. Mite ectoparasites infesting bats in Indonesia were from the Spinturnicidae family, while the ticks were from the Ixodidae and Argasidae families (Fajri *et al.* 2018). Several studies reported ticks and mites ectoparasites infestation on bats in Indonesia (Poerwanto *et al.* 2020; Fajri *et al.* 2018). The results of *Meristaspis* sp. and *Spinturnix* sp. mites from the family Spinturnicidae and ticks larvae from the family Ixodidae were consistent with previous studies on arachnid infestation on bats.

Bat-Ectoparasites Interaction

Ectoparasites in bats spent almost their entire life cycle on the host through blood consumption (Hiller *et al.* 2019; Bordes *et al.* 2008). The intensity and prevalence of ectoparasites were affected by several factors, including host-specificity, habitat, nesting, gender, diet, and social behavior (Nangoy *et al.* 2021; Hiller *et al.* 2019; Ramanantsalama *et al.* 2018). Therefore, their distribution and abundance were associated with the distribution and abundance of the host (Putra, 2014; Ter Hofstede & Fenton, 2005). The colony size of each bat species could also influence social behavior, leading to differences in the prevalence of ectoparasites infestation. Species that shared the same roosting site in larger groups increased the possibility of ectoparasites transmission between the bats (Putra 2014). Bats in genera *Cynopterus*, *Rousettus*, and *Pipistrellus* were generally known to roost from medium to large colonies (Garg *et al.* 2015; Kumar *et al.* 2015; Gay *et al.* 2014). Different roosting behavior was found in *Macroglossus* species, which tended to roost alone or in small colonies occupying different sites (Putra 2014; Gould 1978). The absence of ectoparasites infestation on *Macroglossus* bats in this study was allegedly related to the behavior of the species.

Several reports showed that ectoparasites infestation in bats had an association with sex. In this study, females tended to have a higher intensity of ectoparasites compared to males, but they had few infested individuals due to the small population. The results were consistent with Nangoy *et al.* (2021) on Pteropodid bats in Sulawesi, where females had a higher intensity due to their high susceptibility caused by several factors. Fluctuations in the reproductive cycle, such as pregnancy and lactation, could suppress immunity and increase susceptibility to parasites. During this period, the female species tended to spend more time in roosting sites, increasing contact with ectoparasites or the other infested bats (Tai *et al.* 2022; Nangoy *et al.* 2021; Webber *et al.* 2015).

A previous study reported that extended stay in roosting sites also increased ectoparasite exposure (Lim *et al.* 2020). Compared to the results of this study, Lim *et al.* (2020) found that male *Cynopterus brachyotis* and *Eonycteris spealea* had a higher level of infestation. This was because they spent more time in the roost site due to defense. Grooming activities commonly carried out to expel parasites also affected infiltration among sexes since males spent more time in grooming activities related to ectoparasites consumption compared to females (Ramanantsalama *et al.* 2018). Meanwhile, Godinho *et al.* (2013) found no association between grooming activity and the number of parasites. Due to the variation in literature, further studies must be carried out to investigate ectoparasites infestation in relation to sex.

Ectoparasites in bats generally had certain specific hosts, and could only be found in some species. Bats flies were obligate and specialized parasitic organisms found on the fur and wing membranes. Furthermore, this study found a genus-specific host pattern in ectoparasites. *Leptocyclopodia ferarri* bats flies obtained had specific hosts from the *Cynopterus* spp. bats. These findings were consistent with several studies, where species from the genus *Cynopterus* were primary hosts for *Leptocyclopodia* spp. (Lim *et al.* 2020; Azhar *et al.* 2015). Based on the results, *Meristaspis* mites tended to infest megabats, while *Spinturnix* mites infested microbats. The results were in line with previous studies, which recorded the infestation of *Meristaspis* spp. mites on megabats from the genus *Macroglossus* and *Rousettus* (Fajri & Armiani 2021; Fajri *et al.* 2018). Meanwhile, the study by Zania *et al.* (2022) reported the *Spinturnix* mite infestation on *Rousettus* bats in Banyuwangi. This variation in findings suggested that the mites did not have a genus-specific pattern.

The presence of ectoparasites in bats contributed to the spread of pathogens between individual members in the colony. Some of the parasites had various hosts and could be vectors for certain pathogens. Several studies found *Bartonella* spp. bacteria in *Leptocyclopodia* bats flies collected from the megabats in Malaysia and the Philippines (Low *et al.* 2022; Morse *et al.* 2012). *Bartonella* spp. was a bacteria that caused bartonellosis and was considered zoonosis (Chomel & Kasten 2010). Bacterium *Bartonella* spp. and *Rickettsia* spp. were also found in mites from the genus *Spinturnix* collected from *Myotis myotis* bats in Poland (Szubert-Kruszyńska *et al.* 2019). Feng *et al.* (2017) found the Khaeng Koi virus in *Eucampsipoda sundaica* from *Rousettus leschenaultia* bats in China. The results of bats ectoparasites in Dramaga proved that ectoparasites infestation was a major health problem in bats. The ability of ectoparasites to act as vectors of various pathogens demands further studies on their distribution in each bats species, as well as their public health importance.

CONCLUSION

In conclusion, ectoparasites were one of the causes of health-related problems in bats with health importance due to their ability to act as vectors of various pathogens. Ectoparasites affecting bats in Dramaga were from the family Nycteribiidae, Streblidae, Spinturnicidae, and Ixodidae. The results showed that the total prevalence of infested samples in the study location was 51.7%. Furthermore, the female samples tended to have a higher intensity of ectoparasites compared to males. The species with the highest prevalence of infestation were *Rousettus leschenaultii* and *Myotis muricola*. This study found no ectoparasites infestation on *Macroglossus sobrinus* and *Scotophilus kuhlii*. Based on these findings, further studies were needed as some species of bats required large sample sizes.

REFERENCES

Azhar I, Khan FAA, Ismail N, Abdullah MT. 2015. Checklist of bat flies (Diptera: Nycteribiidae and Streblidae) and their associated bat hosts in Malaysia. Check List 11(5): 1777.

Alvarez JD, Lit Jr IL, Alviola PA. 2015. Bat flies (Diptera: Nycteribiidae) from Mount Makiling, Luzon Island: New host and distribution records, with a checklist of species found in the Philippines. Check List 11(1): 1509.

Baker EW, Delfinado MD. 1964. Spinturnicidae of South East Asia and The Pacific Region. Pacific Insects 6(4): 571-91.

Balitbangkes. 2015. Pedoman Pengumpulan Data Reservoir (Kelelawar) di Lapangan. [Guidelines for Reservoar (Bat) Data Collection in the Field]. Jakarta (ID): Badan Penelitian dan Pengembangan Kesehatan.

Bordes F, Morand S, Ricardo G. 2008. Bat fly species richness in Neotropical bats: correlations with host ecology and host brain. Oecologia 158(1): 109-16.

Brook CE, Dobson AP. 2015. Bats as 'special' reservoirs for emerging zoonotic pathogens. Trends in Microbiology 23(3): 172-80.

Calisher HC, Childs JE, Field HE, Holmes KV, Schountz T. 2006. Bats: Important Reservoir Hosts of Emerging Viruses. Clinical Microbiology Reviews 19(3): 531-45.

Chomel BB, Kasten RW. 2010. Bartonellosis, an increasingly recognized zoonosis. Journal of Applied Microbiology 109(3): 743-50.

Delfinado MD, Baker EW. 1963. Mites of The Family Spinturnicidae From The Philippines (Acarina). Pacific Insects 5(4): 905-20.

Diptyanusa A, Herini ES, Indarjulianto S, Satoto TBT. 2021. The detection of Japanese encephalitis virus in Megachiropteran bats in West Kalimantan, Indonesia: A potential enzootic transmission pattern in the absence of pig holdings. International Journal for Parasitology: Parasites and Wildlife 14: 280-86.

Fajri SR, Armiani S. 2021. A Prevalence, Intensity, and Associated of Ectoparasitic Fauna among Cave-Dwelling Bats from Lombok Island West Nusa Tenggara. Prisma Sains: Jurnal Pengkajian Ilmu dan Pembelajaran Matematika dan IPA IKIP Mataram 9(1): 141-51.

Fajri SR, Primawati SN, Hadi I, Tresnani G. 2018. Ectoparasites diversity of bats species collected from Southern Lombok, West Nusa Tenggara, Indonesia. Journal of Biological Series 1(2): 31-41.

Feng Y, Li Y, Fu S, Li X, Song J, Zhang H, Yang W, Zhang Y, Pan H, Liang G. 2017. Isolation of Kaeng Khoi virus (KKV) from *Eucampsipoda sundaica* bat flies in China. Virus Research 238: 94-100.

Garg KM, Chattopadhyay B, Doss DPS, Kumar AKV, Kandula S, Ramakrishnan U. 2015. Males and Females Gain Differentially from Sociality in a Promiscuous Fruit Bat *Cynopterus sphinx*. PLoS ONE 10(3): e0122180.

Gay N, Olival KJ, Bumrungsri S, Siriaroonrat B, Bourgarel M, Morand S. 2014. Parasite and viral species richness of Southeast Asian bats: Fragmentation of area distribution matters. International Journal for Parasitology: Parasites and Wildlife. 3: 161-70.

Godinho LN, Cripps JK, Coulson G, Lumsden LF. 2013. The effect of ectoparasites on the grooming behaviour of Gould's wattled bat (*Chalinolobus gouldii*): An experimental study. Acta Chiropterologica. 15(2): 463-72.

Gould E. 1978. Foraging Behavior of Malaysian Nectar-Feeding Bats. Biotropica 10(3): 184-93.

Hiller T, Brändel SD, Honner B, Page RA, Tschapka M. 2019. Parasitization of bats by bat flies (Streblidae) in fragmented habitats. Biotropica 52(3): 488-501.

Hoogstraal H. 1955. Bat Ticks of The Genus *Argas* (Ixodoidea, Argasidae) I The Subgenus Chiropterargas. Fieldiana: Zoology 37: 579-600.

Irving AT, Ahn M, Goh G, Anderson DE, Wang LF. 2021. Lessons from the host defences of bats, a unique viral reservoir. Nature 589: 363-70.

Kumar M, Priya YS, Mathur V, Elangovan V. 2015. Roost Selection and Roosting Ecology of Fulvous Fruitbat, *Rousettus leschenaulti* (Pteropodidae). International Journal of Advanced Biological Research 5(1): 62-68.

Lim ZX, Hitch AT, Lee BPYH, Low DHW, Neves ES, Borthwick SA, Smith GJD, Mendenhall IH. 2020. Ecology of bat flies in Singapore: A study on the diversity, infestation bias and host specificity (Diptera: Nycteribiidae). International Journal of Parasitology: Parasites and Wildlife 12: 29-33.

Low VL, Tan TK, Tohiran KA, Lim YAL, AbuBakar S, Nasir DM. 2022. A novel clade of bat-associated *Bartonella* detected in the bat fly *Leptocyclopodia ferrari* (Diptera: Nycteribiidae) parasitizing *Cynopterus brachyotis* (Chiroptera: Pteropodidae). Veterinary Microbiology 264: 109284.

Luis AD, Hayman DTS, O'Shea TJ, Cryan PM, Gilbert AT, Pulliam JRC, Mills JN, Timonin ME, Willis CKR, Cunningham AA, Fooks AR, Rupprecht CE, Wood JLN, Webb CT. 2013. A comparison of bats and rodents as reservoirs of zoonotic viruses: are bats special?. Proceedings of the Royal Society B: Biological Sciences 280: 20122753.

Maa TC. 1962. Records and Descriptions of Nycteribiidae and Streblidae (Diptera). Pacific Insects 4 (2): 417-436.

Maa TC. 1968. Additions to The Cyclopodiinae Part I (Diptera: Nycteribiidae). Pacific Insects 10 (1): 1-23.

Maa TC. 1971. Review of The Streblidae (Diptera) Parasitic on Megachiropteran Bats. Pacific Insects Monograph 28: 213-243.

Maa TC. 1975. On new Diptera Pupipara from The Oriental Region. Pacific Insect 16(4): 465-486.

Maryanto I, Maharadatunkamsi, Achmadi AS, Wiantoro S, Sulistyadi E, Yoneda M, Suyanto A, Sugardjito J. 2020. Checklist of the mammals of Indonesia. Jakarta (ID): Research Center for Biology Indonesian Institute of Sciences (LIPI).

Morse SF, Olival KJ, Kosoy M, Billeter S, Patterson BD, Dick CW, Dittmar K. 2012. Global distribution and genetic diversity of *Bartonella* in bat flies (Hippoboscoidea, Streblidae, Nycteribiidae). Infection, Genetics and Evolution 12: 1717-23.

Mustari AH, Zulkarnain I, Rinaldi D. 2014. Keanakaragaman Jenis dan Penyebaran Mamalia di Kampus IPB Dramaga Bogor. [Species Diversity and Distribution of Mammals in IPB Dramaga Campus, Bogor]. Media Konservasi 19(2): 117-25.

Mustari AH. 2020. Biodiversitas di Kampus IPB University: Mammalia, Burung, Amfibi, Reptil, Kupu-Kupu, dan Tumbuhan. [Biodiversity in IPB University Campus: Mammals, Birds. Amphibians, Reptiles, Butterflies, and Plants]. Bogor (ID): IPB Press.

Nangoy M, Ransaleleh T, Lengkong H, Koneri R, Latinne A, Kyes RC. 2021. Diversity of fruit bats (Pteropodidae) and their ectoparasites in Batuputih Nature Tourism Park, Sulawesi, Indonesia. Biodiversitas 22(6): 3075-82.

Putra MIH. 2014. Hubungan Inang-Ektoparasit pada Kelelawar Pemakan Buah di Kampus Universitas Indonesia Depok. [Host-Ectoparasite Relationship on Fruit Bats in Universitas Indonesia, Depok]. [Undergraduate Thesis]. Retrieved from Universitas Indonesia Repository.

Poerwanto SH, Ridhwan LR, Giyantolin G, Ginawati D, Paramitha DPR. 2020. Keanekaragaman Ektoparasit pada Kelelawar Subordo Microchiroptera di Goa Jepang Bukit Plawangan, Sleman, Yogyakarta. [Ectoparasites Diversity of Microchiroptera Bats Suborder in Jepang Cave, Plawangan Hill, Sleman, Yogyakarta]. Jurnal Veteriner 21(4): 629-36.

Ramanantsalama RV, Andrianarimisa A, Raselimanana AP, Goodman SM. 2018. Rates of hematophagous ectoparasite consumption during grooming by an endemic Madagascar fruit bat. Parasites Vectors 11: 330.

Reeves WK, Beck J, Orlova MV, Daly JL, Pippin K, Revan F, Loftis AD. 2016. Ecology of bats, their ectoparasites, and associated pathogens on Saint Kitts Island. J Med Entomol 53(5): 1218-25.

