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*Journal of*
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## Cover image:

Female specimen of *Hippocampus barbouri*
*(Photo credit: Parivaseni Ravichandran and Chen Cheng Ann)*

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**Research Article**

# A Preliminary Study on Microplastics Contamination in Wild Fishes Caught from Urbanised Sepanggar River of Kota Kinabalu, Sabah

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## ABSTRACT

Urban areas with high population densities generate high levels of plastic waste from human activities, potentially raising microplastic levels in riverine systems. Microplastic pollution in rivers pose serious risks to fish through ingestion, toxicity, and bioaccumulation. Nevertheless, the paucity of previous studies on fish microplastic contamination in Sabah, Malaysia highlights knowledge gaps in this area. Thus, this study aimed to provide a preliminary assessment of microplastic contamination in fish from the urbanised Sepanggar River in Kota Kinabalu, Sabah. A total of 39 fish samples were caught from the river, dissected into muscles and internal organs, and digested with 10% potassium hydroxide (KOH). Microplastics were then extracted using the density separation method in 5M sodium chloride (NaCl) and counted by shape, colour, size, and polymer type. The present study demonstrated that microplastics were detected in 77% of fish caught from the Sepanggar River, with an average of 5.28 ± 6.51 items/fish. Small-sized (97%), fragment (54%) and black colour (40%) were the most prevalent characteristics of microplastics found in fish while rayon (23%) was the most prevalent polymer type. Microplastic abundance in internal organs (3.54 ± 3.63 items/fish) was significantly higher than that in muscles (1.74 ± 5.10 items/fish). The characteristics of ingested microplastics varied significantly by fish species, most likely due to the different feeding habits and diets. This study provides the first confirmation that fish in the Sepanggar River were contaminated by microplastics from adjacent domestic and industrial activities. Improved waste management is needed to monitor and reduce long-term microplastic pollution.

**Keywords:** Fish; internal organs; microplastics; muscles; Sabah; tidal rivers.

## INTRODUCTION

Microplastic pollution in aquatic environments has become ubiquitous in rivers and oceans. Frias & Nash (2019) defined microplastics as insoluble plastic particles with regular or irregular shapes, ranging in size from 1 μm to 5 mm, and come in two forms: primary and secondary microplastics. Primary microplastics are originally manufactured as small particles, such as resin pellets and microbeads, while secondary microplastics are a result of fragmentation of larger plastic items (Idrus et al., 2022; Kwon et al., 2022). Microplastics have been extensively documented not only in river water and sediment (Ismanto et al., 2023; Karing et al., 2023) but also in various aquatic organisms, including fish (Lestari et al., 2023). Fish are ideal representatives for studying the impacts of microplastic pollution on the riverine ecosystem because fish are mobile and inhabit diverse habitats in flowing waters, which allows comprehensive coverage of the entire river area.

Microplastics in rivers are extensively sourced from fisheries (Choong et al., 2021), direct plastic littering and domestic waste disposal (Primus & Azman, 2022), textile washing (Chen et al., 2021) and wastewater leaching (Suardy et al., 2020). These sources would determine the sizes, types, shapes and colours of microplastics in the environment. Once in the environment, the biological, chemical, and physical degradation of microplastics causes surface embrittlement and changes in their colour, elasticity, and strength allowing them to break easily and disperse widely (Syakti et al., 2018; Hwi et al., 2020). Upon their entry, microplastics remain in the water column and are mistakenly ingested by fish because they resemble the fish's natural food sources, such as small zooplankton (Ory et al., 2017). Microplastics could also settle on the bottom sediment depending on their density and surface area, where highly dense fragments and pellets usually sink to the bottom sediment while lighter fibres and films float (Choong et al., 2021; Banik et al., 2024). Once settled on the bottom sediment, these microplastics can also be accidentally ingested by demersal fish while foraging or mistaking them for prey or plankton (Kibria, 2023).

Microplastics ingested by fish expose them to toxicity, tissue damage, and starvation due to digestive tract blockage (Bhuyan, 2022). Microplastics are capable of adsorbing surrounding chemical pollutants onto their surfaces posing toxicity to fish ingesting the contaminated microplastics (Laila et al., 2020). Bioaccumulation and biomagnification of microplastics occur from constant ingestion and microplastics can be transferred to a higher trophic level in the food chain (Yagi et al., 2022). Most studies on microplastic contamination in fish have focused on assessing the risk factors of microplastic ingestion, particularly in the gastrointestinal tract (GIT) and gills (Sarijan et al., 2019; Lim et al., 2023), rather than examining the entire fish, including its tissues. To better understand bioaccumulation in natural environments, studies should also focus on fish tissue, as examining microplastic presence in all parts of the fish offers a comprehensive view of contamination. Additionally, studying microplastics in fish tissue is crucial due to the potential for human consumption, as it raises concerns about food safety if microplastics reportedly accumulate in fish tissue (Daniel et al., 2020; Jitkaew et al., 2024).

The rapid pace of new development and infrastructural changes in Kota Kinabalu, Sabah, as the main city, has pressured expanding developments into the adjacent Sepanggar Town, leading to more mismanaged plastic waste (Dusim, 2021). Since rivers are easily accessible to locals, river resources such as fish are often utilised especially when fish is among a main source of protein for Sabah locals. Recognizing the potential impact on food security and human safety from microplastic contamination in fish (Bhuyan, 2022), it is important to address

the rising issue of microplastics in river fish. However, the status of microplastic contamination in these fish remains unknown due to a lack of documentation and research. Hence, this research aims to close knowledge gaps regarding microplastic contamination of fish in the Sepanggar River, representing an urbanised river in Kota Kinabalu, Sabah. The objective of this study was to collect preliminary data on the occurrence, abundance and characteristics of microplastics in wild fish caught from the river. This baseline data is crucial to serve as an early warning on food safety and security for locals, and establish a foundation for future studies for the development of monitoring, mitigation, and action plans.

## MATERIALS & METHOD

### Study area

Sepanggar River is located in Sepanggar, a sub-district in the West Coast Division (Kota Kinabalu District) of Sabah, Malaysia adjacent to Sepanggar Bay Container Port, Universiti Malaysia Sabah (UMS), Universiti Teknologi MARA (UiTM) Sabah, Taman Indah Permai and Kampong Rampayan. The sub-district of Sepanggar covers an area of 317 $km^2$ with a total population density of 1061 per $km^2$ and annual rainfall of 3456.6 mm recorded in 2020 (Department of Statistics Malaysia, 2023). The expansion of adjacent Kota Kinabalu pressurised the urbanisation of Sepanggar through increased development. The selection of Sepanggar River was due to its proximity to UMS for sampling and analysis in the laboratory. Fish samples were collected on 11$^{th}$ May 2023 during high tide in the (1) downstream and (2) upstream of the river as shown in Fig. 1. Sampling details are summarised in Table 1.

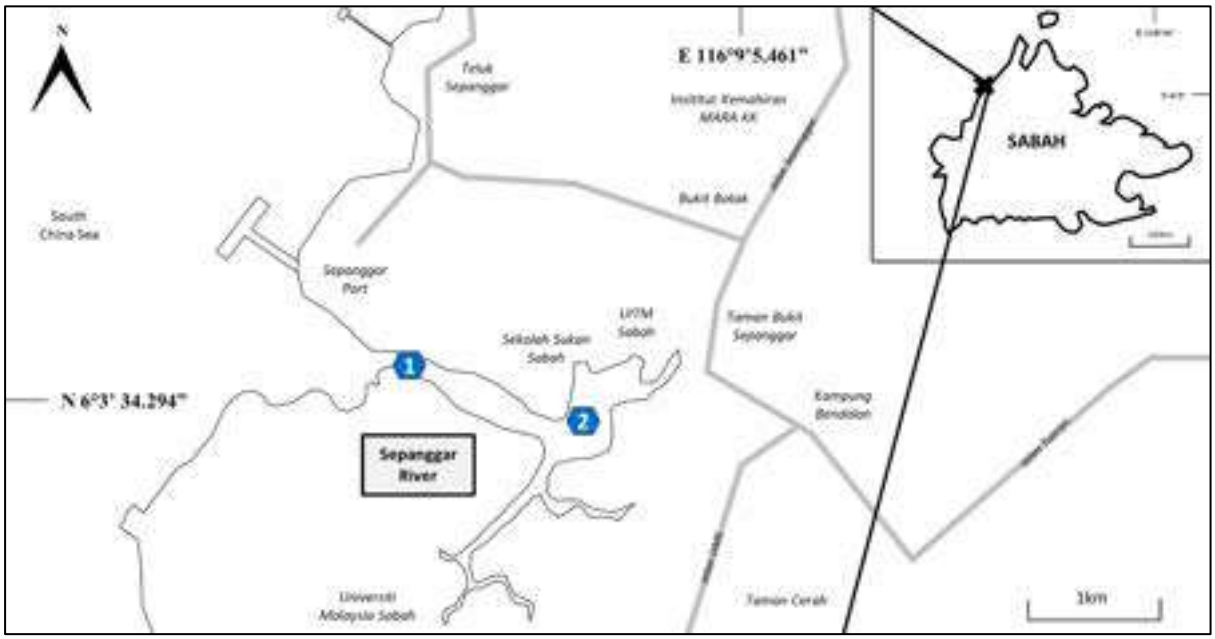


**Figure 1:** Study area map of Sepanggar River. Nets deployment in the river was indicated by a blue circle.

**Table 1:** Description of the sampling area, Sepanggar River.

| Area | Depth (m) | Coordinates | Time | Observation |
|---|---|---|---|---|
| Location 1 - Downstream | 4.0 | 06°03'39.9" N 116°07'46.5" E | 8.45 am | Floating plastics, small wooden village houses along the river, boat jetties and hospital buildings nearby |
| Location 2- Upstream | 1.1 | 06°03'29.9" N 116°08'24.8" E | 9.55 am | |

## Fish collection

Fish collection and proper euthanasia were approved by the Animal Ethics Committee UMS [ref no: AEC0031/2022] prior to sampling. Sampling of fish was carried out using gill nets where three gill nets of 2 inches in mesh size were combined and deployed at (1) downstream and (2) upstream of the river. The nets were pulled up after two hours and all fish caught were collected and immersed in a stainless-steel pail with an overdose of NIKA Transmore solution, adhering to animal ethics guidelines on humane euthanasia with minimal suffering. The fish were placed in aluminium seal bags after confirmation of their death and transported to the Institute for Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah (UMS) laboratory in a cooler box for further analysis. All the collected fishes were cleaned with distilled water in the laboratory to remove impurities before taking morphometric measurements (standard length and total length to the nearest 0.1 cm and wet weight to the nearest 0.1 g) and photographs of individual fish species (Fig. 2). Fish were sorted, counted, and identified to species level where possible following available taxonomic identification books (Mansor et al., 1998; Annie & Albert, 2009; FAO, 2024; Froese & Pauly, 2024) before storing in a freezer at –20°C until further analysis. Information on habitat, feeding guilds, and food sources for each species were obtained from FAO (2024) and Froose & Pauly (2024). A total of 39 individual fishes accounting for eight species were caught from the Sepanggar River and used for microplastic extraction in this study (Table 2).