Sauqi MS, Restiadi TI, Koesdarto S, Hastutiek P, Setiawan B, Wijaya A. 2021. Identification of ectoparasites and endoparasites on fruit bats (Cynopterus brachyotis) in Ketapang Timur Village, Ketapang Sub-District, Sampang District. Journal of Parasite Science 5(2): 35-40.

Sumirto A. 2013. Keanekaragaman Jenis Kelelawar di Desa Cikarawang Kecamatan Dramaga Kabupaten

Bogor Provinsi Jawa Barat. [Bats Diversity in Cikarawang Village Dramaga District Bogor Regency West Java Province]. [Undergraduate Thesis]. Retrieved from IPB University Repository.

Suyanto A. 2001. Panduan Lapangan Kelelawar di Indonesia. [Field Guide of Bats in Indonesia]. Bogor (ID): Puslitbang Biologi LIPI.

Szentiványi T, Christe P, Glaizot O. 2019. Bat Flies and Their Microparasites: Current Knowledge and Distribution. Frontiers in Veterinary Science 6: 115.

Szubert-Kruszyńska A, Stańczak J, Cieniuch S, Podsiadły E, Postawa T, Michalik J. 2019. *Bartonella* and *Rickettsia* infections in haematophagous *Spinturnix myoti* mites (Acari: Mesostigmata) and their bat host, *Myotis myotis* (Yangochiroptera: Vespertilionidae), from Poland. Microbial Ecology 77(3): 759-768.

Tai YL, Lee YF, Kuo YM, Kuo YJ. 2022. Effects of host state and body condition on parasite infestation of bent-wing bats. Frontiers in Zoology 19(1): 1-13.

Ter Hofstede HM, Fenton MB. 2005. Relationship between roost preferences, ectoparasite density, and grooming behaviour of neotropical bats. Journal of Zoology. 266: 333-340.

Theodor O. 1959. A Revision of The Genus *Cyclopodia* (Nycteribiidae, Diptera). Parasitology 49(1-2): 242-308.

Theodor O. 1967. An Illustrated Catalogue of The Rothschild Collection of Nycteribiidae (Diptera) in The British Museum (Natural History). London (UK): British Museum (Natural History).

Tsang SM, Low DHW, Wiantoro S, Smith I, Jayakumar J, Simmons NB, Vijaykrishna D, Lohman DJ, Mendenhall IH. 2021. Detection of Tioman Virus in *Pteropus vampyrus* Near Flores, Indonesia. Viruses 13: 563.

Waldien DL, Wiantoro S. 2021. *Nycteris javanica*. The IUCN Red List of Threatened Species 2021: e.T14932A22013241.

Webber QMR, McGuire LP, Steven B. 2015. Host behaviour, age and sex correlate with ectoparasite prevalence and intensity in a colonial mammal, the little brown bat. Behaviour 152(1): 83-105.

Zania FO, Geraldine AP, Putri CK, Edila R, Yudhana A. 2022. Laporan Kasus Infestasi *Spinturnix* spp. pada Kelelawar Pemakan Buah (*Rousettus* spp.) di Banyuwangi. [Case report of *Spinturnix* spp. infestation on Fruit Bats (*Rousettus* spp.) in Banyuwangi]. Media Kedokteran Hewan 33(3): 233-43.

BIOTROPIA Vol. 30 No. 3, 2023: 374 - 383 DOI: 10.11598/btb.2023.30.3.2005

ANNUAL CARBON CAPTURE POTENTIAL IN BANANA GARDENS OF INDIA

A.N. GANESHAMURTHY

Division of Natural Resources Management, Indian Institute of Horticultural research, Bengaluru 560089. India

Received 9 April 2023 / Revised 13 August 2023 / Accepted 13 August 2023

ABSTRACT

The global interest in increasing the world's carbon stocks is skewed towards perennial woody ecosystems. But a continuous shortage of land stands in the way of achieving the goal. We must therefore aim to explore viable alternatives. The banana as a potential carbon sequester attracted little attention from researchers. Therefore, this study aimed at estimating the potential of bananas in different states of India as potential carbon sequesters. Data was collected from twelve major banana producers between January 2021 and December 2022. One hundred banana gardens were sampled in each of the 12 banana-producing states, covering the major bananas grown (AAA, AAB, and ABB). The above-ground (AGB) and below-ground (BGB) biomass were calculated using the allometric equation with pseudostem volume as the allometric parameter. The dry weight obtained from the allometric equations was then converted to carbon using a dry weight to carbon conversion factor. Sequestered carbon varied with the AAA, AAB, and ABB of bananas. Banana plant carbon stock was also found to be very small, ranging from 2.573 to 6.407 t/ha, compared with very high soil carbon ranging from 39.55 to 77.14t. In all the banana-cultivating states, the proportion of carbon contained in the plant to that in the soil was only 8.286 percent, and that of soil carbon accounted for 91.714%. At the national level, the banana crop sequestered 48.627 million metric tonnes of carbon, with soil carbon accounting for 44.798 metric tonnes and plant carbon accounting for only 3.828 metric tonnes per year. Despite these small amounts of plant carbon, the banana cropping system enriches the soil by enabling much more carbon to be sequestered into the soil in amounts comparable to other perennial plantations.

Keywords: AAA, AAB, ABB, Banana growing states, banana stock estimation, carbon stock, cultivars, plant carbon, SOC.

INTRODUCTION

The giant herb Bananas of the Musaceae family and *Musa* genus are widespread throughout the tropical and subtropical zones (Charrier *et al.* 1997). It has occupied the 6th position in the world's fruit production and is a vital part of human diets in many regions. About 130 countries grow bananas, which they produce (Reay 2019). More than a thousand cultivars' and bred varieties with an array of sizes, colours, and shapes are under cultivation. These include yellow-skinned, red-skinned, and sweet table varieties, as well as starchy plantains used for cooking. They are nutritionally rich in minerals such as potassium and zinc, as well as essential vitamins A and C (Ravi 2013). The genome assemblies that constitute the entire spectrum of edible bananas and plantains, such as AAA, AAB, or ABB, are designated as "groups, while the total set of a basic cultivar and its derived clones form a "subgroup" (Langhe *et al.* 2000). AAA and AAB Group generally have stem diametres of 20–25 cm, grow to a height of 3–4 metres with broad leaves that range from 2 to 3 metres in length, and produce a high yield of medium-sized fruit. The ABB group is smaller, with a stem diameter of 15-20 cm and a leaf size of 2–3 meters in length, and produces high yields of larger fruit.

Banana is cultivated commercially under tropical and subtropical conditions all over the world, both on a commercial scale and by smallholders for home consumption or sale at local markets. The Asia-Pacific region produces

*Corresponding author, email:

55.4% of global production, and India ranks first in production, accounting for nearly 26.3%. Central and South America also contribute significantly to the total world banana basket (FAO 2018, Statista 2022). About a thousand varieties of bananas are produced, but preferences for varieties are generally local. The bananas of the AAA group, mainly the Cavendish type, are the most cultivated and produce about 50 billion metric tonnes, accounting for roughly 47 percent of global production (FAO 2022). (Nkoulou *et al*., 2023). The volume of production and trade of all other local bananas (AAB and ABB groups) is greater than that of Cavendish bananas.

India, with its wide variability of soil and climate, produces a large number of fruits. Among them, eight fruit crops occupy more than 80% of the fruit crop area, and bananas are the third most widely grown after mango and citrus, occupying 20% of the total fruit area and producing one-third (3184,000 metric tonnes) of the total fruits produced in India.

When huge quantities of bananas are produced to meet the livelihoods of millions of people around the world, it has naturally spurred climate-smart actions that include multidimensional and interconnected challenges to minimise GHG emissions and ensure sustainability (UNGA, 2015). This is because banana cultivation in the past concentrated on the need to increase food production and ignored the need to protect the environment, water, and biodiversity, thereby contributing to considerable environmental degradation and the loss of crop diversity. Realising this agricultural diversity and diversity-based farming have been advocated as the major backbones of sustainable agricultural intensification and a sustainable food system (Tutwiler *et al.* 2017). In terms of GHG emissions, a kilogramme of bananas results in the emission of 0.5–1.3 kilogrammes of greenhouse gas, or 100 to 200 grammes per banana (Reay 2019). Parts of the banana's life cycle can be targeted directly to mitigate climate change (WRAP 2012).

The banana crop leaves a large amount of residue. It is estimated that about four metric tonnes of pseudostem remain at the harvest location for each tonne of fruit harvested (Souza *et al.* 2010). Some estimates show that from the total of harvested bananas, 1.5 t of leaves and 2.5 t of pseudostem are generated per tonne of banana produced (Oliveiraa 2013). These residues contribute greatly to carbon sequestration, alcohol production, and natural fibre production. Making use of this biomass for the extraction of fibre and nutrients (particularly potassium), the production of alcohol for fuel, etc., could be a very attractive alternative by not only contributing to the preservation of the environment through carbon sequestration and removing this waste from the land but also by adding value to the fruit production matrix and transforming the residue into a commodity (Soraishram *et al.* 2021; Souza et al., 2010). Banana can be considered a perennial crop; thus, there is relatively permanent standing biomass throughout the year with considerable carbon content in its structure and hence a potential carbon sequester. Given the perennial and morphological nature of a crop like banana, it is worthwhile exploring its contribution to the carbon cycle. We therefore estimated the total annual banana residues produced and tried to work out their carbon sequestration potential in India.

MATERIALS AND METHOD

Selection of gardens and sampling

Banana is grown in many states of India, and the area (880 thousand ha) varies extensively, with large localised pockets (Fig. 1) located in different agroecological regions (Table 1). One hundred banana gardens were sampled in each of the top 12 banana-producing states, covering the major bananas grown (AAA, AAB, and ABB) in these respective states, as presented in Table 1.

A banana plant produces fruits only once during its lifespan. But new stalks are continuously produced from each plant. The fruit is harvested around 9 to 11 months after planting; then, the new stalks produce fruits every 3 to 4 months (Elbehri *et al.* 2015). The selected gardens are commercial-scale monocropping production systems with field histories of banana production spanning more than 20 years. We estimated the annual biomass production per plant and did not include the suckers that go to the next crop cycle.

An extensive survey was conducted in these regions to record allometric data. In each state,

100 gardens at the harvest stage were randomly sampled to obtain a fairly representative sample of the gardens in these states. Given the variability in plant population and yield per hectare across different cultivated varieties, the determination of state-specific plant population per hectare for banana cultivation was guided by productivity considerations. Specifically, states exhibiting productivity levels exceeding 30 tonnes (primarily falling within the AAA group) were assigned a plant population of 4440 plants per hectare, while states with productivity below 30 tonnes per hectare (mainly within the AAB and ABB groups) were allocated a plant population of 2267 plants per hectare. Flowers, flower bracts, and flower stock constitute about This value was used to compute the carbon contribution from this component. Weed biomass in banana gardens is very low, so this parameter was not included in this study.

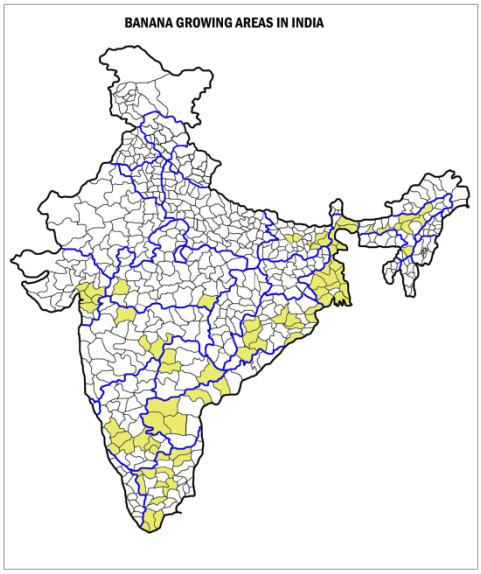

Fig 1 District boundaries of major Banana growing areas in India (Map not to scale)

Table 1 Banana growing States and districts with varieties, area, production and productivity.

State	Districts	Varieties	Area, 000 ha (% share)	Production 000 Mt (% share)	Productivity t/ha
Andhra Pradesh &Telangana	East Godavari, West Godavari, Kurnool, Cuddapah	Dwarf Cavendish, Robusta, Rasthali, Amritpant, Thellachakrakeli, Karpoora Poovan, Chakrakeli, Monthan and Yenagu Bontha	91.25 (10.33%)	5093.10 (16.53%)	56.24
Assam	Goalpara, Nagaon, Sonitpur, foothills of Garo hills	Jahaji (Dwarf Cavendish), Chini Champa, Malbhog, Borjahaji (Robusta), Honda, Manjahaji, Chinia (Manohar), Kanchkol, Bhimkol, Jatikol, Digjowa, Kulpait, Bharat Moni	53.08 (6.00%)	913.27 (2.96%)	17.20
Bihar	Vaishali, Katihar, Kishanganj, Bhagalpur (Naugachia) and Purnia.	Dwarf Cavendish, Alpon, Chinia , Chini Champa, Malbhig, Muthia, Kothia , Gauria	31.07 (3.51%)	1396.39 (4.53%)	44.94
Gujarat	Surat, Vadodara, Anand, Kheda, Junagadh, Narmada, Bharuch	Dwarf Cavendish, Lacatan, Harichal (Lokhandi), Gandevi Selection, Basrai, Robusta, G-9, Harichal, Shrimati	68.15 (7.71%)	4472.32 (14.51%)	65.63
Jharkhand	Ranchi, Sahebganj	Basrai, Singapuri	9.17 (1.03%)	32.06 (0.10%)	3.49
Karnataka	Bangalore, Chitradurga, Shioroga, Hassan, Chikka Mangloor	Dwarf Cavendish, Robusta, Rasthali, Poovan, Monthan, Elakkibale	110.55 (12.50%)	2328.90 (7.56%)	21.07

Table 1 (Continued)

Kerala	Thiruvananthapuram, Kollam, Pathanamthitta, Alappuzha, Kottayam, Idukki, Ernakulam, Thrissur, Palakkad, Malappuram, Kozhikode, Wynadu, Kannur, Kasargod	Nendran (Plantain), Palayankodan (Poovan), Rasthali, Monthan, Red Banana, Robusta	109.26 (12.36%)	1119.16 (3.63%)	10.24
Madhya Pradesh	Khandwa, Badwani, Khargaon, Dhar	Basrai, Grand Naine, Robusta,	26.38 (2.98%)	1834.03 (5.95%)	69.54
Maharashtra	Jalgaon, Ahmednagar, Buldhana, Pune, Wardha, Dhule, Nanded, Parbani, Nandurbar, Satara, Sangli, Osmanabad, Buldhana, Akola, Yeothmal, Amravati, Thane, Kulara, Alibag	Dwarf Cavendish, Basrai, Robusta, Lal Velchi, Safed Velchi, Rajeli Nendran, Grand Naine, Shreemanti, Red Banana	80.88 (9.15%)	4209.27 (13.66%)	52.05
Orissa	Ganjam, Puri, Khurda, Gajpati, Cuttack, Dhenkanal, Angul, Sundargarh, Sambalpur, Bargarh, Deogarh, Koraput, Keonjhar, Raygada, Mayurbhanj	Dwarf Cavendish, Robusta, Champa, Patkapura (Rasthali)	24.20 (2.74%)	449.82 (1.46%)	18.59
Tamil Nadu	Thoothukudi, Tiruchirapalli, Coimbatore, Tirunelveli, Karur, Erode, Kanniyakumari	Virupakshi, Robusta, Rad Banana, Poovan, Rasthali, Nendran, Monthan, Karpuravalli, Sakkai, Peyan, Matti	82.63 (9.35%)	3205.04 (10.40%)	38.79
West Bengal	Hooghly, Nadia, North 24 Parganas	Champa, Mortman , Dwarf Cavendish, Giant Governor, Kanthali, Singapuri	49.30 (5.57%)	1200.00 (3.89%)	24.34
Other states			150.14 (28.77%)	4464.12 (14.49)	29.73
All India			883.77ha	30807.5	34.86

Source: DAC & FW, 2018.

Ganeshamurthy (2023) developed a universal banana allometric equation for nondestructive estimation of the above-ground biomass (AGB) of a standing banana crop at the harvest stage. This involved measuring the volume of the pseudostem as the allometric parameter. Briefly, the allometric equation was developed through destructive sampling of AAA, AAB, and ABB groups at the harvest stage. Allometric parameters such as plant height, diameter of the stem at the base, diameter of the stem at breast height, and volume of the pseudostem were measured. Different statistical models, like linear, exponential, polynomial, and power models, were used to estimate the plant biomass of individual and combined groups of bananas. Based on the best fit, the power model with pseudostem volume as an allometric parameter was used for the estimation of plant biomass (Ganeshamurthy, 2023). The below-ground biomass was estimated using the shoot-to-root ratio of 0.235 suggested by Ganeshamurthy (2023). We used the allometric equation given below for the estimation of banana biomass for computing the CS.

AAA group: Y=0.008X0.513
AAB group: Y=4E-06X1.233
ABB group: Y=4E-06X1.22

Measurement of pseudostem volume

The diameter of the stem at the base above the ground and below the bunch was measured with the help of a Vernier caliper. The pseudostem volume was calculated by multiplying the stem diameter at the base by the diameter below the bunch, as follows:

$$V = 1/3 \times \pi h(R^2 + Rr + r^2)$$

Where:
R = radius at the stem's base

r = radius beneath the bunch such that R > r
h = height from the base of the plant to bunch level expressed in cubic centimeters

Carbon estimation

The carbon content of these plant samples was estimated by using a CHNS analyzer (Elementar) and expressed as a percentage of carbon in the sample.

Soil carbon stocks

Getting representative data on the soil carbon stock of a region is a difficult task. Arriving at a state-wise average SC stock was practically impossible because the data is not available for a political boundary-based average SC as no such effort has been made in India (Ganeshamurthy *et al.* 2019). Further, the available resources were generated from agricultural lands, mainly cultivating annual crops, and hence do not represent a perennial crop system. The Forest Survey of India has made efforts to generate state-wise soil carbon stocks of forests, and the latest data was published in 2017. By and large, banana gardens imitate a disturbed forest ecosystem. Therefore, we used this data to calculate the state average values for SC. The method adopted by FSI is similar to those normally adopted to estimate soil carbon stocks. Briefly, the method followed by FSI for collecting the data on SC is given here.

RESULTS AND DISCUSSION

The volume of the banana pseudostem, the basic growth parameter used for calculating the AGB, varied from state to state (Table 2). This depended mainly on the type of bananas cultivated in these states. The data obtained from Assam, Kerala, Orissa, Tamil Nadu, and West Bengal was above. In Andhra Pradesh, Karnataka, and Telangana states, the recorded pseudostem volume ranged between 50000 and 60000 cm^3. The lowest volumes below 50000 cm^3 were recorded in other states. This variation in the data is mainly accounted for by the type of bananas grown in these states and the climate and management practices adopted by farmers in these regions. Among the states, bananas in Gujarat recorded the lowest mean pseudostem volume (46576 cm^3), and Assam recorded the highest mean value (62731 cm^3). Stevens et al. (2020) reported similar pseudostem volumes for banana plants producing about 2–3 kg of above-ground biomass for two contrasting cultivars.

Table 2 Mean allometric parameters (Vpseudostem, cm^3) and tree carbon sequestered in banana gardens in India

States/UT	Range	Mean	SD	Median	Q2	Q3
Andhra Pradesh & Telangana	45872-63324	54490	5227	54236	52117	56428
Assam	53450-73419	62731	5714	63726	59442	66885
Bihar	32089-63127	45763	1204	45341	41287	47220
Gujarat	31680-62885	46576	1218	46550	42824	49729
Jharkhand	31821-62231	44563	1320	48035	45176	51022
Karnataka	45889-63267	54602	5281	54493	50668	57260
Kerala	53244-73427	62636	5216	63288	59117	67335
Madhya Pradesh	31380-62859	47706	3165	47360	46819	49715
Maharashtra	31416-62865	47631	3052	47256	46358	49021
Orissa	53425-73376	62822	5798	63284	59776	68221
Tamil Nadu	53351-73396	62831	5845	63349	61264	65108
West Bengal	53452-73417	62960	5802	62826	59871	64332
Other states	45927-63316	54721	5108	54774	52285	57064
All India	31380-73427	54618	4150	52268	41220	68376
	Mean of 100 plants					

Danarto and Hapsari (2015) reported that cultivars of banana with "B" genomes (AAB and ABB cultivars) are more vigorous and contribute higher biomass and C-stock than the cultivars having only "A" genomes (AAA group). Our results supported this argument. States like Maharashtra, Andhra Pradesh, Telangana, and Madhya Pradesh, which grow mainly Cavendish bananas (AAA), recorded lower AGB (2.5 kg/plant) and BGB (0.550 kg/plant). Whereas other states cultivating varieties belonging to the AAB and ABB groups recorded higher AGB (>2.5 kg/plant) and BGB (>0.550 kg/plant). Nyombi *et al.* (2009) reported a plant dry weight of 1.44 kg/plant for the pseudostem alone for a similar variety of banana in East Africa. Ortiz-Ulloa *et al.* (2020) also reported similar values for bananas grown in two different regions. Stevens *et al.* (2020) reported similar AGB for two contrasting cultivars. Our values are comparable to those reported in the literature.

The mean carbon content in the pseudostem was 0.465%, and that of the root and corm was 0.471%. The carbon capture in the AGB and BGB was then computed using these values (Table 3). The AGB carbon captured by bananas varied from the lowest at 0.920 kg/plant in Jharkhand to the highest at 1.395 kg/plant, and the BGB carbon varied from the lowest at 0.218 kg/plant in Jharkhand to the highest at 0.332 kg/plant in West Bengal. It is reported that on a per-plant basis, the ABB group sequestered higher carbon, followed by the AAB group, and the least in the AAA group (Nyombi *et al.* 2009; Ortiz-Ulloa *et al.* 2020). Andhra Pradesh, Gujarat, Jharkhand, Madhya Pradesh, Maharashtra, and Telangana mainly cultivate Cavendish-type (AAA group) bananas. Hence, the carbon capture per plant in these states is lower. In Assam, Karnataka, Kerala, Orissa, Tamil Nadu, and West Bengal, we mainly cultivate both AAB and ABB group bananas, capturing the least carbon per plant.

Total carbon capture by bananas per ha depends on the plant population per ha and carbon capture per plant. In carbon capture, two distinct groups can be seen in different states. The differences among states are mainly accounted for by the types of bananas cultivated in these states. The data on total carbon captured per ha (Table 4) obtained from Andhra Pradesh, Bihar, Gujarat, Madhya Pradesh, and Maharashtra (where mainly AAA group bananas are cultivated) were above 5 t/ha. In Assam, Jharkhand, Karnataka, Kerala, Orissa, Tamil Nadu, and West Bengal states (where mainly the AAB and ABB group bananas are cultivated), the recorded carbon capture was below 5 t/ha. This shows that, on a per-ha basis, the AAA group bananas capture more carbon than the AAB and ABB group bananas. It means that all those states cultivating AAA group bananas sequester about 1.3 to 1.5 fold more carbon per hectare than other states growing AAB and ABB group bananas. Ortiz-Ulloa *et al.* (2020) reported that CS by banana in four provinces in Ecuador ranged from 4.18 t/ha to 5.44 t/ha. They accounted for this variability in CS in the plant population. In our study, too, the differences in CS in different states growing different groups of bananas were

Table 3 Biomass and carbon sequestered in banana gardens in India(Mean of 100 plants)

States/UT	AGB, Kg/plant	Major Banana group	BGB Kg/plant	AGB C, Kg/ plant	BGB C, Kg/ plant	Total C, Kg / plant	Total C, t/ha
Andhra Pradesh & Telangana	2.507	AAA	0.589	1.166	0.277	1.443	6.407
Assam	2.994	AAB&ABB	0.704	1.392	0.331	1.724	3.908
Bihar	2.025	AAA	0.476	0.942	0.224	1.166	5.177
Gujarat	2.061	AAA	0.484	0.958	0.228	1.186	5.265
Jharkhand	1.972	AAB&ABB	0.463	0.920	0.218	1.135	2.573
Karnataka	2.512	AAB&ABB	0.590	1.168	0.278	1.446	3.278
Kerala	2.989	AAB&ABB	0.702	1.390	0.331	1.721	3.901
Madhya Pradesh	2.110	AAA	0.496	0.981	0.235	1.215	5.394
Maharashtra	2.108	AAA	0.495	0.980	0.233	1.214	5.390
Orissa	2.998	AAB&ABB	0.705	1.394	0.332	1.726	3.912
Tamil Nadu	2.998	AAA	0.705	1.394	0.332	1.726	3.912
West Bengal	3.000	AAB&ABB	0.705	1.395	0.332	1.727	3.915
Other states	2.517	AAB&ABB	0.591	1.170	0.279	1.449	3.284
All India	2.522		0.593	1.173	0.279	1.452	4.332

mainly due to differences in the plant population per ha. AAA bananas are planted closely, accommodating 4444 plants per ha. Whereas the AAB and ABB group bananas are planted with wider spacing, accommodating 2267 plants per ha. Edible banana cultivars have lower biomass and C-stock values than wild bananas. Danarto and Hapsari (2015) reported a CS of 9.7 kg/plant in Klutuk Wulung, an AAA variety, and 4.74 kg/plant in another variety, Klutuk Ijo, that belongs to the ABB group. These data support the finding that AAA produces lower CS than ABB. However, their values are higher because these varieties are from backyard gardens where solitary plants have grown very robustly. Ours are commercial gardens with high-density planting. Hence, our values reported here are lower than those reported by Danarto and Hapsari (2015). Our values fairly match those reported by Ortiz-Ulloa *et al.* (2020) and Stevens et al. (2020) from commercial gardens.

Soil carbon

Organic C in the soil fluctuates based on the canopy cover over the surface. Quasi-equilibrium (QEV) of SOC is attained over a long period of time, varying from 500 to 1000 years in a forest system. In the agriculture system, this is attained in about 30–50 years after land use change from forest to agriculture and in 30 years for the horticultural system (Arrouays *et al.* 1995; Batjes 2001; Dickson and Crocker 1953; Jenny 1950; Johnson 1995; Naitam and Bhattachaarya 2004). During the process of stabilisation, the SOC shows tooth-like cycles of accumulation and loss during the beginning years and slowly attains an equilibrium level after the accumulation of dry matter and loss of SOC over time. It has been shown that under these tropical land uses, horticultural systems attain QEV in about 25 years (Ganeshamurthy 2012). All the selected banana gardens in this study had a history of banana cultivation spanning more than 20 years. Therefore, the soils under these gardens might have attained the QEV stage after the accumulation of dry matter and loss of SOC over time. As mentioned, it was difficult to obtain representative state averages of soil C stocks under banana gardens. Published information is mainly restricted to agricultural ecosystems and very few to horticultural ecosystems. Since state-wise SOC stock information was available from forest ecosystems and since sites under continuous banana gardens represented forest ecosystems more closely, we used the available data for computing C stocks by banana gardens. The status of SOCs in Karnataka and Kerala is relatively higher because of the favourable climate. Whereas the plant carbon content in Andhra Pradesh and Telangana is higher because of the bright sunshine hours prevailing in these states relative to other banana-growing states.

Table 4 Soil and plant carbon pools in banana gardens in India

States/UT	Soil C t/ha	Plant carbon, t/ha	Area (000 ha)	Total soil carbon stock 1000 tons	Total plant carbon stock 1000 tons	Total CS in Banana gardens,mt
Andhra Pradesh & Telangana	42.09	6.407	91.25	3840.713	584.639	4.425
Assam	39.98	3.908	53.08	2122.138	207.437	2.330
Bihar	39.55	5.177	31.07	1228.819	160.849	1.390
Gujarat	44.04	5.265	68.15	3001.326	358.810	3.360
Jharkhand	43.29	2.573	9.17	396.969	23.594	0.421
Karnataka	77.14	3.278	110.55	8527.827	362.383	8.890
Kerala	75.77	3.901	109.26	8278.630	426.223	8.705
Madhya Pradesh	41.17	5.394	26.38	1086.065	142.294	1.228
Maharashtra	57.23	5.390	80.88	4628.762	435.943	5.065
Orissa	46.50	3.912	24.2	1125.300	94.670	1.220
Tamil Nadu	41.64	3.912	82.63	3440.713	323.249	3.764
West Bengal	59.88	3.915	49.3	2952.084	193.010	3.145
Other states	50.69	3.284	150.14	7610.597	493.060	8.104
All India	50.69	4.332	883.77	44798.301	3828.492	48.627
% contribution to the total CS				91.714	8.286	100.000

Total C sequestered in orchards

The soil and plant carbon pools in banana gardens are presented in Table 5. The soil carbon pool is very large compared to the plant carbon pool. It contributed more than 86% of the total CS in banana gardens. The mean contribution of SOC varied from 86.8% in Andhra Pradesh to 95.9% in Jharkhand. At the national level, the SOC contributed 91.71% to the total CS.

The plant carbon pool, on the other hand, is very small compared to the soil carbon pool. The plant carbon pool contributed less than 15% in all the states. The lowest contribution from plant C to the total CS was recorded in Karnataka (4.08%) and Kerala (4.90%), and the highest contribution was recorded in Andhra Pradesh (13.21%) and Madhya Pradesh (11.58%). At the national level, Plant C contributed 8.29% to the total CS.