## Sample analysis

### *Extraction of microplastics*

Throughout the microplastic extraction and identification process, precautions were taken to reduce contamination of collected samples by airborne plastic particles, as proposed by Prata et al. (2019). All apparatus was made of glass or metal, and it was acid-washed when necessary and rinsed with distilled water. The surfaces of the bench and the working table were regularly cleaned by wiping these with 70% ethanol. Synthetic clothing was avoided during laboratory analysis, opting for cotton clothing. Distilled water was used as blanks and subjected to the same extraction and analysis processes as the samples for quality control purposes.

All fishes obtained were used for microplastic extraction. Each fish sample was dissected into (1) muscle consisting of fish skin and fillet, and (2) internal organs of heart, lungs, liver, stomach, intestines and gills and placed in two cleaned conical flasks (Fig. 3, Daniel et al., 2020). Internal organs and fish muscles were first digested with 10% potassium hydroxide (KOH) at the optimum temperature of 40°C for 72 hours and 60°C for 24 hours, respectively. The digested samples were then filtered into 100 ml glass beakers using a 10 mm stainless steel sieve. Microplastics were then extracted following density separation method where 5M of sodium chloride (NaCl) were poured into the beakers and left overnight for settling. The supernatant obtained the following day was vacuum filtered through a 1.2 µm pore size glass microfiber filter (Whatman GF/C) and placed into a clean petri dish with cover. This process was repeated thrice.

**Table 2:** Summary of fish species caught from Sepanggar River for microplastic extraction and associated morphometric measurements expressed in (mean and standard error), habitat, feeding habit, and diet source.

| Fish Species | Total, n | TL (cm) | SL (cm) | Weight (g) | Habitat/ Environment | Feeding guilds | Diet sources |
|---|---|---|---|---|---|---|---|
| *Glossogobius* sp. | 5 | 21.5 ± 1.3 | 17.5 ± 0.7 | 61.0 ± 5.9 | Benthopelagic, amphidromous | Carnivorous | Mosquito larvae, earthworm, fish |
| *Plotosus lineatus* | 5 | 20.3 ± 0.6 | NA | 49.6 ± 6.0 | Demersal, amphidromous | Carnivorous | Fish, molluscs, crustaceans, worms |
| *Pennahia* sp. | 2 | 15.0 ± 0.0 | 12.0 ± 0.1 | 35.7 ± 2.45 | Benthopelagic, oceanodromous | Carnivorous | Invertebrates, small fishes |
| *Diapterus auratus* | 1 | 9.2 ± 0.0 | 7.4 ± 0.0 | 10.9 ± 0.0 | Demersal | Omnivorous | Plant, ostracods, copepods, nematodes, invertebrates, |
| *Leiognathus equula* | 3 | 8.1 ± 1.0 | 6.6 ± 0.6 | 8.1 ± 2.6 | Demersal, amphidromous | Omnivorous | Worms, crustaceans, polychaetes, fish |
| *Karalla daura* | 2 | 7.1 ± 0.1 | 5.9 ± 0.1 | 5.4 ± 1.4 | Demersal | Omnivorous | Sponges, crustaceans, bivalves, polychaetes, |
| *Nemapteryx caelata* | 2 | 21.9 ± 1.1 | 17.7 ± 0.6 | 70.8 ± 1.8 | Demersal, amphidromous | Carnivorous | Invertebrates, fishes |
| *Arius venosus* | 19 | 17.2 ± 2.2 | 14.2 ± 2.0 | 40.3 ± 17.0 | Demersal | Carnivorous | Invertebrates, fishes |

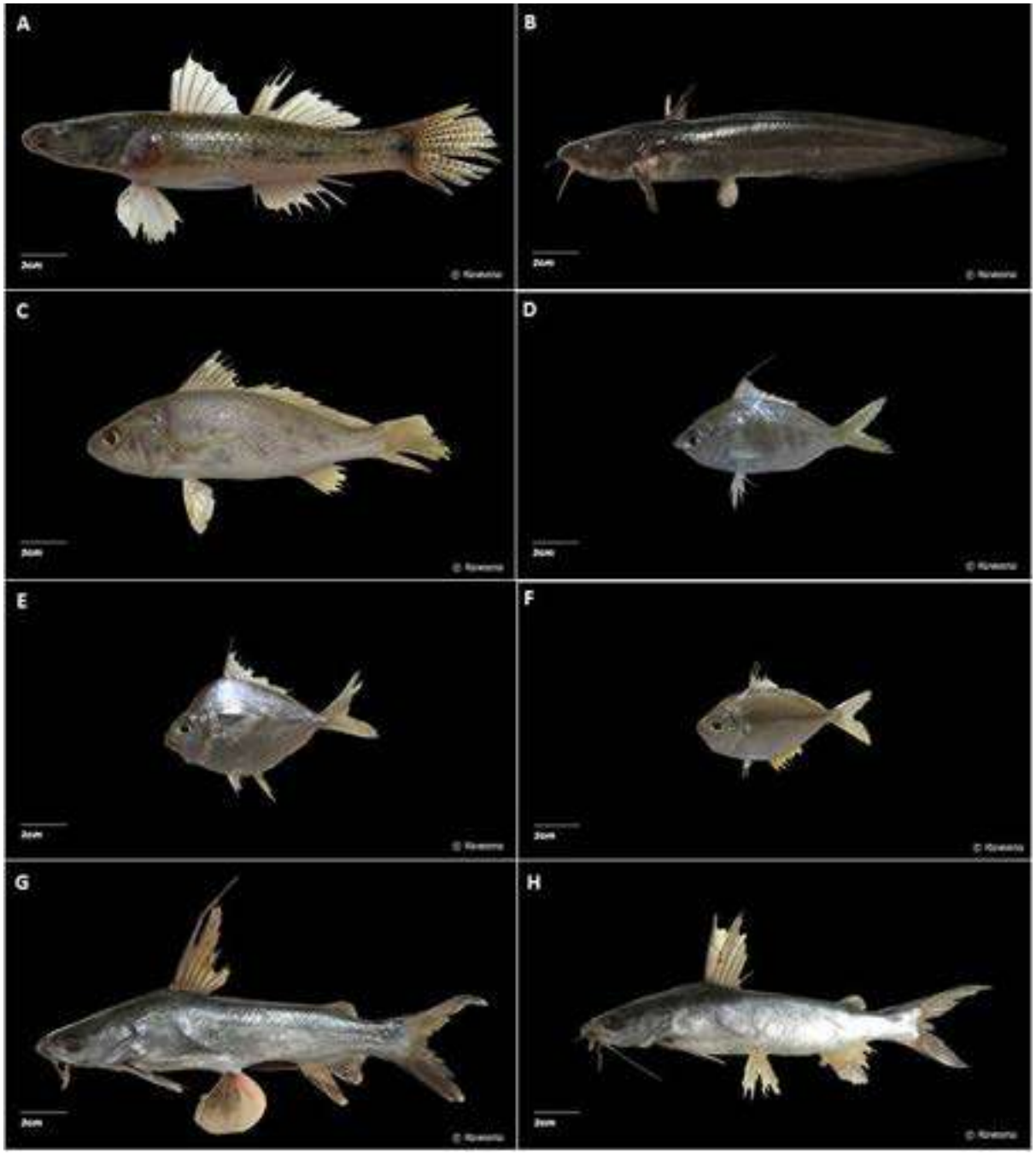


**Figure 2:** Fish species caught in Sepanggar River; **A.** *Glossogobius* sp., **B.** *Plotosus lineatus*, **C.** *Pennahia* sp., **D.** *Diapterus auratus*, **E.** *Leiognathus equula*, **F.** *Karalla daura*, **G.** *Nemapteryx caelata*, and **H.** *Arius venosus*.

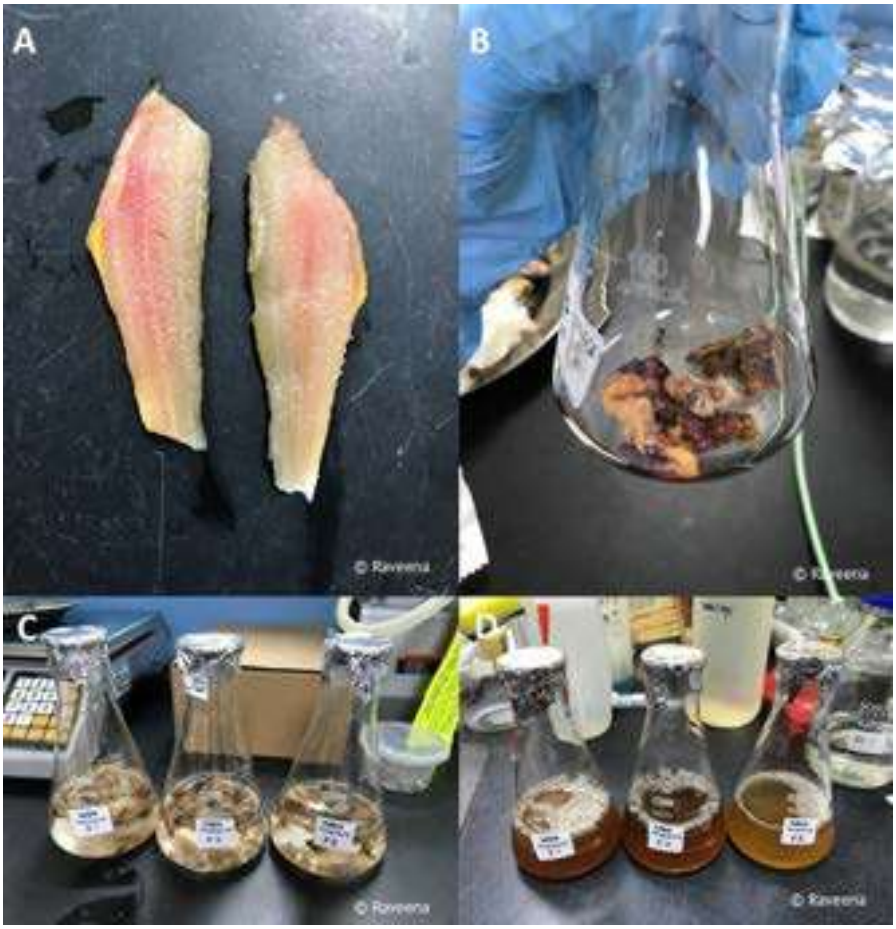


**Figure 3:** Microplastics extraction process; dissection of fish into **A.** Fish muscles and **B.** Internal organs, **C.** Pre-digestion, and **D.** Post-digestion of samples.