The carbon content of banana plants accounted for only 8.286 percent of the total CS in banana gardens. The contribution of each state to the total carbon sequestered by banana plants alone depended upon the area and varieties of bananas under cultivation in these states and the quantity of SOC. Andhra Pradesh with the highest area under banana (91.25 thousand ha) cultivating mainly the AAA group bananas sequestered 584639 metric tonnes of carbon, whereas Jharkhand with the lowest area under banana (9.17 thousand ha) cultivating mainly the AAB and ABB group bananas sequestered the lowest plant carbon (23594 metric tonnes). On an all-India basis, the total plant carbon sequestered accounted for 3828492 metric tonnes. The study clearly indicated that under banana gardens, SC is the major C pool, accounting for 91.7%, and plant carbon is only a small portion, accounting for 8.29% of the total CS.

Kamusingize et al. (2017) reported that the SOC contribution to total CS under bananas exceeded 90% in Ugandan banana gardens. In their study, the total SOC stock beneath all cultivars was considerably high, ranging from 81 to 92 mg ha^{1}. The plant carbon in these studies was found to be very small, ranging between 0.37 and 1.64 mg ha^{1}. Danarto and Hapsari (2015) reported that, on average, various Indonesian bananas captured around 2.26 kg (or 0.98 tonne) per hectare. Thus, banana crops enrich soil by investing carbon into the soil through huge root biomass and over time during photosynthesis as carbon moves from the vegetative canopy into the soil (Turner, 2003; Hairiah et al., 2010). Published information on SOC in banana gardens in India is not available for a fair comparison. Therefore, it can only be made from the data published on other fruit crop systems such as mango, sapota, guava, and forest systems. Gupta (2011) reported that the soil C stock was 41 tonne ha^{1} at the surface and 50 cm deep. Chabra *et al.* (2003) also reported that the soil C sequestered in Indian forest soils ranged from 37.5 tonne ha^{1} in tropical dry deciduous forests to 92.1 tonne ha1 in littoral swamp forests. Our values are for 100-cm-depth soil profiles and are fairly similar to those reported in the literature for different regions. The banana cropping system enables much more carbon to be stored in the soil, despite the fact that banana cultivars contain small average amounts of plant carbon stocks. Kamusingize *et al.* (2017) and Danarto and Hapsari (2015), in their study, reported that the proportion of carbon contained in the plant to that in the soil across all cultivars was in the range of 0.4–2%. Large soil carbon stocks in banana cropping systems could perhaps be attributed to the sustainable agricultural land management practices employed by farmers, such as mulching, the use of trenches to minimise erosion, and minimal and no tillage (Lal 2011, Paswel *et al.* 2012, Joris *et al.* 2013). Investing in the proper management of banana plantations is invaluable for contributing to SOC as a major carbon pool in banana systems.

The crop demography in India is changing fast towards perennial horticulture. This results in land-use change in the region. Therefore, we must examine the related changes in C fluxes derived from such land-use change patterns in different regions to formulate viable strategies for climate change mitigation. The present study generated unique information on banana gardens in India. This involved a comprehensive effort in the integration of different methodologies for field work and data processing. But on a national scale, there is a need to generate information on all such perennial horticulture systems, such as other fruit crop orchards, coffee and tea estates, and plantations in India, with larger sample sizes to be able to determine the percentage of carbon sequestered in perennial horticultural crops in the country as a whole.

The land-use change patterns are occurring mainly on prime agricultural lands due to increased demand for bananas. Bihar, Jharkhand, Odisha, Madhya Pradesh, and Chhattisgarh have large tracts of tribal land. These regions are suitable for banana cultivation. Such regions are to be encouraged for productive banana cultivation. Similar efforts may be made in other states as well, which reasonably imitate forests and sequester carbon in similar quantities and can augment climate change risks. The information generated in this study may be used by the administrators in these regions to claim carbon credits to benefit the farmers and the local population.

CONCLUSIONS

This study showed a significant difference in total plant carbon stock across different cultivars and states in India. There was a significant difference between groups of bananas in their ability to sequester carbon. Soil carbon stocks were very high,ranging from 39.55 to 6.407 t/ha. But plant carbon stocks were found to be very small,ranging from 2.573 to 6.407 t/ha. In all banana-cultivating states, the proportion of carbon contained in the plant to that in the soil was only 8.286 percent. Soil carbon accounted for the majority of the total CS, accounting for 91.714%. Despite these small amounts of plant carbon, the banana cropping system enriches the soil by enabling much more carbon to be sequestered into the soil. Apart from augmenting fruit production, expanding the area under banana farming systems helps to rehabilitate the landscape, decrease carbon emissions in the form of biomass and carbon stocks, and meet the economic needs of the region.

REFERENCES

Arrouays D, Isabelle V, Luckicin J. 1995. Spatial analysis and modelingof top soil carbon storage in temperate forest humic loamy soil ofFrance. Soil Science. 159, 191-198.

Batjes N.H. 2001.Options for increasing carbon sequestration in West African soils: An exploratory study with special focus on Senegal. Land Degradation and Development. 12, 131-142.

Charrier A, Jacquot M, Hamon S, Nicolas D. 1997. *L'amélioration des plantes tropicales*. Montpellier (FRA) ; Paris : CIRAD ; ORSTOM, 624 p. (Repères). ISBN 2-87614-292-9. ISSN 1251-7224.

Chhabra A, Palria S, Dadhwal A. K. 2003. Soil organic carbon pools in Indian forests. For. Ecol. Manage. 2003. 173: 187-199.

DAC & FW 2018. Horticultural Statistics at a Glance 2018, Horticulture Statistics Division, Department of Agriculture, Cooperation & Farmers Welfare (DAC&FW), Ministry of Agriculture & Farmers Welfare, Government of India.

Danarto S.A, Hapsari L. 2017. Biomass and carbon stock estimation inventory of indonesian bananas (musa spp.) and its potential role for land rehabilitation. Biotropia. 22: 102-108.

Dickson B.A, Crocker R.L. 1953. A chronosequence of soils andvegetation near Mt. Shasta, California, I and II. Soil Science. 4: 142-154.

Elbehri A, Calberto G, Staver C, Hospido A.2016. *Ecuador's Banana Sector Under Climate Change*. Food and Agriculture Organization of the United Nations. http://www.fao.org/3/a-i5697e.pdf

FAO. 2018. *Reducing Carbon and Water Footprints in Banana Plantations*. Food and Agriculture Organization of the United Nations. http://www.fao.org/world-banana-forum/projects/reducing-carbon-and-water-footprints-in-banana-plantations/en/

FAO. 2022. Banana: 2022. https://www.fao.org/markets-and-trade/commodities/bananas/en/#:~:text=More%20than%201%20000%20varieties,volume%20of%2050%20million%20tonnes

FSI, 2017. Carbon stocks in India's forests. In India State of Forest Report 2017 Chapter 8.0, Forest Survey of India.

Ganeshamurthy 2012. Annual Report, ICAR-Indian Institute of Horticultural Research, Bengaluru

Ganeshamurthy A.N, Ravindra V, Rupa T.R, Bhat R.M. 2019.Carbon Sequestration Potential of Mango Orchards in India Current Sci. 117(12): 2006-2013.

Gupta M. K.2011. Soil organic carbon pools under different land use in Haridwar district of Uttarakhand. Indian For., 137: 1-8. 28.

Hairiah K, Dewi S, Agus F, Velarde S, Ekadinata A, Rahayu S, Van N.M. 2010. Measuring carbon stocks across land use systems: A Manual, Bogor, Indonesia. World Agroforestry Centre (ICRAF), SEA Regional Office. 155p.

Jenny H. 1950. Causes of high nitrogen and organic matter content ofcertain tropical forest soils. Soil Science. 69: 63-69.

Johnson M.G. 1995. The role of soil management in sequestering soilcarbon. In 'Soil management and greenhouse effects'. (Eds R Lal,JM Kimble, RF

Follet, BA Stewart) pp. 351–363. (Lewis Publishers,Boca Raton, FL).

Joris A, Leo D.N, Anne G. 2013. Valuing the carbon sequestration potential for European agriculture. Land Use Policy 31: 584-594.

Kamusingize D, Mwanjalolo J.M, KomutungaE, Tumwebaze S, NowakundaK, Namanya P Kamu J.K. 2017. Carbon sequestration potential of East African Highland Banana cultivars (Musa spp. AAA-EAHB) cv. Kibuzi, Nakitembe, Enyeru and Nakinyika in Uganda. Journal of Soil Science and Environmental Management. 8: 44-51.

Lal R. 2011. Sequestering carbon in soils of agro-ecosystems. Food Policy 36:S33-S39. De Langhe E. 2000. Diversity in the genus Musa, its significance and its potential. ActaHort. 540: 81-86.

Naitam, R and Bhattachaarya, T. Quasi-equilibrium of organic carbon in shrink–swell soilsof the subhumid tropics in India under forest, horticulture, andagricultural systems. Australian Journal of Soil Research. 2004. 42, 181-188

Nkoulou L.F.M, a b f, Ninla L.A.T, Cros,D, Martin G, Ndiang Z, Houegban J, Ngalle H.B, Bell J.M, Achigan-Dako E.G. 2023. Analysis of genetic diversity and agronomic variation in banana sub-populations for genomic selection under drought stress in southern Benin. Gene. 859, 147210

Nyombi K, Van Asten P.J.A, Leffelaar P.A, Corbeels M, Kaizzi C.K, Giller K.E. 2009. Allometric growth relationships of East Africa highland bananas (Musa AAA-EAHB) cv. Kisansa and Mbwazirume. Ann. Appl. Biol. 155: 403-418.

Oliveira C.D, Donato S.L.R, Mizobutsi G.P, Silva J.D, Mizobutsi E.H. 2013. Características pós-colheita de bananas 'Prata-Anã'e' BRS Platina'armazenadas sob refrigeração. Revista Brasileira Fruticultura. 35(3): 891-897.

Ortiz-UlloaJ.A, Abril-Gonzalez M.F, Pelaez-Samaniego M.R, Silvana T. 2020. Biomass yield and carbon a batement potential of banana crops (*Musa* spp.) in Ecuador. Environmental Science and Pollution Research. https://doi.org/10.1007/sl1356-020-09755-4.

Paswel M, Ephraim N, Wei X, Jose D, Edward K. 2012. Which policy would work better for improved soil fertility management in subSaharan Africa, fertilizer subsidies or carbon credits? Agric. Syst. 110: 162-172.

Ravi I, Uma S, Vaganana M, Mustafa M. 2013. Phenotyping bananas for drought resistance. Frontiers in Physiology 4(9): 1-14.

Reay D.2019. Climate-Smart Food. eBook.https://doi.org/10.1007/978-3-030-18206-9.

Saranya S, Aneesa Rani M.S, Raveendran M, Kalaiyarasi R and Auxcilia J. 2021. Molecular characterization of diploid and triploid banana germplasms through DNA finger printing using ISSR markers.The Pharma Innovation Journal 2021; 10(10): 1876-1882.

Soraisham L.D, Gogoi N, Mishra L, Basu G. 2021. Extraction and Evaluation of Properties of Indian Banana Fibre (Musa Domestica Var. Balbisiana, BB Group) and Its Processing with Ramie. J.Natural Fibewrs. https://www.researchgate.net/deref/https%3A%2F%2Fwww.tandfonline.com%2Floi%2Fwjnf20

Souza E.L, Sellin N, Marangoni C, Souza O. 2010. The Influence of Different Strategies for the Saccharification of the Banana Plant Pseudostem and the Detoxification of Concentrated Brothon Bioethanol Production. Appl Biochem Biotechnol. 183: 943-965.

Statista 2022. Volume of banana produced across India from financial year 2015 to 2021, with an estimate for 2022. https://www.statista.com/statistics/1038905/india-production-of-banana/

Stevens B, Diels J, Brown A, Bayo S, Ndakidemi P.A, Swennen R. 2020. Banana Biomass Estimation and Yield Forecasting from Non-Destructive Measurements for Two Contrasting Cultivars and Water Regimes. Agronomy 10: 1435.

Turner D.W. 2003. Factors affecting the physiology of the banana root system. In: Turner DW, Rosales FE (Eds) Banana Root System: Towards a better understanding for its productive management. Proceedings of an international symposium held in San Jose, Costa Rica. pp. 107-113.

Tutwiler M.A, Bailey A, Attwood S, Remans R, Ramirez M. 2017. Agricultural biodiversity and food system sustainability Transformation. In book: Mainstreaming Agrobiodiversity in Sustainable Food Systems.

UNFCC 2020.The clean development mechanism. Mechanisms under the Kyoto Protocol, In https://unfccc.int/process-and-meetings/the-kyoto-protoco1/mechanisms-under-the-kyoto-protocoI/th e-cIean- development-mechanism.

WRAP. 2013. Household food and drink waste in the United Kingdom 2012. Waste and Resource Action Programme. http://www.wrap.org.uk/sites/files/wrap/ hhfdw-2012-main.pdf.pdf .

BIOTROPIA Vol. 30 No. 3, 2023: 384 - 394 DOI: 10.11598/btb.2023.30.3.2027

EVALUATION OF METHODS FOR TOTAL RNA EXTRACTION FROM THE ENDOSPERM OF COCOS NUCIFERA VAR. MAKAPUNO IN VIETNAM FOR MOLECULAR ANALYSIS

THANH-DIEM NGUYEN AND HUYEN-TRANG VU*

[1]*Faculty of Biotechnology, Nguyen Tat Thanh University, 298A-300A Nguyen Tat Thanh Street, Ward 13, District 4, Ho Chi Minh 72820, Vietnam*

Received 25 May 2023 / Revised 15 August 2023 / Accepted 15 August 2023

ABSTRACT

Sap coconut (*Cocos nucifera* L. var. *makapuno*) in Vietnam is a mutant coconut variant; coconut water is in the state of lotus glue, and coconut rice is like cream. For high-quality transcriptome, sequencing, quality, purity and concentration of RNA are the key factors. However, coconut endosperm tissue has higher stiffness and fatness than the leaf tissue, which complicates the extraction process. Moreover, RNA is much more difficult to preserve than DNA. In this study, various RNA extraction methods were examined in Vietnamese waxy coconut endosperm tissue samples. Optimum extraction and preservation of the RNA using the simplest possible chemicals was the objective of this study. The modified CTAB method with LiCl and the TRI reagent method were tested and evaluated. The purity, concentration and quality of RNA after storage were improved. The findings indicated that the TRIsure extraction method with the addition of NaCl and β-mercaptoethanol yielded optimum RNA quality. The RNA concentration was 159 ng/μL, with a purity ratio of 1.94 ± 0.04 for A260/A280 and 1.58 ± 0.02 for A260/230. RNA samples remained stable for up to 3 weeks when stored in absolute ethanol at 8°C–10°C, which significantly reduced their degradation during transportation. This study facilitated the use of simple chemicals for high-quality RNA extraction from coconut endosperm and its preservation for applications in high throughput sequencing.

Keywords: *Cocos nucifera var. makapuno*, extraction method, total RNA

INTRODUCTION

Coconut (*Cocos nucifera* L.) is a tree with high economic value, and coconut water is a beverage that is preferred by many people. Coconut is also an important perennial oil crop around the world. In particular, the Sap coconut variant originating from the Philippines, called *Makapuno coconuts*, is considered a specialty, with the coconut sap in a state of gelatinous creamy paste and high in fat content. Interestingly, there are normal fruits with solid endosperm like other coconut variants, and some fruits contain waxy endosperm on the same coconut tree (Arellano *et al.* 2019).

RNA isolated from tissue samples is important for several research projects. Ribosomal RNA sequencing is often used for the identification, classification, or assessment of biodiversity. Furthermore, messenger RNA (mRNA) is used for gene expression research, novel gene discovery, or gene isoform analysis. Short fragments can be sequenced to assess the expression of certain genes, or whole mRNAs can be encoded for comparative transcriptomics. mRNA is used in reverse transcription PCR (polymerase chain reaction) to synthesise cDNA for sequencing, which helps identify gene variants and is also useful in gene expression studies. In addition, mRNA is used in Northern blotting to analyse gene expression over time or under specific conditions based on the size and amount of mRNA. However, for RNA sequencing, reverse transcription PCR and quantitative real-time PCR, an intact RNA with high purity is essential.

*Corresponding author, email:

Extraction of RNA from coconut endosperm tissue for mRNA transcriptome sequencing helps to evaluate the differences in gene expression between waxy and normal endosperm tissues, thereby aiding in future fruit characterisation studies. However, obtaining high quality RNA from endosperm tissue is more challenging than obtaining it from leaf tissue. The reason is that coconut endosperm possesses numerous polysaccharides, proteins, polyphenols, and lipids that bind to nucleic acids, thereby posing a challenge in the isolation of RNA (Angeles *et al.* 2005, Zhang *et al.* 2022). Moreover, sequencing results are affected owing to inadequate preservation during the transportation process over an extended period. Hence, obtaining of RNA samples that are highly concentrated and pure is of utmost importance in ensuring the success of RNA sequencing and genetic analysis (Gallego Romero *et al.* 2014, Scholes *et al.* 2020).

Several studies have been performed to enhance the RNA separation techniques to facilitate subsequent molecular investigations. Most of these studies were based on guanidine thiocyanate as the main lysate. In 2012, MRIP (Methods for RNA Isolation from Palms) extraction buffer, which contains a series of different components, i.e. ammonium thiocyanate, guanidine thiocyanate, sodium acetate, phenol and glycerol, was first used for RNA isolation from palm leaves. Higher purity and integrity of the RNA molecules were achieved in comparison with CTAB, TRIzol methods and Tiangen RNA plant kit. The electrophoresis results from MRIP showed clear lines of 28S, 18S and 5S rRNA whereas the other techniques did not. Nonetheless, the concentration of the RNA product from each method was not discussed and the study was performed on the leaf only. Subsequently, Iqbal et al. (2019) provided an improved method, QRREM (Quick and Reliable RNA Extraction Method), which was similar to MRIP in all aspects except for the addition of β-mercaptoethanol (2%) and polyvinylpyrrolidone-40 (3%). Intact RNA was efficiently isolated, and high RNA concentration (16.2 μg/80 mg) was obtained from stored coconut endosperm compared with CTAB (0.1), TRIzol (10.4) and RNA plant kit (0.5). This method had many advantages over other methods in terms of time and cost efficiency (Iqbal *et al.* 2019). Later, in 2020, the research group of Iqbal *et al.* proposed another improved RNA extraction method, IRCM (Isolation of RNA from Complex Matrices), by halving the concentrations of β-mercaptoethanol (1%) and polyvinylpyrrolidone-40 (1.5%) in comparison with QRREM. The protocol was applied for coconut endosperm, coconut mortise and coconut buds, and the results were higher when compared with CTAB, TRIzol and plant RNA extraction kit (Iqbal *et al.* 2020). In all the above methods, the main lysis component was guanidine thiocyanate. Although it was more effective than CTAB and TRIzol, the components were quite complex and acquiring the products from the suppliers was a time consuming process, which sometimes affected the progress of the project. However, TRI reagents, the major component of which is guanidine thiocyanate, are often accessible from vendors, making them more convenient to purchase.

Contrary to guanidine thiocyanate-based methods, Souza-Perera *et al.* (2018) enhanced the CTAB extraction method for RNA from various tissue samples, such as leaves, inflorescences and primary and secondary roots based on the CTAB extraction protocol for DNA. This study examined the characteristics of grade, zygote embryos, and solid endosperm in mature coconut trees. The authors successfully isolated RNA from coconut endosperm tissue with higher integrity compared with the TRIzol method and without any carbohydrate or protein contamination (Souza-Perera *et al.* 2018). Therefore, various RNA isolation methods should be evaluated in coconut endosperm tissue samples and optimised to obtain high quality RNA. This study aimed to develop a protocol for the extraction, preservation, and transportation of total RNA to minimise RNA quality loss.

MATERIALS AND METHODS

Collection of coconut tissue samples

Throughout the study, the waxy endosperm (WE) sample from coconut was the main target for the investigations, i.e. evaluation of RNA extraction efficiency, purity improvement test, lysis temperature test and RNA preservation test during transportation. Particularly, in the

evaluation of RNA extraction efficiency of the two methods CTAB-LiCl and TRIsure, additional samples of non-waxy endosperm (NWE) tissues and leaf (L) tissues from the same coconut cultivar were used as comparison controls. All samples were collected from Tra Vinh province, Vietnam.

The morphology of coconut shells and skulls of waxy and non-waxy fruits indicated no discernible differences in their shape or coloration (Figure 1). The only external difference between the two types of fruits was the characteristic sound upon shaking. The waxy fruit made a rattling sound owingto its thick, viscous, overdeveloped endosperm. However, this sound may also arise during the growing stages of non-waxy fruit. Hence, precise differentiation is possible only by identifying the endosperm tissues. The endosperm of waxy coconut is pliable and contains colloidal water and a disorganised tissue arrangement. In contrast, non-waxy fruit exhibits high water content and a dense and rigid endosperm.

Therefore, for waxy samples, sterile spoons were used to collect viscous endosperm from the non-cut contact area, which was then promptly transferred to labelled sterile bags. The non-waxy endosperm and the leaf tissue were prepared by partially cutting and cleaning them with distilled water and 70% alcohol. After drying, the samples were aseptically transferred to labelled sterile bags. All pretreated samples were promptly utilised or preserved at −80°C.

Extraction of total RNA

Guanidine thiocyanate and CTAB methods have been extensively evaluated but have shown varying results in different studies. Hence, in this study, these two approaches were tested for high-performance RNA extraction from the coconut endosperm tissue. As the composition of lytic compounds is quite complex in IRCM and QRREM and orders for these chemicals are time consuming, the main lysate guanidine thiocyanate via the TRIsure reagent was used with some improvements. The total RNA extraction methods investigated included CTAB-LiCl (Chang *et al.* 1993, Ghangal *et al.* 2009, White *et al.* 2008) and TRIsure (Thermo Fisher 2010).

After assessing the extraction efficiency, several follow-up investigations were conducted to improve the quality of the RNA product. For enhancing the purity of RNA, the addition of NaCl and β-mercaptoethanol to the lysis process was tested and the RNA washing step was repeated thrice. For increasing the efficiency of tissue resolution, different incubation temperatures (room temperature, 50°C, 55°C and 60°C) were used during the lysis process. As RNA is easily degraded, it was stored under conditions similar to transportation conditions to determine its degradation time. Each treatment was performed in triplicate.

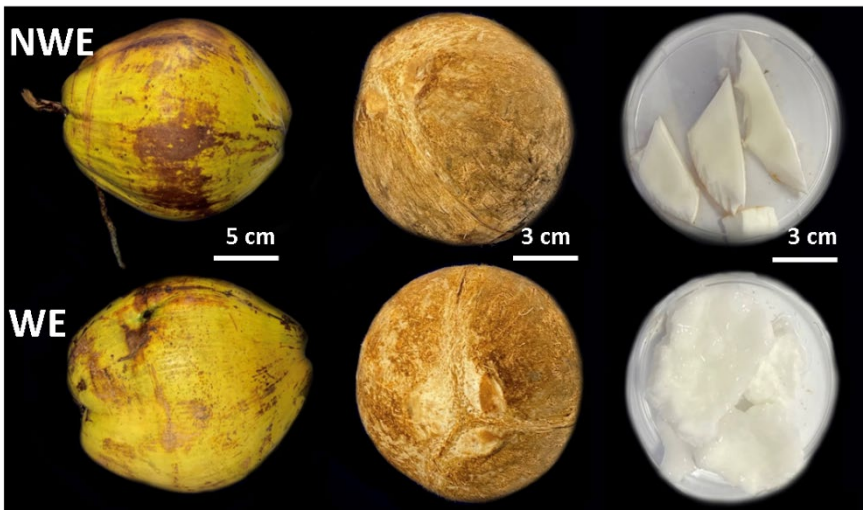

Figure 1 Coconut samples collected in the study
Notes: top row: non-waxy coconut; bottom row: waxy coconut. From left to right: unpeeled fruit; fruit skull, endosperm samples in petri dish.

Investigation of the preservation process under transportation conditions

RNA is inherently unstable and prone to degradation. This experiment aimed to examine the preservation of total RNA post-extraction. Two methods were used to preserve total RNA: (i) Total RNA was stored at −20°C in diethylpyrocarbonate (DEPC)-water after dissolution. (ii) Total RNA was mixed with 0.1× volume of 3M sodium acetate CH_3COONa (pH 5.5) and twice the volume of 100% ethanol after dissolution in DEPC. It is a chemical compound that inactivates RNase, the enzyme that degrades RNA. DEPC is used to preserve RNA integrity in laboratory settings during various experimental procedures. Meanwhile, sodium acetate acts as a co-precipitant in the presence of ethanol. When added to an RNA sample, it neutralises the negative charges on the RNA molecules, facilitating their aggregation and precipitation, which in turn condenses the RNA structure and resists degradation. Both methods are often used for RNA preservation. RNA samples were then stored at −20°C. The examination was performed over a period of 1 month, with weekly testing.

Quality assessment of the extracted RNA

For transcriptome sequencing, the concentration and purity of RNA are of primary concern. RNA purity and total electrophores concentration were checked using the NanoDrop™ - Thermo Fisher Scientific spectrophotometer at wavelengths A260, A280 and A230. For Illumina sequencing, the minimum required weight is 500 ng and concentration should be >10 ng/μL (Hong *et al.* 2020). As for purity, the A260/A280 ratio should be in the range of 1.8–2.0 and A260/A230 should be between 2.0 and 2.2.

Electrophoresis was also used to assess the relative concentration and integrity of RNA bands of different sizes. The extracted RNA samples were electrophoresed on a 2% (w/v) agarose gel together with a DNA scale. However, the size of the mRNA varied greatly depending on the length of the corresponding gene; hence, the number or position of the lanes on the electrophoresis gel was not evaluated. The DNA scale preliminarily assessed the length of the RNA lines that appeared. Brighter the bands, higher the concentration of RNA; clear bands represented intact RNA molecules of certain lengths, whereas smeared bands represented different sizes of RNA, which could be attributed to degradation. To enhance the objectivity while analysing the electrophoresis images, the GelAnalyzer 19.1 software (Lazar Jr. *et al.*) was used in combination.

Statistical analysis

The data obtained from the above experiments were statistically analyzed using the MiniTab 17 software (Minitab Inc. 2020). The data were statistically evaluated using ANOVA with the Turkey algorithm.

RESULTS AND DISCUSSION

Evaluation results of RNA extraction procedures using CTAB-LiCl and TRIsure

The WE sample was used as the main target for investigating the effects of the two extraction methods CTAB-LiCl and TRIsure on the quality of the extracted RNA. In addition, the NWE and L samples from the same plant were included as control samples. In total, three types of tissue samples were used for the two extraction methods. Each treatment was repeated thrice for calculating statistical reliability.

With regard to the total RNA concentration, the results showed the statistical difference between the two methods (P value < 0.05) in all types of tissues. The total RNA concentrations obtained from WE tissue samples were 142.00 ± 2 ng/μL and 157.00 ± 1.7 ng/μL for the CTAB-LiCl and TRIsure methods, respectively (Table 1). In both methods, there was no statistically significant difference between WE and NWE samples. However, both endosperm tissues (statistically classified as B) had significantly lower extraction efficiency than leaf tissues (statistically classified as A), and the difference was significant (P value < 0.05) (Table 1). There was no interaction between the two factors and tissue types [P value > 0.05 (0.422)].

Table 1 Total RNA concentrations extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	142.00 ± 2^{bB}	142.33 ± 1.5^{bB}	155.00 ± 2^{bA}
TRIsure	157.00 ± 1.7^{aB}	156.67 ± 0.5^{aB}	167.67 ± 0.5^{aA}
P value (Extraction method)	0 (<0.05)		
P value (Tissue type)	0 (<0.05)		
P value (two-factor interaction)	0.422 (>0.05)		

Note: Two-way ANOVA analysiswas used to investigate the influenceeffects of different extraction methods (CTAB-LiCl versusvs TRIsure) and different tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on RNA product concentration. The lowercase letters a and b: represent the differences between the extraction methods; the uppercase letters A and B represent the differences between the tissue types. The differences are significant when the P value is <0.05.

As for the OD assessment to determine sample purity, both CTAB-LiCl and TRIsure methods yielded an A260/A280 OD ratio within the optimal range of 1.8–2.0 (Table 2). In contrast, the OD A260/A230 ratio exhibited a distinct disparity. Samples extracted with CTAB-LiCl had a ratio of 1.8–1.9, whereas those extracted with TRIsure had a significantly lower ratio of 0.99–1.02 (Table 3). The optimal purity range for A260/A230 is 2.0–2.2. Macrogen company does not necessitate high-quality samples for sequencing as the issue can be resolved by purifying the sample before sequencing. The index of samples extracted using CTAB-LiCl was acceptable at 1.8–1.9, despite not reaching 2.0–2.2. On the contrary, the A260/A230 ratio of TRIsure-extracted samples fell very much below the standard threshold and should be improved.

Table 2 A260/A280 ratio of samples extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	1.99 ± 0.03^{aA}	1.99 ± 0.01^{aA}	2.00 ± 0.05^{aA}
TRIsure	1.99 ± 0.01^{aA}	2.00 ± 0.03^{aA}	2.01 ± 0.01^{aA}
P value (Extraction method)	0.482 (>0.05)		
P value (Tissue type)	0.526 (>0.05)		
P value (two-factor interaction)	0.928 (>0.05)		

Note: Two-way ANOVA was used to investigate the effects of different extraction methods (CTAB-LiCl vs TRIsure) and tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on the A260/A280 ratio of the RNA product. The lowercase letters a and b represent the difference based on the extraction method; the uppercase letters A and B represent the difference based on the tissue type. The differences are significant when the P value is <0.05.