### *Identifications of MPs*

Microplastics on filter paper were identified under a stereo microscope (Leica EZ24) according to shape, colour and size (Table 3). The heated needle test was used when the identification of microplastics was uncertain; if the object melted and curled when it came into touch with the heated needle, it was determined to be plastic. Microplastic concentration was expressed in unit of items/fish.

**Table 3:** Categories used in the description and identification of microplastic.

| Characteristic | Categories | Description | References |
|---|---|---|---|
| **Shape** | Fibre | A very thin threadlike straight structure | Singh et al. (2022) |
| | Filament | A thicker and harder straight structure | |
| | Foam | A sponge-like lightweight structure, | |
| | Fragment | An irregular edge of hard structures | |
| | Pellet | A round spherical hard structure | |
| | Film | A thin layer plan of flimsy structure | |
| **Colour** | Black | Black, transparent black, grey and white-striped black | Peng et al. (2017) |
| | Blue | Deep blue, light blue, deep green, light green | |
| | Red | Red, purple, pink | |
| | White | Opaque white, silver | |
| | Yellow | Yellow, brown, orange | |

| | | |
|---|---|---|
| | Transparent | Colourless |
| **Size** | SMP | < 1 mm |
| | LMP | 1 – 5 mm |

For polymer type identification, samples of four fish species of which three or more individuals were obtained were selected and only individuals with detectable microplastics in both internal organs and muscles were analysed, i.e., *Arius venosus* and *Glossogobius* sp. (5 individuals each), *Plotosus lineatus* (3 individuals), *Leiognathus equula* (2 individuals). Due to low microplastic count in fish, microplastics from the same fish species were pooled together to ensure a sufficient concentration for the subsequent polymer type analysis. Microplastics from the same fish species were pooled together through sonification with distilled water at 50 HZ for 10 minutes, followed by filtration into a new 1.2 µm pore size glass microfiber filter (Whatman GF/C). The filter papers were sent to ALS Technichem laboratory in Shah Alam, Malaysia for analysis using micro-FTIR (Nicolet iN10 MX). In the lab, microplastics on filter paper were sonicated with ultrapure water at 50HZ for 10 minutes, followed by organic matter digestion with Fenton reagent for 24 hours, and filtered on a 0.2 µm alumina oxide filter membrane. The filter membrane was then placed under the instrument to produce single spectra based on the functional groups of the particle. The spectrum obtained was compared with available libraries on established databases on polymer type with quality matching more than 80% with a size detection limit of 20 um for identification, counting and reporting.

**Data analysis**

All parameters studied were checked for outliers using boxplots, tested for normality using Shapiro-Wilk's test, and examined for equality of variances using Levene's test prior to statistical analysis. Non-parametric tests were used due to the violation of normality and equal variance of data. Kruskal Walis-H test was used to compare microplastic concentration and characteristics between fish species ($n \geq 3$) while the Mann-Whitney U-test was carried out to compare if there was any significant difference in microplastic concentration between internal organs and fish muscles at $p$ value < 0.05.

## RESULTS

**Microplastic occurrence and abundance in river fishes**

In this study, 77% of fish samples caught from the Sepanggar River, 30 out of a total of 39 fish samples were found to contain microplastics, with an average abundance of 5.28 ± 6.51 items per fish. *Leiognathus equula* (n = 3) had the highest average microplastic count at 12.67 ± 16.23 items per fish, followed by *Glossogobius* sp. (n = 5) each with 10.00 items per fish. *Arius venosus* (n = 19) exhibited the lowest microplastic count, with an average of 2.95 ± 2.50 items per fish. However, microplastic concentrations were not significantly different between the studied fish species ($n \geq 3$, $\chi^2(3)$, H = 6.628, $p$ = 0.009) when tested with the Kruskal-Wallis H test (Table 4).

**Table 4:** Comparison of microplastic concentration and characteristics among fish species ($n \geq 3$) using the Kruskal-Walis H test.

| Parameters | Test statistic, H | Asymptotic *p* value | Retain/Reject |
|---|---|---|---|
| Microplastic Concentration | 6.628 | 0.09 | Retain |
| Microplastic Type | | | |
| **Fibre** | **10.029** | **0.02** | **Reject** |

| | | | |
|---|---|---|---|
| Foam | 0.684 | 0.88 | Retain |
| Fragment | 5.182 | 0.16 | Retain |
| Film | 7.764 | 0.05 | Retain |
| Microplastic Colour | | | |
| **Black** | **9.301** | **0.01** | **Reject** |
| Blue | 6.101 | 0.32 | Retain |
| **Red** | **15.539** | **0.02** | **Reject** |
| Yellow | 16.210 | 0.56 | Retain |
| White | 9.556 | 0.15 | Retain |
| Transparent | 27.316 | 0.68 | Retain |
| Microplastic Size | | | |
| LMP | 27.288 | 0.70 | Retain |
| SMP | 18.041 | 0.07 | Retain |

* The significant difference was indicated in bold, $p$ value < 0.05.

Comparing microplastic concentrations between internal organs and fish muscles, the internal organs of fish showed a higher count, with 3.54 ± 3.63 items per fish, compared to 1.74 ± 5.10 items per fish in the muscles. Statistically, a Mann-Whitney U test indicated significant difference in microplastic concentrations between these two parts. The mean rank for microplastic concentration in internal organs, 48.56 was significantly higher than the mean rank of 30.44 for fish muscles, U = 78, z = 3.671, p = 0.0002.

**Variation of microplastic characteristics in river fishes**

Microplastics extracted from fish samples caught from the Sepanggar River exhibited variations in shape, colour, size, and polymer type composition. Fig. 4 depicts the percentage composition of microplastic shapes, colours, sizes, and polymer types found in all fish samples caught from the Sepanggar River, while Fig. 5 illustrates the percentage composition of these microplastics for each fish species (n ≥ 3). Microplastics in those fishes were primarily small-sized (< 1mm), constituting 97% of the total microplastics (Fig. 4). The pattern of small microplastics dominating was similar across different fish species as well, with microplastic extracted from *P. lineatus* and *L. equula* accounting for 100% of small-sized microplastics. Among these, fragments were the most prevalent, making up 54% of the microplastics, followed by films at 36%. This dominance of fragment was also observed in most individual fish species, except for *L. equula*, where film was the highest at 76%. In terms of microplastic colours, the fishes exhibited the highest percentage of black microplastics, accounting for 40%, followed by blue (21%), red (19%), yellow (16%), transparent (3%), and white (1%). All fish samples caught from the Sepanggar River predominantly contained black microplastics, except for *A. venosus* which recorded the highest percentage of yellow microplastics (32%).

Polymer type analysis by micro-FTIR revealed that microplastics in this study consisted of nine different polymer types. Fig. 6 shows the spectra of polymer types identified from extracted microplastics from fish samples in this study. Rayon accounted for the highest polymer type (23%), followed by polyurethane (PU) at 20%, and both polyamides (PA) and polypropylene (PP) at 13% each. Similarly, microplastics in *Glossogobius* sp. (43%) and *P. lineatus* (22%) were also predominantly rayon. However, *L. equula* and *A. venosus* ingested the highest amounts of PA (24%) and PU (54%), respectively.

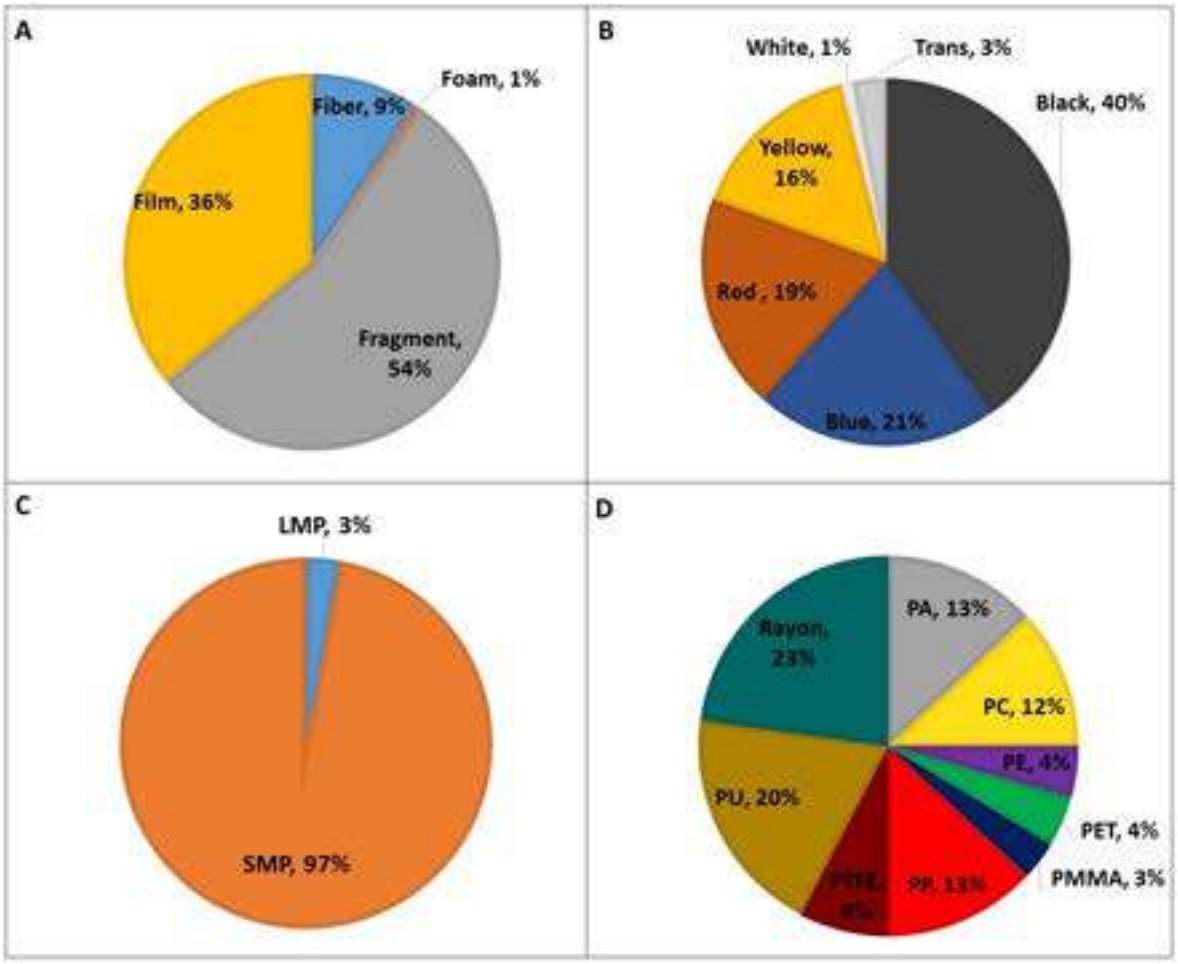


**Figure 4:** Composition of microplastic **A.** Shape, **B.** Colour, **C.** Size and **D.** Polymer type in fish samples caught from the Sepanggar River.