Table 3 A260/A230 ratio of samples extracted using CTAB-LiCl and TRIsure

Extraction method	Tissue type		
	Waxy endosperm sample (WE)	Non-waxy endosperm sample (NWE)	Leaf sample (L)
CTAB-LiCl	1.87 ± 0.005^{aA}	1.89 ± 0.01^{aA}	1.87 ± 0.02^{aA}
TRIsure	1.01 ± 0.03^{bA}	0.99 ± 0.02^{bA}	1.02 ± 0.06^{bA}
P value (Extraction method)	0.00 (<0.05)		
P value (Tissue type)	0.936 (>0.05)		
P value (two-factor interaction)	1.87 ± 0.02^{aA}		

Note: Two-way ANOVA was used to investigate the effects of different extraction methods (CTAB-LiCl vs TRIsure) and tissue types (waxy endosperm, non-waxy endosperm and leaf tissue) on the A260/A280 ratio of the RNA product. The lowercase letters a and b represent the difference based on the extraction method; the uppercase letters A and B represent the difference based on the tissue type. The differences are significant when the P value is <0.05.

In electrophoretic separation, RNA samples extracted using CTAB-LiCl exhibited limited bands, including two 23S rRNA subunits at approximately 2900 bp, 18S rRNA subunits at approximately 1900 bp and various other bands at approximately 250–500 bp (Thermo Fisher 2006) (Figure 2). On the contrary, electrophoretic separation of RNA samples extracted using the TRIsure method displayed multiple bands in the range of 250–3000 bp in addition to the 23S and 18S rRNA bands, and these bands were more distinct and intense. To evaluate the results of electrophoresis more objectively, a combination of electrophoretic images and the image analysis software Gelanalyzer was used. A significant difference was observed (Figure 3). According to the analysis results, RNA samples extracted using the TRIsure reagent had clearer peaks and were better in terms of quantity than those extracted using CTAB-LiCl (seven peaks vs two peaks).

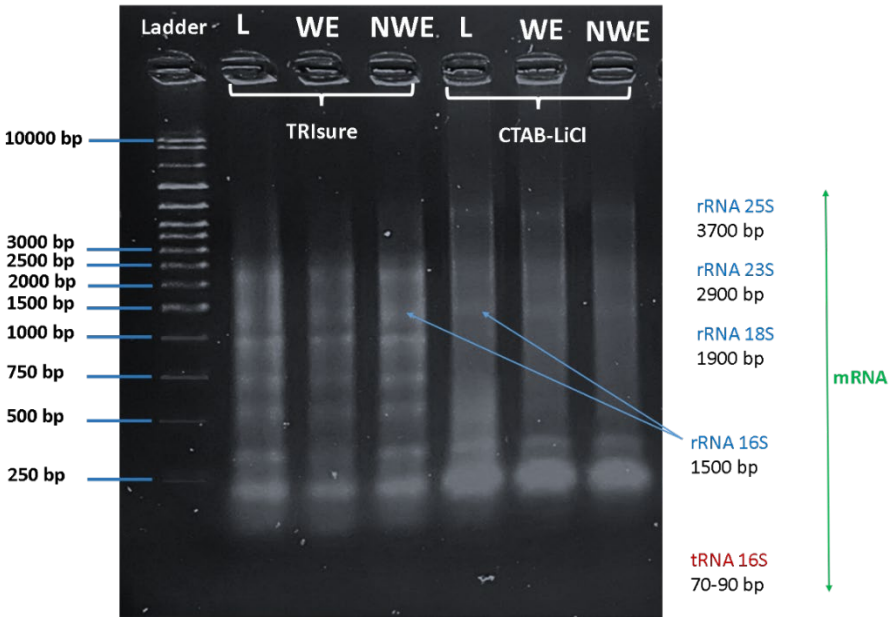

Figure 2 Results of electrophoresis on 2% agarose gel with a scale of 10 kbp; WE: waxy endosperm, NWE: non-waxy endosperm, L: leaf tissue

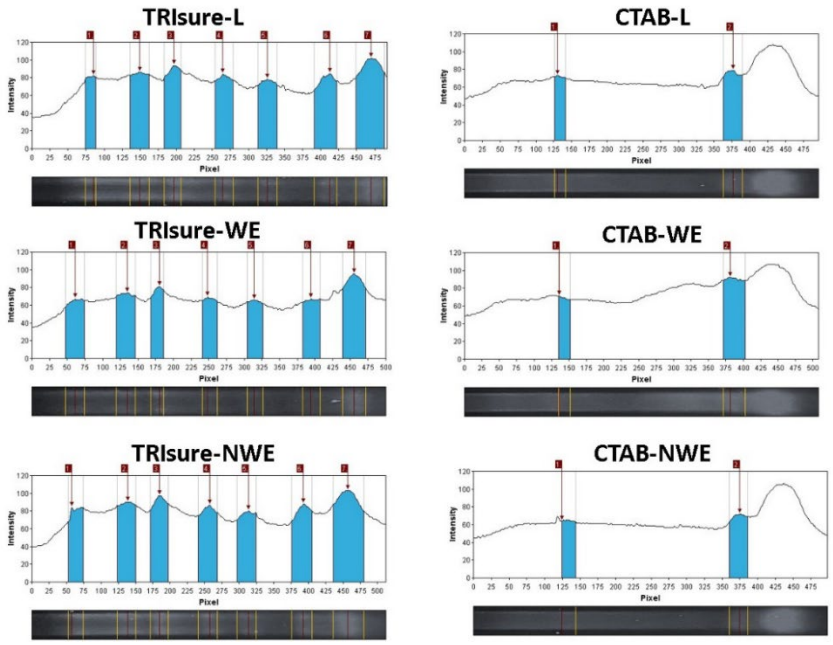

Figure 3 Results of electrophoretic analysis using the GelAnalyzer software
Note: Intensity axis: brightness level based on background; pixel axis: coordinate position on the electrophoresis membrane. The blue area shows the location of the marked RNA bands.

The results indicated that the concentration of total RNA in leaf tissue samples was greater than that in endosperm tissues, specifically in WE and NWE. This finding is consistent with those from previous studies (Iqbal, Yang, Qadri, Wu, Li, Shah, Hamayun & Hussain 2019, Iqbal, Yang, Wu, Li, Hamayun, Hussain & Shah 2020, Souza-Perera *et al.* 2018). Coconut endosperm tissue is hard and has a high lipid content, making it harder to grind than the leaf sample. Furthermore, endosperm is a significant component of seeds and comprises tissues that contain high levels of various polysaccharides (such as starch, cellulose, arabinoxylan, β-glucan, and fructan), proteins, lipids, and secondary metabolites (Angeles, Laurena & Tecson-Mendoza 2005, Ghangal, Raghuvanshi & Chand Sharma 2009, Zhang *et al.* 2019, Liu *et al.* 2018). Most of these components share structural similarities with nucleic acids, including polysaccharides that resemble the ribose sugar of RNA. Consequently, these compounds can bind to RNA and undergo degradation. Low RNA extraction yield was observed because of its encapsulation during centrifugation and its subsequent removal during phase separation using chloroform (White, Venter, Hiten & Burger 2008, Li *et al.* 2005, Wang *et al.* 2012). In addition, small polysaccharide molecules were distributed in the liquid phase during phase separation and co-precipitated with RNA, which reduced the RNA yield and affected subsequent applications (Liu, Han, Yu, Zhang, Xing, Xie & Peng 2018, Li & Trick 2005, Wang *et al.* 2010). Furthermore, a low A260/A230 ratio was detected, which indicated potential guanidine thiocyanate contamination as inferred from the warning messages from the meter. This component was present as a key element in the TRIsure mixture during the extraction procedure (Iqbal, Yang, Wu, Li, Hamayun, Hussain & Shah 2020).

Particularly, for coconut endosperm tissue, RNA extraction yielded mixed results in previous studies. The present study had some interesting findings. The concentration of the product extracted using TRIsure was approximately 157 ng/μL (corresponding to 47.1 μg/g), which was not as high as the QRREM results of Iqbal et al. (2019) (16.2 μg/80 mg corresponding to 202.5 μg/g) or the IRCM results of Iqbal *et al.* (2020) (150 μg/g). Moreover the results were not even comparable to those of the TRIzol method in the aforementioned studies (130 μg/g from Iqbal et al. 2019 and 145 μg/g from Iqbal *et al.* 2020). However, the product from CTAB-LiCL of approximately 142 ng/μL (equivalent to 42.6 μg/g) was significantly higher than the CTAB results of Iqbal *et al.* (2019) (1.25 μg/g) and Iqbal et al. (2020) (7.5 μg/g) and even higher than the results of Souza *et al.* (2018) (28.3 μg/g) in which CTAB was rated as the best reagent. However, in the end, the RNA concentration obtained from TRIsure was still higher than that from CTAB-LiCl in a statistically significant manner [*P* value 0.00 (<0.05)].

The integrity of the RNA fragments is important in assessing the quality of the total RNA obtained. Total RNA differs from DNA as it includes several types of RNA (rRNA, tRNA, mRNA, etc.) that vary in size. The size of rRNA in plants is usually fixed at 25S (3700 bp), 23S (2900 bp), 18S (1900 bp) and 16S (1500 bp) (Thermo Fisher 2006). In contrast, the size of mRNA varies widely because it depends on the length and specificity of the coding gene. Transcriptome sequencing focuses on mRNA molecules, which carry genetic information from the gene and encode functional proteins. mRNA sequencing provides valuable insights into the expressed functional gene and its expression level, which may be significantly lower than that of rRNA. Identifying specific mRNA bands on electrophoresis is challenging owing to variations in size and expression levels. Relative estimates of band quantity and tape compactness may indicate the presence of more intact RNAs and fewer fragmented RNAs (Wang *et al.* 2009, Kukurba *et al.* 2015).

The results demonstrated that the TRIsure method yielded superior electrophoresis and concentration outcomes than the CTAB-LiCl method. Furthermore, the TRIsure procedure required a total extraction time of merely 1 h, which was significantly shorter than the 15 h required for the other methods. This step aimed to determine the relative efficiency of the endosperm tissue compared with the leaf samples, rather than to enhance the extraction efficiency. Therefore, in the next step, the TRIsure method was employed to optimise the extraction of RNA from WE tissue as it offered superior RNA integrity and can potentially overcome the limitations of the current methods.

Improving purity valuethe A260/230 ratio, an indicator of purity, in RNA extraction process with TRIsure

During the extraction of plant tissue samples, A260/230 values are often low owing to the presence of polysaccharides (Wang & Stegemann 2010, Orek 2018). To overcome this problem, nucleotide lysis buffers can be supplemented with NaCl and β-mercaptoethanol to limit the amount of polysaccharides remaining in the sample. NaCl effectively removes polysaccharides during chloroform cleavage (Chang, Puryear & Cairney 1993), and β-mercaptoethanol is a potent reducing reagent that aids in denaturing RNase (Chang, Puryear & Cairney 1993, Mommaerts *et al.* 2015) thereby preventing undesired RNA degradation.

In this study, 0.1 mL of 2M NaCl and 1% β-mercaptoethanol were added simultaneously to the TRIsure buffer, as recommended by Chang *et al.* (1993). The amount of chloroform was increased to a ratio of 1:1 (v/v) for the sample aliquot obtained in the phase separation step. The RNA washing step with 75% alcohol was repeated thrice to eliminate any residual guanidine thiocyanate in the sample. Subsequently, only WE and NWE samples were utilised, with no further inclusion of leaf samples.

The results of statistical analysis using ANOVA signified no difference in the RNA concentration (*P* value 0.067), A260/280 (*P* value 0.124). Furthermore, the A260/230 value was significantly improved when using TRIsure supplemented with NaCl + β-mercaptoethanol (1.58 ± 0.02) compared with the use of TRIsure only (1.01 ± 0.03) [*P* value 0.000 (<0.05)] (Table 4).

RNA quality was measured using the absorption spectrometric ratios of the components present in the sample: pure RNA (Amax = 260 nm), proteins (Amax = 280 nm) and polysaccharides (Amax = 230 nm) (Iandolino *et al.* 2004). The addition of NaCl and the strong reducing agent β-mercaptoethanol to the extraction buffer enhanced the solubility of polysaccharides, decreased their co-precipitation with RNA in later steps and denatured ribonucleases and proteins. Other contaminants were reduced during extraction (Iandolino, Goes Da Silva, Lim, Choi, Williams & Cook 2004, Lodhi *et al.* 1994, Fang *et al.* 1992), thereby improving the purity ratio A260/230.

Testing of the sample incubation temperature atduring the lysis step for improvingto improve RNA concentration

Following the extraction with TRIsure as recommended by the manufacturer of Bioline, the lysate was incubated at room temperature for 5 min. However, coconut endosperm tissues are often hard and contain several lipids. Hence, the grinding process is more difficult than that of the leaf tissue, leading to lower extraction efficiency. In the CTAB method, the sample is usually incubated at a higher temperature (approximately 65°C) to augment the activity of the tissue-degrading enzyme. Therefore, this study tested the effect of increased incubation temperature (50°C, 55°C and 60°C) on the lysis of WE samples for 15 min to determine whether temperature affects the ability to obtain total RNA from coconut endothelial cells in the TRIsure reaction.

The results of ANOVA showed that the WE sample had total RNA concentrations of 157.67 ± 0.57, 156.67 ± 2, 157.00 ± 1 and 156.67 ± 2.5 ng/μL (Table 5), which corresponded to the incubation temperatures of room temperature (approximately 25°C–28°C), 50°C, 55°C and 60°C. However, there was no difference in yield

Table 4 Investigation of the improvement in A260/230 ratio when adding NaCl and β-mercaptoethanol in the lysis step

Buffer	Concentration	A260/280	A260/230
TRIsure	157.33 ± 0.5 [a]	1.99 ± 0.01 [a]	1.01 ± 0.03 [a]
TRIsure + NaCl + β-mercaptoethanol	159.00 ± 1 [a]	1.94 ± 0.04 [a]	1.58 ± 0.02 [b]
P value	0.067 (>0.05)	0.124 (>0.05)	0.000 (<0.05)

Note: One-way ANOVA was used to investigate the influence of the two extraction methods (original TRIsure vs modified TRIsure) on the A260/A230 ratio of the RNA product from the endosperm. The lowercase letters a and b represent the difference in the extraction methods. The differences are significant when the P value is <0.05.

among the investigated temperatures (*P* value 0.875). The OD results were also not different. The A260/A280 ratio was in the range of 1.8–2.0, and the 260/A230 ratio was in the range of 1.58–1.6. The electrophoresis results of the three treatments were also not different (Figure 4). These results revealed that increasing the sample incubation temperature did not increase the amount of total RNA obtained in the TRIsure procedure.