Although the characteristics of microplastic composition varied between fish species, only fibre as well as black and red colour microplastics were statistically significant ($p < 0.05$) among the fish species as presented in Table 4. Table 5 summarizes the significant pairwise comparisons of fish species for multiple comparisons for microplastic characteristics. *Plotosus lineatus* ingested significantly ($p < 0.05$) more fibres than *A. venosus*. Microplastics in *Glossogobius* sp. on the other hand were of higher black ($p = 0.046$) and red colour ($p = 0.049$) compared to *A. venosus*. Red colour microplastics in *Glossogobius* sp. were also significantly higher than *P. lineatus* at $p = 0.037$.

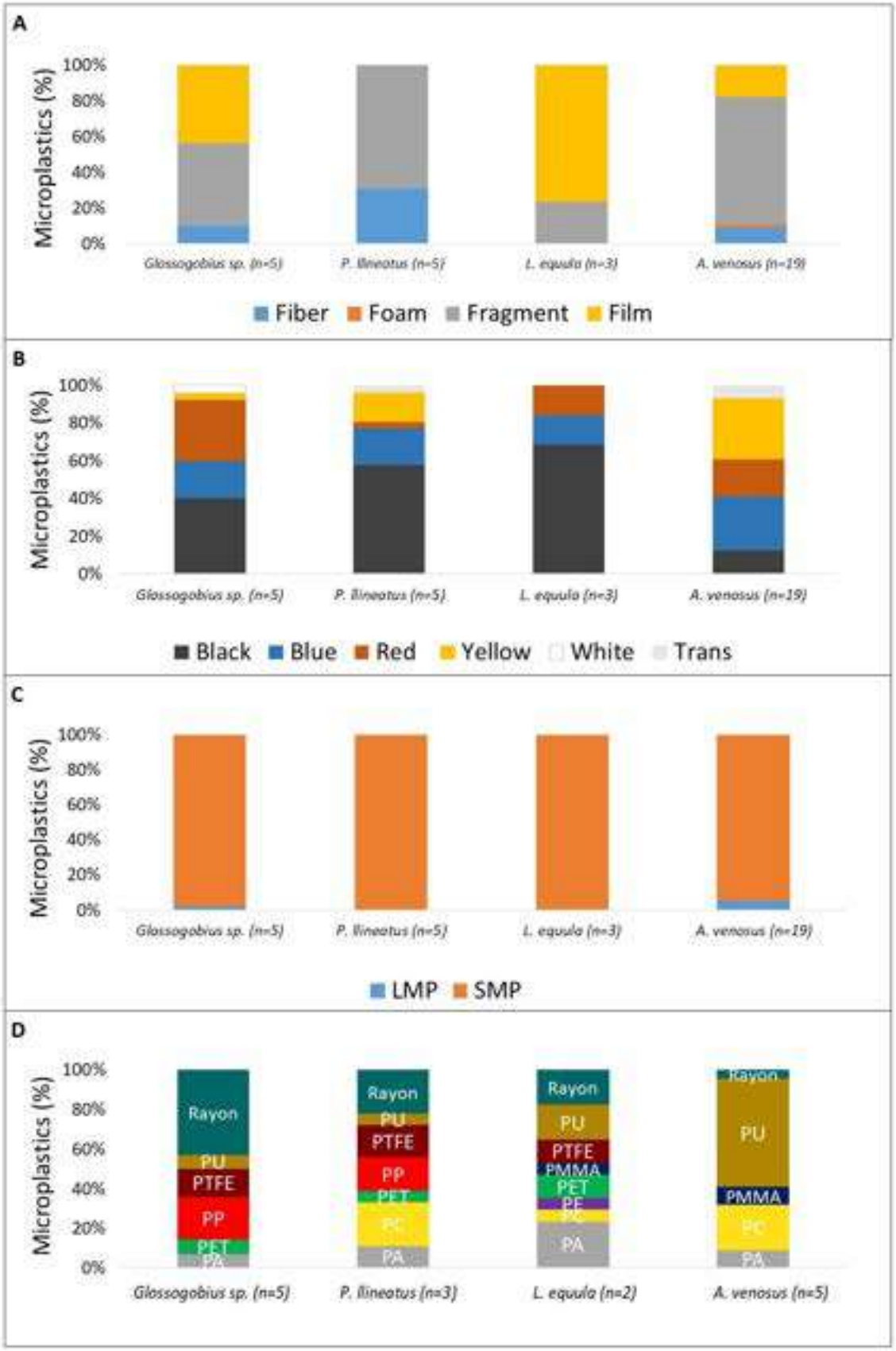


**Figure 5:** Composition of microplastic **A.** Shape, **B.** Colour, **C.** Size and **D.** Polymer type in selected fish species caught from the Sepanggar River.

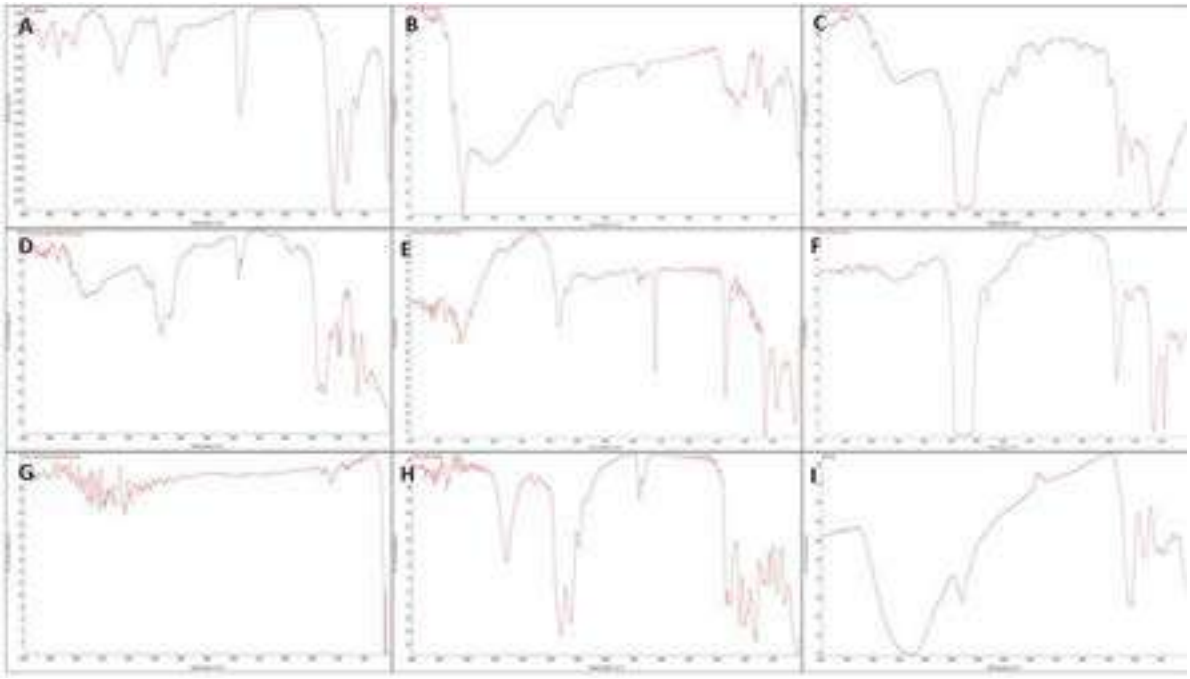


**Figure 6:** Micro-FTIR spectrum of polymer type **A.** Polyamides (PA), **B.** Polycarbonate (PC), **C.** Polyethylene (PE), **D.** Polyethylene Terephthalate (PET), **E.** Poly Methyl Methacrylate (PMMA), **F.** Polypropylene (PP), **G.** Polytetrafluoroethylene (PTFE), **H.** Polyurethane (PU) and **I.** Rayon.

**Table 5:** Pairwise comparisons using Dunn's (1964) procedure with a Bonferroni correction for multiple comparisons for significant parameters.

| Parameter | Fish species | Test Statistics | Adjusted $p$ value |
|---|---|---|---|
| Fibre | *Plotosus lineatus* > *Arius venosus* | 10.521 | 0.049 |
| Black | *Glossogobius* sp. > *Arius venosus* | 11.563 | 0.046 |
| Red | *Glossogobius* sp. > *Plotosus lineatus* | 15.000 | 0.037 |
| | *Glossogobius* sp. > *Arius venosus* | 11.500 | 0.049 |

*Higher mean rank is indicated by the symbol (>)

## DISCUSSION

### Microplastic ingestion by fish in the Sepanggar River

Based on a single sampling event, 77% of fish collected from Sepanggar River (n = 39) contained microplastics. This study provides the first confirmation that fish in the Sepanggar River were contaminated with microplastics, highlighting the global challenge of escalating microplastic contamination in aquatic organisms, including fish. With no national or standardised regulations addressing microplastic contamination in river fish, the concentrations were compared with other studies to gauge the overall microplastic contamination status in river fish. Previous research has predominantly focused on microplastic ingestion in separate parts of the fish, i.e., the gastrointestinal track and gills, with only a few that analysed fish tissue (Table 6), instead of the whole fish. In comparison to previous studies, this study analysed microplastics in internal organs separately from the fish tissue (muscles). Similarly, various available literature also shows ranges of percentages of microplastics in their studied river fishes. Overall, fish samples caught from the Sepanggar River were moderately polluted compared to fish in other studies. This study detected microplastics in both fish muscles and internal organs of the fish; however, the concentrations of microplastics in fish muscles were

significantly lower than those in their internal organs such as gills, stomach, and intestines ($p < 0.05$). Likewise, fish in Songkhla River in Thailand also demonstrated a lower abundance of microplastics in the fish muscles compared to their internal organs (Pradit et al., 2023; Jitkaew et al., 2024).

All fish species that were caught from Sepanggar River in this study are demersal fish that feed on invertebrates such as small crustaceans, molluscs, worms, and tiny fish from demersal habitat except for *Pennahia* sp. and *Glossogobius* sp. which are benthopelagic habitat fish species (Table 2). Sarijan et al. (2019) and Sultan et al. (2023) reported that bottom-feeding species inhabiting benthic-pelagic and demersal habitats exhibited the highest microplastic ingestion rate. It is postulated that the bottom sediment contains higher microplastic abundance compared to the water column (Ismanto et al., 2023; Karing et al., 2023). Therefore, these bottom-feeding fishes may mistakenly ingest microplastics directly from sediment when scavenging or feed on benthic organisms inhabiting in the benthic realm that were contaminated with microplastics (James et al., 2021).

Although not statistically significant, the highest microplastic ingestion by *L. equula* ($12.77 \pm 16.26$ items/fish) corresponds to it being an omnivorous fish as omnivorous fish have a greater likelihood and higher risks of ingesting microplastics compared to herbivorous and carnivorous fish due to their diverse habitat interaction (James et al., 2021; Yasaka et al., 2022; Sutan et al., 2023). Similarly, *Diapterus auratus* is also an omnivorous fish that had high microplastic count ($9.20 \pm 0.00$ items/fish) likely due to microplastics resembling items in its diet, which includes organisms such as ostracods, copepods, and nematodes. *Glossogobius* sp. were among the second highest most ingested microplastic fish as they inhabit and feed in both benthic and midwater zones, making them susceptible to microplastic contamination from the water column as well as settled microplastics in bottom mud sediments (Kibria, 2023).

**The potential sources of microplastic ingested by fish**

Microplastics extracted from fish samples caught from the Sepanggar River exhibited a diverse range of characteristics, including variations in shape, colour, size and polymer type. All microplastics ingested by these fish were secondary microplastics originating from the fragmentation of larger plastics, as evidenced by the absence of pellets in their internal organs or muscles. The diverse array of microplastic characteristics extracted from these fish offers insight into their origins. The presence of secondary microplastics in the river fish suggests that they originate from various activities along the Sepanggar River, contributing to variations in their ingested compositions of microplastic characteristics. The major sources of microplastics identified in this study were materials released from domestic waste discharge and industrial activities along the upstream.

Sepanggar River is situated within an urbanised area characterised by dense residential zones and is influenced by upstream rivers passing through active industrial areas, automotive workshops, tyre shops, furniture shops, and densely populated residential neighbourhoods. Additionally, plastic waste such as wrappers, bags, and sachets were seen floating on the surface waters during field sampling, likely discarded from the adjacent residential areas. The highest percentages of fragments (54%) in fish were therefore possibly released from the fragmentations of hard plastic such as plastic bottles, drums, containers, and other materials derived from those residential and industrial activities (Singh et al., 2022; Sultan et al., 2023). Previous studies also reported fragments as highly ingested microplastics in river fishes sourced from residential activities (Karbalaei et al., 2019; Yasaka et al., 2022).

**Table 6:** Summary of the microplastics concentration (Items/fish) in different parts of tidal river fishes in Southeast Asia.

| Location | Method | Fish part | Number of species, (n= ind.) | MP occurrence (%) | Concentration (Items/fish) | Reference |
|---|---|---|---|---|---|---|
| Barangay Britania, Philippines | Fishers | GIT | 5(n=180) | 12 | 4.00 ± 2.00 | Gomez et al. (2020) |
| Lubuk Yu River, Pahang | Fish net | GIT | 8(n=32) | 33-50 | NA. | Harith et al. (2021) |
| Melayu River, Johor | Captured wild | GIT | 3(n=14) | NA. | 4.50 ± 4.50 | Primus & Azman (2022) |
| Nam Pong River, Thailand | Trawling/Netting | GIT | NA. | 38 | 7.60 ± 17.70 | Yasaka et al. (2022) |
| Kuala Selangor | Fishers | GIT | 1(n=12) | NA. | 1.75 ± 1.92 | Lim et al. (2023) |
| Kuantan, Pahang | Fishers | GIT | 1(n=32) | NA. | 2.34 ± 1.98 | Lim et al. (2023) |
| Mukah, Sarawak | Fishers | GIT | 1(n=5) | NA. | 4.00 ± 0.63 | Lim et al. (2023) |
| Sungai Besar, Selangor | Fishers | GIT | 1(n=13) | NA. | 2.62 ± 0.61 | Lim et al. (2023) |
| Songkhla, Thailand | Fishers | Gills, stomach | 1(n=40) | 81 | 2.60 ± 0.28 | Pradit et al. (2023) |
| Songkhla Lagoon, Thailand | Fishers | Gills, stomach | 1(n=35) | 100 | 3.06 ± 0.42 | Jitkaew et al. (2024) |
| Nam Pong River, Thailand | Trawling/ Netting | Tissue | NA. | 0 | 0.00 ± 0.00 | Yasaka et al. (2022) |
| Songkhla, Thailand | Fishers | Tissue | 1(n=40) | 81 | 1.55 ± 0.19 | Pradit et al. (2023) |
| Songkhla Lagoon, Thailand | Fishers | Tissue | 1(n=35) | 100 | 1.97 ± 0.19 | Jitkaew et al. (2024) |
| Sepanggar River | 3-layer net | Gills, organs | 8(n=39) | 74 | 3.54 ± 3.63 | Present study |
| Sepanggar River | 3-layer net | Tissue | 8(n=39) | 44 | 1.74 ± 5.10 | Present study |

The film, which is the second-highest microplastics (36%) ingested by fish samples in this study, likely originated from food packaging and plastic bag manufacturing released from the above activities (Sang et al., 2021). The small proportions of fibres ingested by fish samples may derive from synthetic fibres potentially released into watercourses through domestic wastewater discharge, from wear and tear during clothes washing or fibre materials from residential areas (Anuar et al., 2023).

The sources of microplastics that are ingested by fish samples in the Sepanggar River are supported by the polymer type analysis. The highest polymer type obtained; rayon (23%) in the present study suggested that these semi-synthetic cellulose-based polymer fibres could be released from washing activities while the second highest polyurethane (PU) polymer type (20%) suggested that these materials potentially stem from manufacturing factories, such as those used in shoe soles, car seats, furniture, and mattresses. The polyethylene (PE), polypropylene (PP), and polytetrafluoroethylene (PTFE) polymer types collectively accounted for 25% of the analysed polymer types from the extracted microplastics in fish are commonly found in household items. For example, PE is extensively used in the manufacturing of various items such as plastic bags and films, releasing film-shape microplastics, while PP materials used as containers for plastic bottles, pipes, plumbing components, as well as toys and household goods, release fragment-shape microplastics (He et al., 2020). Conversely, PTFE is a microplastic polymer commonly found in non-stick coatings for both cookware and labware, while PP is frequently utilised in the production of plastic materials for packaging and fishing gear (Hwi et al., 2020).

**Study limitations and future directions**

The present study serves as an initial effort to delve into microplastic contamination in wild fish caught from the Sepanggar River in the Sabah region. Although the results offer solid evidence of fish contamination by microplastic in Sabah and insightful information on possible food safety for the local population, it is important to acknowledge a number of limitations that could affect how the findings are interpreted. The primary drawback of the present study is its one-time sampling strategy, which might not adequately represent the dynamic fluctuations of microplastic contamination in fish throughout the year. Microplastic levels in aquatic environments can fluctuate due to seasonal changes, weather events, and human activities (Nithin et al., 2022; Johnson et al., 2020). Additionally, the small sample size of 39 fish may not fully represent the microplastic contamination levels present in the Sepanggar River, potentially skewing the findings. Unequal sample sizes between species also make it difficult to conclude microplastic ingestion across different fish species. Certain species may have been under-represented, and the variability in feeding habits may not have been adequately reflected. This singular snapshot may not represent the broader patterns of contamination, limiting our ability to generalise the results to the entire fish population in the Sepanggar River.

Future research should aim to address these limitations by conducting multiple samplings across different seasons and tidal conditions to capture temporal variations in microplastic concentrations. Increasing the sample size will enhance the robustness of the findings and allow for more comprehensive comparisons of microplastic ingestion among various fish species inhabiting the Sepanggar River. Such data will also allow for more meaningful comparisons of microplastic concentrations among different feeding guilds. Furthermore, research may look into the relationship between microplastic contamination and environmental factors, such as water quality and sediment composition, which may influence microplastic levels and their accumulation in fish tissues. Integrating findings across water, sediment, and fish will provide

a more holistic understanding of the dynamics of microplastics in riverine systems (Sayed et al., 2021; Blankson et al., 2022).

## CONCLUSION

This study marked the first investigation into the microplastic contamination in fish from the Sepanggar River, an urbanised river in Kota Kinabalu, Sabah. The findings of this study demonstrated that fish from the Sepanggar River were contaminated with microplastics, with significantly higher microplastics found in internal organs than those in muscle. Microplastics ingested by the fish samples were all small-sized secondary microplastics consisting primarily of fragments, film, fibres and foam shape potentially sourced from residential waste disposal and industries activities nearby the river. Therefore, proper waste management for domestic household discharge and industrial activities is recommended to reduce the microplastic contamination risk on Sepanggar River fish in the long run.

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## DECLARATIONS

**Research permits.** This study was conducted with the approval of the Sabah Biodiversity Council [Access License Ref. - JKM/MBS.1000-2/2 JLD.16 (53–55)].
**Ethical approval/statement.** This study was conducted with the approval from Animal Ethics Committee UMS (Ref no: AEC0031/2022).
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Morphological and Genetic Characterisation of Seahorse Species (Syngnathidae: *Hippocampus* spp.) in the Waters of Sabah, Malaysia.

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## ABSTRACT

Seahorse are teleosts belonging to the genus *Hippocampus* which consists of 57 species. Among the 57 species, only 12 species exist in Malaysian waters and 11 species in the coastal waters of Sabah. These records and checklists pre-date 2015, and no known studies or field surveys have been conducted in Sabah since then. In this study, a field survey was conducted in selected areas of Sabah waters to document the species composition through morphological and genetic identification. Out of the 11 species that were recorded previously, only two species (*Hippocampus barbouri and Hippocampus comes*) were found and identified using taxonomic keys. CO1 mitochondrial gene was used for genetic identification and phylogenetic tree reconstruction of Maximum Likelihood (ML). The dataset comprises sequences of 11 species from Malaysian waters (excluding *H. satomiae*). The genetic distances, i.e., p-distances, for *H. barbouri* and *H. comes* were recorded to be less than 1% inter-species and more than 10% intra-species, which confirmed the distinct species genetically. Furthermore, findings highlight the urgency of implementing conservation strategies to protect the remaining populations, in light of limitations of this study.

**Keywords:** Syngnathids; Barbour's seahorse; tiger-tail seahorse; morphology; phylogeny; Borneo.

## INTRODUCTION

Seahorses, the members of the genus *Hippocampus* are a group of captivating marine fishes belonging to the family Syngnathidae. This genus consists of 57 species, all of which form a diverse group. A total of 12 species in the Hippocampinae family namely, *Hippocampus barbouri, Hippocampus bargibanti, Hippocampus comes, Hippocampus denise, Hippocampus histrix, Hippocampus kelloggi, Hippocampus kuda, Hippocampus satomiae, Hippocampus spinosissimus, Hippocampus mohnikei, Hippocampus pontohi* and *Hippocampus trimaculatus* were recorded in Malaysia. Eleven species were found in Sabah but the presence of *H. mohnikei* has not been confirmed. The *Hippocampus* species has a body that is encased in a ring like rigid plates where the body is maintained in a vertical posture with the head bent towards the front forming a 90° sharp angle (Kuiter, 2000). Some general characteristics alone are not enough to identify them to species level but small details such as number of rings, height of coronet, and sharpness of spines are one of morphological characteristics that distinguish one species from another. The rings are divided into two: trunk rings which are the uppermost rings seen from the dorsal view to the ring immediately above the anal fin, and tail rings which are counted from the ring just below the anal fin to the ring before the tip of the (Wilson et al., 2001). Beside the morphological traits, seahorses also have a unique reproductive character, where males carry the fertilized eggs in a specialised brood pouch until the young are ready to be born (Stölting & Wilson, 2007). Seahorses inhabit subtropical and tropical shallow coastal waters, including threatened habitats, such as seagrass beds, coral reefs, mangroves, and river mouths (Lourie et al., 2004). They exhibit a range of vibrant colours and intricate patterns, which not only add to their allure but also serve as camouflage against predators (Wallis, 2004). Seahorses play a vital regulatory role in the marine ecosystem as both prey and predator. As predators, they control populations of small crustaceans and plankton. This in turn helps maintain balance in the food web by regulating the abundance of these organisms (Trewhella & Hatcher, 2017). Simultaneously, as prey, they provide sustenance for larger marine species, creating a critical link in the trophic chain (Trewhella & Hatcher, 2017). The presence of seahorses can therefore serve as an indicator of the overall health and integrity of these valuable marine ecosystems (Delunardo et al., 2015).