Testing the effectiveness of RNA preservation during transport

In many cases, the sample needs to be sent elsewhere for sequencing, which can take several days to a week, which affects RNA quality. To minimise RNA loss during transport, tests were performed using two preservation methods: DEPC solubilisation and absolute alcohol precipitation. The sample was kept chilled in a Styrofoam container filled with gel ice. The temperature of the container was maintained between 8°C and 10°C, which is similar to the transportation condition. RNA quality during this preservation process was evaluated on a weekly basis using electrophoretic analysis.

After 1 week, in samples that were stored in DEPC and precipitated with absolute alcohol, the electrophoresis bands showed minimal changes. However, from the 2nd week, the electrophoresis band of the RNA product precipitated with DEPC began to become more blurred, and by the 4th week, it was completely blurred and had several streaks (Figure 5). On the contrary, the electrophoresis band of the RNA stored in absolute alcohol did not show many changes compared with the initial time; nonetheless, it began to fade after the 3rd week. The results showed that storage in absolute alcohol is more optimal for RNA preservation than that in DEPC, which could be maintained for up to 3 weeks.

Table 5 Investigation of the possibility of improving the A260/230 ratio when increasing the annealing temperature in the lysis step

Annealing temperature	Concentration	A260/280	A260/230
Room temperature (25°C–28°C)	157.67 ± 0.57 [a]	1.98 ± 0.02 [a]	1.6 ± 0.01 [a]
50°C	156.67 ± 2 [a]	2.00 ± 0.03 [a]	1.58 ± 0.01 [a]
55°C	157.00 ± 1[a]	2.01 ± 0.01 [a]	1.6 ± 0.001 [a]
60°C	156.67 ± 2.5 [a]	1.98 ± 0.02 [a]	1.6 ± 0.02 [a]
P value	0.875 (>0.05)	0.281 (>0.05)	0.205 (>0.05)

Note: One-way ANOVA was used to investigate the influence of four different temperatures in the lysis step on the A260/A230 ratio of the RNA product from the endosperm. The lowercase letter a represents the difference between the screening temperatures. P value >0.05 shows lack of statistical significance.

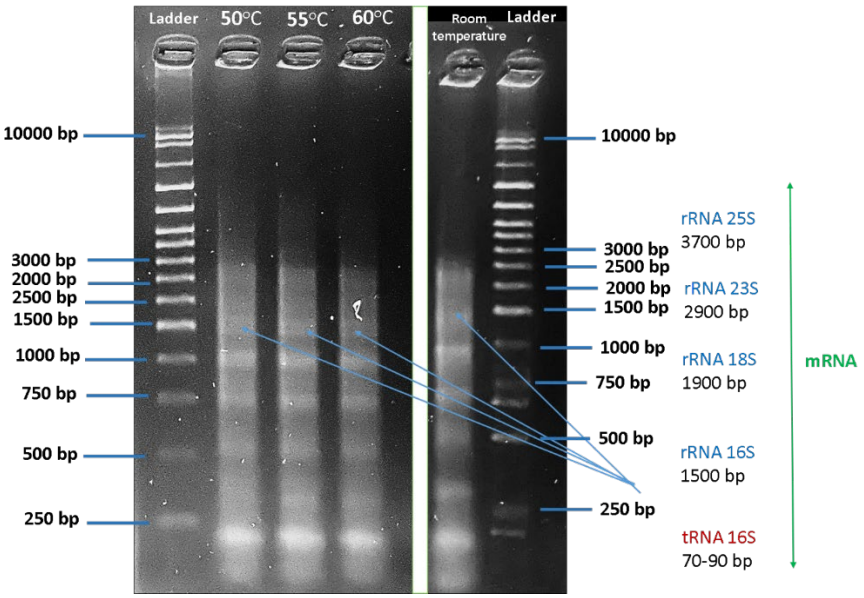

Figure 4 Results of electrophoresis of RNA extracted from WE samples at different temperatures

After RNA extraction, its preservation is crucial. Because RNA is rapidly influenced by temperature and nucleases in the media, it becomes unstable following extraction. This instability could be attributed to the fact that the RNA is often single-stranded and that the hydroxyl group renders the structure unstable, making the nucleotides prone to hydrolysis and destruction (Fordyce *et al.* 2013). Moreover, samples are often sent to sequencing firms located abroad, leading to extended transit times. Unfortunately, this prolonged transportation can immensely affect the quality of the RNA. In such circumstances, precipitation with pure alcohol is a technique that can help prolong the preservation of RNA. Unlike DNA, RNA in its single-stranded structure is inherently more challenging to preserve over an extended period. By precipitating RNA with 99.5% alcohol, the RNA structure condenses, strengthening the bonds and eliminating the enzymes that catalyse hydrolysis reactions. Consequently, RNA becomes significantly more resilient, facilitating its storage, transportation and utilisation for further research.

CONCLUSION

The concentration and purity of RNA, as reflected by the 260/280 ratio, is of utmost concern in next-generation sequencing. Even some sequencing companies now have strict requirements in terms of quality and concentration. This is because prior to sequencing, the extract will be purified so that the product is of the desired purity, but the concentration will be lost. Concentration is also significantly lost during transportation if the time is too long. Therefore, improving the concentration of the extracted RNA is essential. We recommend the use of TRIsure in combination with NaCl and β-mercaptoethanol for efficient RNA extraction instead of the CTAB-LiCl method. Additionally, RNA could withstand temperatures of 8°C–10°C for 3 weeks when stored in a 99.5% ethanol solution, which helps to reduce its degradation when shipping samples over a long distance for an extended period prior to sequencing.

ACKNOWLEDGMENTS

This work is supported by Nguyen Tat Thanh University under Grant No. 2022.01.135/HĐ-KHCN.

REFERENCES

Angeles, J. G., A. Laurena and E. Tecson-Mendoza (2005). "Extraction of genomic DNA from the lipid-, polysaccharide-, and polyphenol-rich coconut (Cocos nudfera L.)." Plant Molecular Biology Reporter - PL MOL BIOL REP **23**: 297-298.

BIOLINE. (2023). "TRIsure™ Reagent User Guide." from https://www.bioline.com/mwdownloads/download/link/id/953/trisure_pro-.

Chang, S., J. Puryear and J. Cairney (1993). "A simple and efficient method for isolating RNA from pine trees." *Plant Molecular Biology Reporter* **11**(2).

Cruz, R. and V. J. D. Bugayong (2016). "Mutations in the alpha-D-galactosidase gene suggest molecular basis of the mutant" makapuno" coconut (*Cocos nucifera* L.) phenomenon." *Philippine Agricultural Scientist* **99**(4): 321-325.

Fan, H., Y. Xiao, Y. Yang, W. Xia, A. S. Mason, Z. Xia, F. Qiao, S. Zhao and H. Tang (2013). "RNA-Seq analysis of Cocos nucifera: transcriptome sequencing and de novo assembly for subsequent functional genomics approaches." *PloS one* **8**(3): e59997.

Fang, G., S. Hammar and R. Grumet (1992). "A quick and inexpensive method for removing polysaccharides from plant genomic DNA." *BioTechniques* **13**(1).

fisher, T. (2010). "TRIzol™ Reagent User Guide." from https://tools.thermofisher.com/content/sfs/manuals/trizol_reagent.pdf.

Fordyce, S. L., M. L. Kampmann, N. L. van Doorn and M. T. P. Gilbert (2013). Long-term RNA persistence in postmortem contexts. *Investigative Genetics*. **4**.

Ghangal, R., S. Raghuvanshi and P. Chand Sharma (2009). "Isolation of good quality RNA from a medicinal plant seabuckthorn, rich in secondary metabolites." *Plant Physiol Biochem* **47**(11-12): 1113-1115.

Hong, M., S. Tao, L. Zhang, L.-T. Diao, X. Huang, S. Huang, S.-J. Xie, Z.-D. Xiao and H. Zhang (2020). "RNA sequencing: new technologies and applications in cancer research." *Journal of Hematology & Oncology* **13**(1): 166.

Iandolino, A. B., F. Goes Da Silva, H. Lim, H. Choi, L. E. Williams and D. R. Cook (2004). "High-quality RNA, cDNA, and derived EST libraries from grapevine (Vitis vinifera L.)." *Plant Molecular Biology Reporter* **22**(3).

Iqbal, A., Y. Yang, R. Qadri, Y. Wu, J. Li, F. Shah, M. Hamayun and A. Hussain (2019). "QRREM method for the isolation of high-quality RNA from the complex matrices of coconut." *Bioscience Reports* **39**(1).

Iqbal, A., Y. Yang, Y. Wu, J. Li, M. Hamayun, A. Hussain and F. Shah (2020). "An easy and robust method for the isolation of high quality RNA from coconut tissues." *Electronic Journal of Biotechnology* **48**.

Kumar, K. D., R. Gautam, I. Ahmad, S. D. Roy and A. Sharma (2015). "Biochemical, genetic and molecular basis of the novel and commercially important soft endosperm Makapuno coconut-A review." *Journal of Food, Agriculture & Environment* **14**(1): 61-65.

Lado, J. P., B. Z. Arellano, C. A. L. Osio, B. J. T. Tendero, C. de la Vina, M. A. O. Torio, R. P. Laude and M. G. Q. Diaz (2019). "Early differential expression of galactomannan biosynthesis genes in 'Makapuno' coconut (*Cocos nucifera* L.) revealed by the de novo assembly and analysis of endosperm transcriptome." *Philipp Agricul Sci* **102**: 6-24.

Li, Z. and H. N. Trick (2005). "Rapid method for high-quality RNA isolation from seed endosperm containing high levels of starch." *BioTechniques* **38**(6).

Liu, L., R. Han, N. Yu, W. Zhang, L. Xing, D. Xie and D. Peng (2018). "A method for extracting high-quality total RNA from plant rich in polysaccharides and polyphenols using Dendrobium huoshanense." *PLOS ONE* **13**(5): e0196592.

Lodhi, M. A., G. N. Ye, N. F. Weeden and B. I. Reisch (1994). "A simple and efficient method for DNA extraction from grapevine cultivars and Vitis species." *Plant Molecular Biology Reporter* **12**(1).

Minitab Inc. (2020). "Minitab 17 Statistical Software." from http://www.minitab.com/.

Mommaerts, K., I. Sanchez, F. Betsou and W. Mathieson (2015). "Replacing β-mercaptoethanol in RNA extractions." *Analytical Biochemistry* **479**.

Mujer, C. V., D. A. Ramirez and E. M. T. Mendoza (1984). "α-D-Galactosidase deficiency in coconut endosperm: its possible pleiotropic effects in Makapuno." *Phytochemistry* **23**(4): 893-894.

Mutz, K.-O., A. Heilkenbrinker, M. Lönne, J.-G. Walter and F. Stahl (2013). "Transcriptome analysis using next-generation sequencing." *Current opinion in biotechnology* **24**(1): 22-30.

Nguyễn Văn Đồng, Đinh Thị Thu Ngần, Tống Thị Hường, Nguyễn Hữu Kiên, Nguyễn Thị Hòa, Lê Thị Mai Hương, Đinh Thị Mai Thu, Nguyễn Nhất Linh and Phạm Thị Phương Thúy (2021). "Nghiên cứu nâng cao tỷ lệ tạo cây hoàn chỉnh in vitro trong quá trình nuôi cấy phôi dừa Sáp (Makapuno coconuts)." *Tạp chí Nông nghiệp & Phát triển nông thôn* **4**: 16-22.

Orek, C. O. (2018). "An Optimised Cetyltrimethylammonium Bromide (CTAB)-Based Protocol for Extracting RNA from Young and Old Cassava Leaves." *Journal of Advances in Biology & Biotechnology* **19**(4).

Phạm Thị Phương Thúy, Lê Trúc Linh, Đoàn Văn Hâu and Nguyễn Ngọc Trai (2016). "Báo cáo tổng kết đề tài "Nhân giống dừa Sáp bằng phương pháp nuôi cấy phôi tại tỉnh trà Vinh"."

Samonte, J. L., E. M. T. Mendoza, L. L. Ilag, B. Norberto and D. A. Ramirez (1989). "Galactomannan degrading enzymes in maturing normal and makapuno and germinating normal coconut endosperm." *Phytochemistry* **28**(9): 2269-2273.

Souza-Perera, R. A., N. Torres-Hernández and J. J. Zúñiga-Aguilar (2018). *Extraction of high quality RNA from hard tissues of adult coconut palms*.

Sukendah, S., H. Volkaert and S. Sudarsono (2009). "Isolation and analysis of DNA fragment of genes related to kopyor trait in coconut plant." *Indonesian Journal of Biotechnology* **14**(2).

ThermoFisher, S. (2023). "Ribosomal RNA Sizes." from https://www.thermofisher.com/vn/en/home/references/ambion-tech-support/rna-isolation/general-articles/ribosomal-rna-sizes.html.

Trần Thị Ngọc Thảo (2010). "Báo cáo tổng kết đề tài "Nghiên cứu cải tiến qui trình công nghệ nhân giống dừa sáp"." *Trường ĐH Trà Vinh*.

Trương Quốc Ánh (2012). "Báo cáo tổng kết đề tài "Nghiên cứu ứng dụng phương pháp nuôi cấy phôi soma từ chối mầm để nhân giống nh sát đặt ruột tại tỉnh Trà Vinh."

UCONN Health. (2017). "Total RNA extraction using Trizol reagent." from https://health.uconn.edu/li-lab/wpcontent/uploads/sites/129/2017/06/total_rna_extraction.pdf.

Võ Minh Hải, Phạm Thị Phương Thuý, Lê Vĩnh Thúc and Nguyễn Bảo Toàn (2021). "Nghiên cứu ảnh hưởng của một số yếu tố nuôi cấy in vitro đến quá trình tạo rễ cây dừa Sáp (Makapuno coconut) cấy phôi." Tạp chí Khoa học Trường Đại học Cần Thơ **57**(2B): 127-134.

Wang, G., G. Wang, X. Zhang, F. Wang and R. Song (2012). "Isolation of high quality RNA from cereal seeds containing high levels of starch." *Phytochem Anal* **23**(2): 159-163.

Wang, L. and J. P. Stegemann (2010). "Extraction of high quality RNA from polysaccharide matrices using cetlytrimethylammonium bromide." *Biomaterials* **31**(7).

White, E. J., M. Venter, N. F. Hiten and J. T. Burger (2008). "Modified Cetyltrimethylammonium bromide method improves robustness and versatility: The benchmark for plant RNA extraction." *Biotechnology Journal* **3**(11): 1424-1428.

Zhang, Y., J. Kan, M. Tang, F. Song, N. Li and Y. Zhang (2022). "Chemical Composition, Nutritive Value, Volatile Profiles and Antioxidant Activity of Coconut (Cocos nucifera L.) Haustorium with Different Transverse Diameter." **11**(7).