Despite their enchanting appearance and ecological importance, seahorses face significant threats. Habitat destruction due to coastal development, pollution, and destructive fishing practices have severely impacted their populations (Lim et al., 2011). Additionally, seahorses are often caught for the aquarium trade and traditional medicine, further endangering their numbers (Lourie et al., 1999). The combination of these factors makes seahorses vulnerable to population decline, highlighting the need for conservation efforts. The seahorse records and checklist data pre-dates to 2015, with Lim et al. (2011) conducting a study in 2011 on the diversity, habitats, and conservation threats of syngnathid fishes in Malaysia, while in 2015, Shapawi et al. (2015) explored the species and size composition of seahorses in the coastal waters and local markets of Kota Kinabalu, Sabah, Malaysia. As mentioned by Chen et al. (2021), there has been a lack of recent comprehensive surveys of seahorse populations in Sabah waters.

This study therefore aims to fill critical knowledge gaps by incorporating both morphological and genetic identification techniques to accurately document the *Hippocampus* species diversity in Sabah waters. Additionally, as previous research has primarily focused more on Peninsular Malaysia species (Lim et al., 2011; Ng et al., 2024), this study provides valuable data that further

builds our understanding of seahorses in Sabah. By combining traditional and modern approaches, this study not only enriches the knowledge of seahorse taxonomy but also sets the stage to support future biodiversity management and conservation initiatives in Sabah.

## MATERIALS AND METHODS

Fig. 1 shows the sampling sites that were covered in the present study. A total of 21 sampling stations were selected to carry out this field survey guided by occurrence information from past literature (Lim et al., 2011; Shapawi et al., 2015; Chen et al., 2021). These locations were chosen and prioritised, as this maximises the likelihood of encountering *Hippocampus* species by considering habitats and environmental conditions favourable for seahorses. In Table 1, detailed information on the sampling stations is recorded. The samples were collected using several non-destructive techniques during sampling to minimize the impact to habitat destructions: (i) SCUBA diving for seahorse surveys based on information from literature, dive centres, and local communities; (ii) purchasing samples from local fishermen, who provided confirmation that the samples were collected from the region with an estimated known location; and iii) using scoop net and dip nets to obtain seahorse species. On-site photographs of the specimens were taken prior to preservation to facilitate accurate species identification as the colouration of fish specimen often fades or becomes discoloured after preservation in ethanol (Carter, 2003). All collected samples were submerged in 99% undenatured ethanol solution after dipping them into chilled freshwater for DNA barcoding.

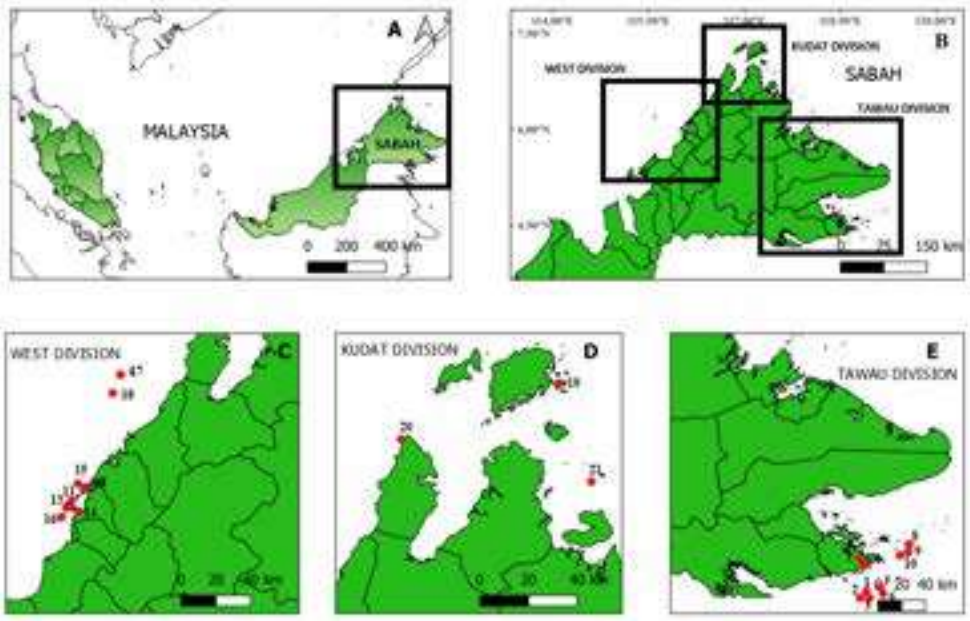


**Figure 1:** Sampling sites of seahorses in Sabah, Malaysia (1–21). **A**. Map of Malaysia with Sabah marked out. **B**. Map of Sabah with divisions marked out. **C**. Sampling locations in the West Division. **D.** Map outline of Kudat Division **E.** Map outline of Tawau Division. Sampling sites are plotted in red dots on three respective divisions.

Procedures were carried out according to ethical standards of Researcher's Guidelines on Code of Practice for the Care and Use of Animal for Scientific Purposes (JHEUMS) and deposited in the collection of the Borneo Marine Research Institute (IPMB).

For morphological identification, meristic and morphometric measurements were measured up to the nearest centimetre (cm) in the laboratory (Lourie et al., 2004). The sex of collected specimens were distinguished by the presence of the brood pouch, which would indicate that the specimen was a male (Shapawi et al., 2015). The identification of seahorses was carried out using published taxonomic keys (Lourie et al., 1999; Kuiter, 2000; Lourie et al., 2004). Morphological traits such as trunk rings, tail rings, and spines are key taxonomic features for distinguishing seahorse species, as they vary among species (Lourie et al., 1999; Kuiter, 2000; Lourie et al., 2004).

For DNA extraction, approximately 5–10 mg of tissue from the specimen's tail was cut and inserted into a 1.5 ml microcentrifuge tube. The extraction was performed using the Toyobo MagExtractor™ Genome (NPK–101). The mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (universal primer pair FishF1: 5'- TCA ACC AAC CAC AAA GAC ATT GGC AC -3'; FishR1: 5'- TAG ACT TCT GGG TGG CCA AAG AAT CA -3'), was used as a genetic marker for seahorses and pipefishes for the present study (Zhu et al., 2013; Chao et al., 2014; Chaiphongpachara et al., 2022). Gene amplification was performed using the protocol of Taq Polymerase (Vivantis PL1204, Malaysia) with 25µl reaction containing 13.3µl sterile distilled water, 2.5µl ViBuffer A (10x), 2µl dNTPs Mix (2.5 mM each), 1µl MgCl2 (50 mM), 2µl of each primer (10µM), 0.2µl Taq polymerase (5 u/µl) and 2µl DNA template. Thermal cycling was performed with initial denaturation at 94°C for 2 minutes, denaturation at 94°C for 30 seconds, followed by 30 cycles annealing at 54°C for 30 seconds, and elongation at 72°C for 30 seconds, with an additional extension step of 7 min at at 72°C. Amplicons were visualized on a 1.8% agarose gel after the electrophoresis. Purification of amplicons was carried out using Monarch® Genomic DNA Purification Kit, and sequencing was done using Sanger Sequencing (Apical Scientific Sdn. Bhd). All obtained sequences were deposited in GenBank (Accession no. PP859224–PP859234).

The obtained nucleotide sequences were used to construct a phylogenetic tree based on Maximum Likelihood (ML). The COI dataset for the present study included only 11 sequences out of 12 recorded seahorse species found in Malaysian waters (except for *Hippocampus satomiae* that was unavailable in GenBank). *Corythoichthys haematopterus* (messmate pipefish) was used as an outgroup. ClustalX was used for multiple sequence alignment as proposed in (Thompson et al., 2003) and (Ng et al., 2022, 2023). The Bayesian Information Criterion (BIC) in jModelTest v.2.1.10 (Darriba et al., 2012) identified the Tamura-Nei model as optimal for the present dataset. The Maximum Likelihood tree was generated with 500 bootstraps, and intra- and inter-species p-distances were determined using MEGA-X (Kumar et al., 2018) and (Ng et al., 2022, 2023).

**Table 1:** List of sampling stations along with their respective coordinates, habitat type of that station, number of species count, and type of *Hippocampus* spp, and the sex of each obtained species.

| Station | Division | Name of Location | Habitat Type | Count | Species | Sex | Survey Date |
|---|---|---|---|---|---|---|---|
| 1 | Tawau Division | Mabul House Reef | Reef | — | — | — | 06 September 2023 |
| 2 | | Mabul Paradise 2 | Reef | — | — | — | |
| 3 | | Mabul Paradise 1 | Reef | — | — | — | |
| 4 | | Tampi-Tampi Island | Sandy Area | 2 | *Hippocampus comes* | 1 male<br>1 female | |
| 5 | | Kapalai House Reef 1 | Reef | — | — | — | |
| 6 | | Kapalai House Reef 2 | Reef | — | — | — | 07 September 2023 |
| 7 | | Semporna mangrove | Mangrove | — | — | — | |
| 8 | | Mataking Island | Sandy - Reef | — | — | — | 08 September 2023 |
| 9 | | Timba- Timba Island | Reef | — | — | — | |
| 10 | | Pandanan Island | Sandy area | — | — | — | |
| 11 | West Division | ODEC, UMS | Sandy Area | 1 | *Hippocampus comes* | 1 male | 17 April 2023 |
| 12 | | Mengakabong | Mangrove | — | — | — | |
| 13 | | Pulau Sepanggar | Sandy Area | — | — | — | |
| 14 | | Tentera Laut Diraja Malaysia (Seagrass Patch) | Seagrass | 2 | *Hippocampus barbouri* | 2 female | 22 March 2023 |
| 15 | | Kibagu Island | Sandy - Seagrass patch | 1 | *Hippocampus barbouri* | 1 female | 22 March 2023 |
| 16 | | Pulau Gaya | Sandy Area | 2<br>1 | *Hippocampus barbouri*<br>*Hippocampus comes* | 1 male<br>1 female<br>1 female | 08 June 2022 |
| 17 | Kudat Division | Pulau Mantanani | Sandy - Reef | — | — | — | 05 November 2022 |
| 18 | | Pulau Pandan | Sandy - Reef | — | — | — | |
| 19 | | Pulau Sibogo | Reef | — | — | — | |
| 20 | | Simpang Mengayau | Reef | — | — | — | 23 July 2022 |
| 21 | | Pulau Tigabu | Reef | — | — | — | |

Note: '—' = No individual that belongs to the genus *Hippocampus* was found in that specific location.