GUIDE FOR AUTHOR

BIOTROPIA, The Southeast Asian Journal of Tropical Biology, is a scientific publication of the Southeast Asian Ministers of Education Organization (SEAMEO) – Southeast Asian Regional Center for Tropical Biology (BIOTROP). It publishes articles in the broad areas of tropical ecosystems and environmental impacts, biodiversity conservation and sustainable development and biotechnology.

Since 2012, BIOTROPIA has been indexed by SciVerse SCOPUS (Elsevier), CrossRef, DOAJ and Google Scholar.

Articles published in the journal are papers reporting results of original research, short communication in which advances in knowledge of significance are briefly announced, and reviews on specific topics not available elsewhere in up-to-date form.

Submission of a paper is taken to imply that it has not been submitted to other journals and that, if accepted, will not be published elsewhere. All papers submitted for publication in BIOTROPIA are evaluated by subject matter experts and go through editorial revision. Authors may suggest names of potential reviewers. BIOTROPIA uses anonymous reviewing, in which the identities of authors and reviewers are kept confidential.

MANUSCRIPT SUBMISSION

BIOTROPIA publishes the following article types:

- Full Research article

A full research article is expected to present an original research focus with a clear articulation of its significance, a scientifically acceptable methodology, and a critical analysis and discussion of the findings. The components of the article consist of Introduction, Materials and Methods, Results and Discussion, and References (see Manuscript Format in details). The article should be typed in A4 size paper and within 15 pages or 4,000 – 5,000 words (including figures and tables). The article should be written in standard English, typed in Times New Roman font at 12-point size, one and a half spaced with margins on all four sides of 2 cm on a standard A4 paper.

- Short communication

This differs from a full paper on the basis of scope or completeness, rather than quality of research. It may report significant new data arising from problems with narrow, well-defined limits, or important findings that warrant rapid publication before broader studies are complete. It should neither exceed 1,500 words (approximately 6 pages of typescript) nor be divided up into conventional sections. When submitting a Short Communication, authors should make it clear that their work is to be treated as such.

- Review article

A review article summarizes previously published studies and the current state of understanding on a topic rather than reporting new facts or analysis. Its text should neither exceed 1,500 words (approximately 6 pages of typescript) nor be divided up into conventional sections. When submitting a Review Article, authors should make it clear that their work is to be treated as such. Review article should be written by at least three authors, so as to obtain an objective review.

Submission Declaration

Submission of an article implies that the work described has not been published previously, that it is not under consideration for publication elsewhere, that its publication is approved by all authors and tacitly or explicitly by the responsible authorities where the work was carried out, and that, if accepted, it will not be published elsewhere including electronically in the same form, in English or in any other language, without the written consent of the copyright holder.

Corresponding/First Author should fill out the enclosed declaration form and sign it together with all the rest of the author(s) before sending the form to BIOTROPIA. Please note that the publication process will not be performed until the declaration form has been provided by the Author(s). The form can be accessed at the Template section of BIOTROPIA Open Journal System.

English Language Editing

The language of the manuscripts is English in passive form. Author(s) are not required to have their manuscripts edited by a professional English language editor during the submission process; however, the Author(s) need to ensure that the language quality of the manuscript is not lower than 85% when being checked with Grammarly application.

Manuscript with checking result of more than 85% shall be processed to the next stage. However, if the checking result is lower than 85%, the Editorial Board shall assess the content of the manuscript. If the content of the manuscript is scientifically significant, the manuscript shall be given back to the Author(s) for language improvement through proofreading process by professional language editing service or editor assigned by the Editorial Board and the Author(s) later need to provide BIOTROPIA with a certification from the same language institution when done together with the edited version of the manuscript. All editing costs will be borne by the Author(s). Nevertheless, if the content of the manuscript is not scientifically significant, the manuscript shall be rejected.

Manuscript in the Microsoft Word file should be submitted to BIOTROPIA Open Journal System (OJS) address at: http://journal.biotrop.org/index.php/biotropia. The manuscript declaration form should also be sent to BIOTROPIA OJS as supplementary file.

MANUSCRIPT PREPARATION

Overview of Manuscript Sections for Research Article and Short Communication

Manuscripts for Research Article and Short Communication submitted to BIOTROPIA should be divided into the following sections (in this order):

- **Title**
- **Abstract**
- **Keywords**
- **Introduction**
- **Materials and Methods**
- **Results and Discussion**
- **Conclusion**
- **Acknowledgments**
- **References**

Title

The title should:

- provide the title of the article, maximum of 14 words
- list the full names, institutional addresses, email addresses and contact number for all authors
- indicate the corresponding author
- indicate a running title

Structure for institutional address of authors:

- for university:
  name of department/study program, name of faculty, name of university, city with postal code, country
- for institution:
  name of center/division/laboratory, name of institution, city with postal code, country

Abstract

The Abstract of the manuscript should not exceed 350 words and consists of **Introduction** (i.e., the context and purpose of the study), **Methods** (i.e., the main methods and experimental design), **Results** (i.e., the main findings) and Conclusion (i.e., brief summary and potential implications). Please minimize the use of abbreviations and do not cite any references in the abstract.

Keywords

Three to five keywords represent the main content of the article. Keywords should be arranged alphabetically.

Introduction

The Introduction section should be written in a way that is understandable to other researchers without specialist knowledge in that area and must clearly state - and, if helpful, illustrate - the background of the research and its aims. The section should end with a brief statement of what is being reported in the article. Standard chemical symbols and abbreviations may be used in the text, but full terms should be given at the first mention. Units of measurements should be spelled out except when preceded by a numeral. If no-metric measurement units are used, the metric equivalent should be mentioned. The complete scientific name of every organism must be cited when it is first mentioned in the text. The generic name may be abbreviated thereafter, except when there are references to other genera with the same initial. The use of common names must be accompanied by the correct scientific name on first use. Example:

a. Sourgrass (*Paspalum conjugatum*)
b. *P. conjugatum*
c. *Paspalum conjugatum* and *Pinus merkusii*

Materials and Methods

The materials section should explain in detail the materials used in the study, the sources, measurements, and quantity used.

The methods section should include a clear description of the design of the study, including population and sampling, and the type of analysis used, to enable replication.

For studies involving human participants, a statement detailing ethical approval and consent should be included in the methods section.

Results and Discussion

The Results and Discussion should be incorporated into a single section. Each result must be followed by its discussion. This section may be broken into subsections with short, informative headings.

Conclusion

The Conclusion section should clearly state the main conclusions of the research and explain their importance and relevance. Summary illustrations may be included.

Acknowledgments

Acknowledgments must be given to anyone who substantially contributed to the conception, design, and data acquisition analysis and interpretation related to the research, or who was involved in drafting the manuscript or revising it critically for important intellectual content, but who does not meet the criteria for authorship. Please also include the source(s) of funding for each author, and for the manuscript preparation. Authors must describe the role of the funding body, if any, in design, in the collection, analysis, and interpretation of data; in the writing of the manuscript; and in the decision to submit the manuscript for publication. Please also acknowledge anyone who contributed materials essential for the study. If a language editor has made significant revision of the manuscript, proper acknowledgment is also recommended.

Authors should obtain permission to acknowledge all those to be mentioned in the Acknowledgments section.

References

References cited in the text should be presented according to the CBE (Council of Biology Editors) Style Manual, latest edition. The list of References should be given at the end of the paper, immediately following the section on Acknowledgment, if any. **The references cited from journals should be within the past ten years.** The journal's name should be written in its abbreviation style.

If the reference consists of more than seven authors: the first six authors are listed, all subsequent authors except the last are omitted and replaced with an ellipsis and then the name of the last author is listed.

If the same author published multiple works in the same year: the sequence is determined by the publication time, earliest to latest. If it cannot be determined, the references are put in alphabetic order by article title.

If the title of the reference is not in English, the original title should be written, then followed by the translated version in English. For example:

Mirleau-Thebaud V, Dayde J, Scheiner JD. 2017. Influencia de la compactación del suelo y laboreo de conservación en el sistema radical de Helianthus annuus L. [The influence of soil compaction and conservation tillage on sunflower's (*Helianthus annuus* L.) below ground system]. Phyton 86:53-67.

Examples of BIOTROPIA reference style

Article within a journal

Andrade CC, Young KI, Johnson WL, Villa ME, Buraczyk CA, Messer WB, Hanley KA. 2016. Rise and fall of vector infectivity during sequential strain displacements by mosquito-borne dengue virus. J Evol Biol 29:2205-18.

Zhang J. 2016a. Coinfection with type 1 and type 2 PRRSV. Vet Rec 178(12):288-90.

Zhang J. 2016b. Porcine delta corona virus: Overview of infection dynamics, diagnostic methods, prevalence and genetic evolution. Virus Res 226:71-84.

Article within a journal supplement

Meharena HS, Fan X, Ahuja LG, Keshwani MM, McClendon CL, Chen AM, … Taylor SS. 2016. Decoding the interactions regulating the active state mechanics of eukaryotic protein kinases. PLoS Biol. Suppl:1-23.

In-press article

Voong LN, Xi L, Sebeson AC, Xiong B, Wang JP, Wang XZ. Insights into nucleosome organization in mouse embryonic stem cells through chemical mapping. Cell, in press.

Published abstract

Rijsdijk KF, de Louw PGB, Meijer HJM, Claessens LPAM, Florens FBV, Baider C, … de Boer EJ. 2016. Restoring and conserving the remains of the ecosystem of the Dodo: Lessons from a 4200 year old multitaxic bone bed [Abstract]. Island Biology 2016:279-80.

Article within conference proceedings

Cao H, Guo W, Qin H, Xu M, Lehrman B, Tao Y, Shugart YY. 2016. Integrating multiple genomic data: Sparse representation based biomarker selection for blood pressure. In: Rowles T, editor. BMC proceedings 2016. Genetic analysis workshop 19. p. 283-8.

Tran Q, Gao S, Vo NS, Phan V. 2016. Repeat complexity of genomes as a means to predict the performance of short-read aligners. In: BICOB 2016. Proceedings: 2016 Apr 6-4; Las Vegas. Winona (US): International Society for Computers and Their Applications (ISCA). p. 135-42.

Book chapter, or article within a book

Faith DP. 2016. The PD phylogenetic diversity framework: Linking evolutionary history to feature diversity for biodiversity conservation. In: Pellens R, Grandcolas P, editors. Biodiversity Conservation and Phylogenetic Systematics. Cham (ZG): Springer International Publishing. p. 39-56.

Complete book

Reddy PP. 2016. Sustainable intensification of crop production. Singapore (SG): Springer Singapore.

Monograph or book in a series

O'Toole D, Sondgeroth KS. 2016. Histophilosis as a natural disease. In: *Histophilus somni*. Edited by Inzana TJ. Cham (ZG): Springer International Publishing. p. 15-48. [CompansRW, Honjo T, *et al.* (Series Editors): Current Topics in Microbiology and Immunology, Vol. 396].

Book with institutional author

Food and Agriculture Organization. 2016. The State of Food and Agriculture 2016 (SOFA): Climate change, agriculture and food security. Rome (IT): Food and Agriculture Organization of the United Nations.

Dissertation

Ayloo S. 2016. Molecular and cellular approaches toward understanding dynein-driven motility [Dissertation]. Retrieved from University of Pennsylvania Repository. (Paper AAI10124524).

Electronic Journal Article with DOI

Morzillo AT, Kreakie BJ, Netusil NR, Yeakley JA, Ozawa CP, Duncan SL. 2016. Resident perceptions of natural resources between cities and across scales in the Pacific Northwest. Ecol Soc [Internet]. [cited 2016 Nov 30]; 21(3):14. Available from: http://www.ecologyandsociety.org/vol21/iss3/art14/doi: 10.5751/ES-08478-210314

Electronic Journal Article Non DOI

Hamzah A, Hapsari RI, Wisnubroto EI. 2016. Phytoremediation of cadmium-contaminated agricultural land using indigenous plants. Int J Environ Agric Res [Internet]. [cited 2016 Dec 20]; 2(1):8-14. Available from: http://www.ijoear.com/Paper-January-2016/IJOEAR-JAN-2016-2.pdf

Webpage

Michigan State University [Internet]. 2016. Just add water: New discovery in plant-disease mechanism. Rockville, US: ScienceDaily; [updated 2016 Nov 23; cited 2016 Dec 10]. Available from: https://www.sciencedaily.com/releases/2016/11/161123140238.htm

Webpage with Personal Author

Cowie S [Internet]. 2016. 'The Pantanal is national heritage': Protecting the world's largest wetlands. London (UK): The Guardian.com; [updated 2016 Nov 12; cited 2016 Dec22]. Available from: https://www.theguardian.com/global-development-professionals-network/2016/nov/12/pantanal-conservation-wetlands- brazil-paraguay-bolivia

Preparing illustrations and figures

Illustrations should be embedded in the text file for clarity purposes. However, we also require authors to submit separate JPEG files of the illustrations and figures for printing purposes with minimum size of 3 MB as supplementary file, so that the clarity of illustrations and figures can be maintained during the printing process. Each figure should include a single illustration and should fit on a single page in portrait format. If a figure consists of separate parts, it is important that a single composite illustration file be submitted which contains all parts of the figure. There is no charge for the use of color figures.

Graph should not contain gridlines.

All figures and tables should be embedded in text, not being placed after references section.

Figure legends

The legends should be included in the main manuscript text file at the end of the document, rather than being a part of the figure file. For each figure, the following information should be provided: Figure number (in sequence, using Arabic numerals - i.e., Figure 1, 2, 3 etc.); short title of figure (maximum 15 words); detailed legend, up to 300 words.

Please note that it is the responsibility of the author(s) to obtain permission from the copyright holder to reproduce figures or tables that have previously been published elsewhere.

PROOFS

Proofs will be sent by email to the corresponding author who is expected to proofread the article carefully. The corrected proof should be received by the administration within three working days.

MANUSCRIPT SUBMISSION AND PUBLICATION FEE

There is no charge for the submission and publication. Four off-prints are given free of charge to the identified recipient Author(s). Please note that we will not give the journal unless a request from the Author(s).

Submission Preparation Checklist

As part of the submission process, authors are required to check off their submission's compliance with all of the required items, and submissions may be returned to authors that do not adhere to these guidelines.

Privacy Statement

The names and email addresses entered in this journal site will be used exclusively for the stated purposes of this journal and will not be made available for any other purposes or to any other parties.

How to Cite BIOTROPIA

Master J, Tjitrosoedirdjo S, Qayim I. 2016. Abiotic factor influencing mantangan (*Merremia peltata*) invasion in Bukit Barisan Selatan National Park. BIOTROPIA 23(1):21-7.

A Publication of the Southeast Asian Ministers of Education Organization
Southeast Asian Regional Centre for Tropical Biology
SEAMEO BIOTROP
Bogor, Indonesia

Vol. 30 No. 3 December 2023

CONTENTS

Research Papers

Indexed by

ISSN 1907-770X