## RESULTS

Two species of seahorses were collected from six of the 21 surveyed stations around Sabah, i.e., *Hippocampus barbouri* (# IPMB- I 01.00185 — IPMB- I 01.00190) and *H. comes* (# IPMB- I 01.00191, IPMB- I 12.00314, and IPMB- I 12.00315) (see Tables 1 and 2). The results of this study highlight key findings on morphological and genetic information of *H. barbouri* and *H. comes* from Sabah waters. Significant variations in physical traits and colouration were observed between species alongside information on the habitat. Phylogenetic analysis based on mitochondrial DNA (CO1), further clarifies the relationship between these two species and among other species in the same genus.

### Systematics

Family **SYNGNATHIDAE** Bonaparte, 1831

Genus ***Hippocampus*** Rafinesque, 1810

***Hippocampus barbouri*** Jordan & Richardson, 1908
(Figs. 2–3)


**Figure 2:** Female specimen of *Hippocampus barbouri* (IPMB-I 01.00185) collected from Pulau Gaya, Sabah, Malaysia.

**Material examined:** IPMB-I 01.00185, female, total length 6.7cm, location Pulau Gaya, habitat type sandy area, depth 1–4m, collection date 08 June 2022; IPMB-I 01.00187, male, total length 7.8cm, location Pulau Gaya, habitat type sandy area, depth 1–4m, collection date 08 June 2022; IPMB-I 01.00190, female, total length 7.9cm, location Kibagu Island, habitat type sandy-seagrass patch, depth 1–4m, collection date 22 March 2023; IPMB-I 01.00188, male, total length 10.5cm, location Tentera Laut Diraja Malaysia habitat type seagrass patch, seagrass area, depth 3m, collection date 22 March 2023; IPMB-I 01.001889, male, total length 5.4cm, location Tentera Laut Diraja Malaysia seagrass patch, habitat type seagrass area, depth 3m, collection date 22 March 2023.

**Description**

11 trunk rings; 33 tail rings were recorded for the observed specimens; two cheek spines and 1 sharp eye spine. Dorsal fin rays 17; pectoral fin rays 15; distinct raised high coronet (5 sharp spines); 2 + 1 rings supporting the dorsal fin; zebra- striped snout. The anal fin is absent; well-developed spine throughout the body; first dorsal trunk spine much longer than others and curved backwards; spines of different lengths in a regular series (e.g., long, short, long, short respectively). Snout appears narrow and loses its colour once submerged in ethanol, giving it a translucent look; double spines below eye; and body often covered with black spots. Fine lines radiate from the eye. Supraorbital spines are prominent, simple and acute. The nasal spine appears sharp.

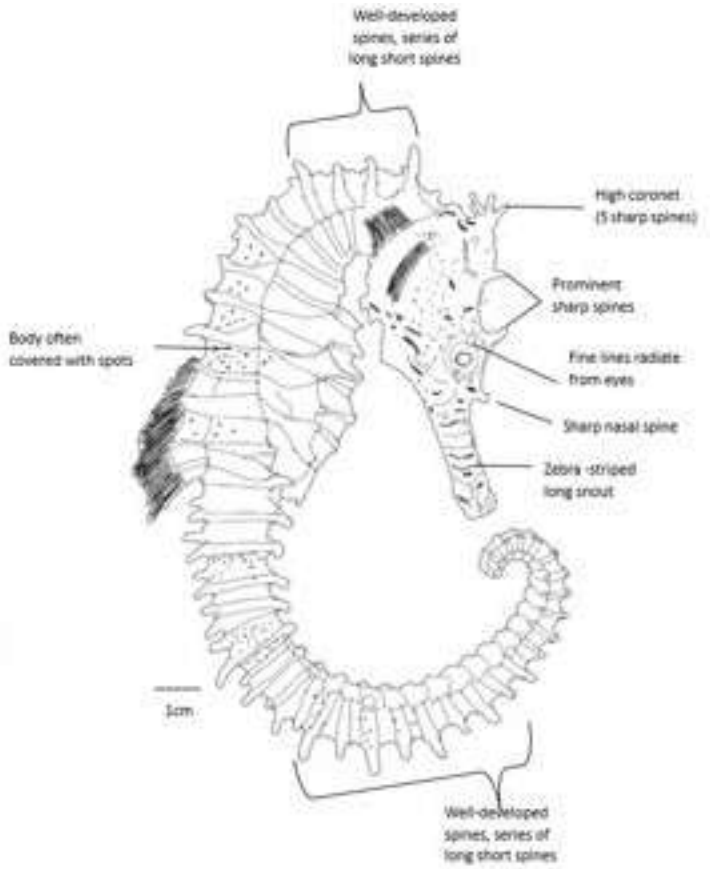


**Figure 3:** A detailed sketch of a female specimen of *Hippocampus barbouri* (IPMB-I 01.00185) collected from Pulau Gaya, Sabah, Malaysia. Scale bar, 1cm

**Measurements:** The mean total length (TL) of *H. barbouri* is 7.66 ± 3.60 (Standard deviation, SD) while mean snout length is 0.88 ± 0.28 (SD) for the collected samples. As for comparison, the highest recorded total length of *H. barbouri* to date is 15cm while length of snout falls in the range of 2.0–2.6 (Lourie et al., 1999). Additional meristic and morphometrics data are recorded in (Table 2).

**Colour in life:** Overall *H.barbouri* has an opaque colour ranging between white, pale yellow and pale brown (Fig. 2). Reddish, brown spots are present throughout the body. Dusky brown lines are present on body. Fine lines radiating from eye and stripes on the snouts range in colour shades of pale brown to dark brown. The tip of the spines is white in colour. However, after submerging in 99% undenatured ethanol, the specimen loses its colouration and became pale. Stripes on the snout became more visible. Black dots throughout the body became much more visible after being preserved for a period of time (Fig. 2).

**Habitat:** All individuals of *H. barbouri* for this field survey were collected in shallow waters, to a maximum depth of 5m. They were found in shallow seagrass patches parallel with previous reports (Lourie et al., 2005; Unsworth, 2021). Generally, the maximum reported depth for *H. barbouri* is at 10m (Kuiter, 2000). *Hippocampus barbouri* were often found clinging on to shallow seagrass beds using their prehensile tails; a common behaviour that has also been observed elsewhere (Lourie et al., 2016).

***Hippocampus comes* Cantor, 1849**
(Figs. 4–5)


**Figure 4:** Male specimen of *Hippocampus comes* (IPMB-I 12.00314), collected from Tampi-Tampi Island, Sabah, Malaysia.

**Material examined:** IPMB-I 12.00314, male, total length 15.1cm, location Tampi-Tampi Island, habitat type sandy area, depth 6m, collection date 6 September 2023**;** IPMB-I 12.00315, female, total length 15.1cm, location Tampi- Tampi Island, habitat type sandy area, depth 6m, collection date 06 September 2023; IPMB-I 01.00186, female, total length 7.3cm, location Pulau Gaya, habitat type sandy area, depth 1–4m, collection date 08 June 2022; IPMB-I 01.00191, male, total length 14.2cm, location ODEC, UMS, habitat type sandy area, depth 5m, collection date 17 April 2023.

**Description**

12 trunk rings; 32 tail rings were recorded for the observed specimens; two cheek spines and two eye spines. Dorsal fin rays 16; pectoral fin rays 18; 2 + 1 rings supporting the dorsal fin. This species possesses striped tail; small anal fin; pectorals appear to be shorter than dorsal, and anal fin is the shortest of all. All the spines of the salient angles are surmounted by oval or appear knob-like and blunt. A single spine appears to be present in between the head and the eyes. The coronet appears to be lower than the other species with five distinct rounded and knob-like spines. Possess a long and slender snout, and sometimes striped. The nose spine is sharp; double cheek spines and double spines below the eye. The body is heptagonal and its vertical diameter slightly exceeds the length of the snout to the nostrils. The tail is quadrangular, which is tapering into a point. The anus is situated nearly in the middle of the total length, opposite the posterior third of the dorsal.

**Measurement:** The mean total length (TL) of *H. comes* is 12.2 ± 5.51 (SD) while mean snout length is 1.23 ± 0.42 (SD) for the collected samples. As for comparison, the highest recorded total length of *H. comes* is 18.7cm (Project Seahorse, 2022) while length of snout falls in the range of 0.9 – 1.5 (Shapawi et al., 2015). while length of snout falls in the range of 0.9 – 1.5 (Shapawi et al., 2015). Number of pectoral fin rays and number of dorsal fin rays recorded are (16-18) and (17-19) respectively. Additional meristic and morphometrics data are recorded in (Table 2).

**Colour in life:** Overall, *H. comes* appears in hues of yellow and black, sometimes alternating; striped tail (although this may not be visible in dark specimens); molted or blotched pattern on body; may have fine white lines radiating from eye and rarely striped snout (Fig. 4).

**Habitat:** Individuals of *H. comes* were collected in shallow waters, to a maximum depth of 6m. They are typically found in a depth less than 10m and maximum reported depth of 20m (Kuiter, 2000). *Hippocampus comes* were found clinging on to artificial structures under the jetty using its prehensile tail. Past literature also recorded that *H. comes* inhabiting sandy areas clinging on to floating sargassum (Perante et al., 2002).

The morphological differences between *H. barbouri* and *H. comes* are central to distinguishing these closely related species, in particular, the individuals in this study showed key differences in characters, including in the snout, spine and colouration (see Table 3).

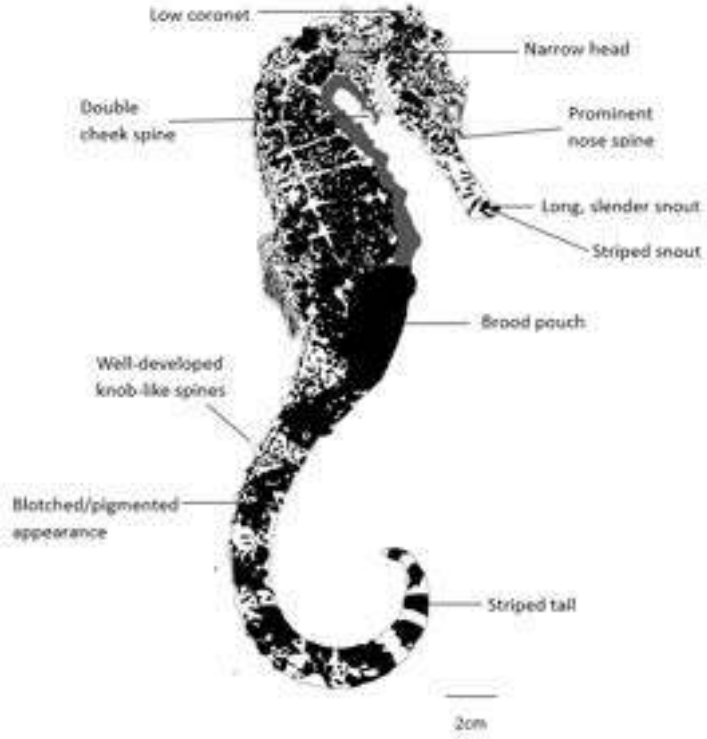


**Figure 5:** A detailed sketch a male specimen of *Hippocampus comes* (IPMB-I 12.00314), collected from Tampi-Tampi Island, Sabah, Malaysia. Scale bar, 2cm.

**Molecular characterization:** The Maximum Likelihood tree of COI (Fig. 6) consisting of 11 species that are found to exist in Malaysia including *H. barbouri* (observed species) and *H. comes* (observed species) was constructed. Overall, the ML tree of COI comprised one major clade as shown in Fig. 6. The observed specimen *H. barbouri* and *H. comes* in this study grouped together with the reference sequences of *H. barbouri* and *H. comes* with strong bootstrap value of 100% and 99%. *H. barbouri* grouped as sister species with *H. comes* in one sub clade with strong bootstrap value of 87%. The p-distance within *H. barbouri* is less than 1% while the distance of *H. barbouri* with molecularly closely related (*H. comes*) is less than 10% and morphologically closely related (*H. histrix*) is more than 10%. As for *H. comes*, the p-distance within *H. comes* is less than 1% while the distance with molecularly closely related (*H. barbouri*) is less than 10% and morphologically closely related (*H. kuda*) is 10%. Accordingly, based on comparison to published sequences of *Hippocampus*, the collected specimens in this study are confirmed as *H. barbouri* and *H. comes*, respectively.

**Table 2:** Meristic and morphometric characters examined in *Hippocampus* spp. seahorses collected from Sabah, Malaysia.

| **Species** | ***Hippocampus barbouri*** | | | | | | ***Hippocampus comes*** | | |
|---|---|---|---|---|---|---|---|---|---|
| **Accession number** | IPMB-I 01.00188 | IPMB-I 01.00189 | IPMB-I 01.00187 | IPMB-I 01.00185 | IPMB-I 01.00190 | IPMB-I 01.00186 | IPMB-I 01.00191 | IPMB-I 12.00314 | IPMB-I 12.00315 |
| **Location** | Tentera Laut Diraja Malaysia (Port Seagrass patch) | | Gaya Island | | Kibagu Island | Gaya Island | ODEC, UMS | Tampi- Tampi Island | |
| **Morphometrics (cm)** | | | | | | | | | |
| Total length | 10.5 | 5.4 | 7.8 | 6.7 | 7.9 | 7.3 | 14.2 | 15.1 | 15.1 |
| Height of coronet | 0.5 | 0.5 | 0.4 | 0.5 | 0.3 | 0.1 | 0.1-0.2 | 0.2 | 0.1 |
| Head length | 2.8 | 2.5 | 2.9 | 2.8 | 2.3 | 2.7 | 2.7 | 3.1 | 2.9 |
| Snout length | 0.9 | 0.8 | 1.1 | 0.9 | 0.7 | 0.9 | 1.1 | 1.5 | 1.4 |
| Eye diameter | 0.5 | 0.4 | 0.5 | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 |
| Tail length | 7.1 | 6.9 | 5.7 | 5.5 | 6.7 | 6.8 | 8.5 | 9.2 | 8.8 |
| Snout depth | 0.3 | 0.3 | 0.3 | 0.3 | 0.2 | 0.2 | 0.2 | 0.2 | 0.2 |
| Pectoral fin base length | 0.5 | 0.5 | 0.5 | 0.5 | 0.4 | 0.5 | 0.5 | 0.5 | 0.4 |
| Dorsal fin base length | 1.2 | 1.2 | 1.3 | 1.2 | 1.1 | 1.2 | 1.3 | 1.3 | 1.2 |
| **Meristic** | | | | | | | | | |
| Dorsal fin soft rays | 19 | 18 | 19 | 17 | 14 | 16 | 17 | 16 | 17 |
| Pectoral fin rays | 17 | 14 | 16 | 15 | 17 | 16 | 16 | 18 | 19 |
| Trunk rings | 12 | 11 | 11 | 11 | 11 | 11 | 11 | 12 | 12 |
| Tail rings | 35 | 31 | 33 | 33 | 32 | 35 | 30 | 32 | 35 |
| No. rings supporting dorsal fin | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring | 2 trunk ring and 1 tail ring |

Note: IPMB-1 = Code for Borneo Marine Research Institute Ichthyological collection; 01–12 = Collection locality code; .000* = Specimen collection number collected from the collection locality code.

**Table 3:** Comparative morphological traits of *Hippocampus barbouri* and *Hippocampus comes* in Sabah, Malaysia.

| **Key Traits** | ***Hippocampus barbouri*** **(Barbour's Seahorse)** | ***Hippocampus comes*** **(Tiger tail Seahorse)** |
|---|---|---|
| Height of Coronet | High | Low |
| Spines | Sharper and more prominent | Knob-like and rounded |
| Snout | Long, narrow and striped snout | Long, slender and rarely striped |
| Cheek spines | Double cheek spine | Double cheek spine |
| Body rings | More distinct, with sharper segments (long, short, long short spine series) | Fewer spines and more blunt trunk rings |
| Tail | No stripes | Striped/blotched/ pigmented appearance |
| Colouration | Pale to yellow or brownish shade | Dark brown to black shade |

**Table 4:** Pairwise p-distances of *Hippocampus barbouri and H. comes* with other closely related species.

| **Species** | ***H. barbouri*** | ***H. comes*** | ***H. kuda*** | ***H. kellogi*** | ***H. spinossisimus*** | ***H. pontohi*** | ***H. denise*** | ***H. trimaculatus*** | ***H. bargibanti*** | ***H. histrix*** | ***H. mohnikei*** | **Outgroup** |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| ***H. barbouri*** | < 0.01 | 0.06 - 0.07 | 0.11 - 0.12 | 0.10 - 0.11 | 0.11 - 0.12 | 0.16 - 0.17 | 0.16 - 0.17 | 0.11 - 0.12 | 0.16 - 0.17 | 0.09 - 0.10 | 0.11- 0.12 | 0.24 - 0.25 |
| ***H. comes*** | 0.06 - 0.07 | < 0.01 | 0.11 - 0.12 | 0.11 - 0.12 | 0.11 - 0.12 | 0.14 - 0.15 | 0.15 - 0.16 | < 0.10 | < 0.16 | 0.07- 0.08 | 0.11- 0.12 | 0.24 - 0.25 |

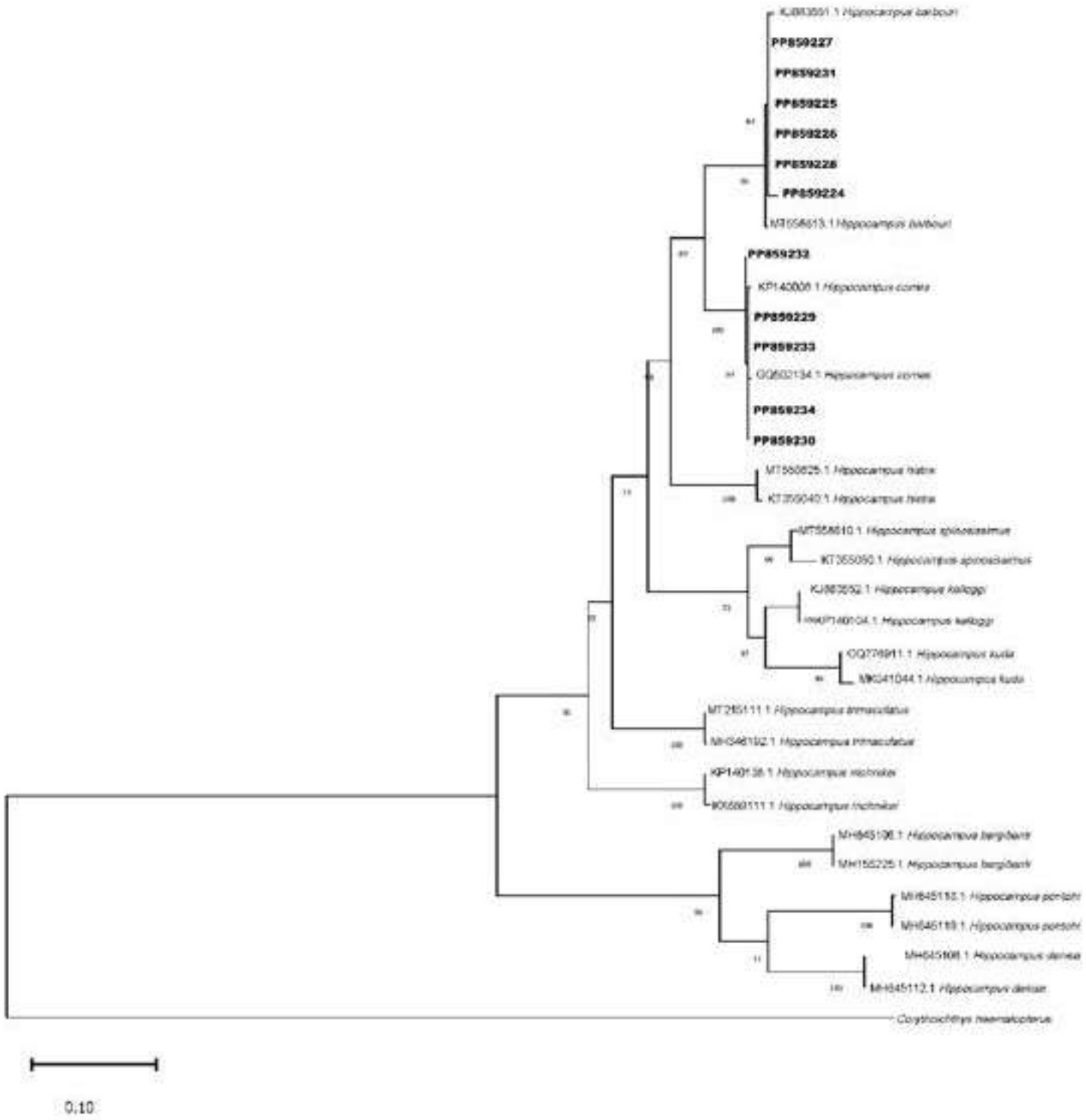


**Figure 6:** Maximum Likelihood (ML) tree inferred from the partial mitochondrial COI sequences of 11 species of *Hippocampus*, with *Corythoichthys haematopterus* as an outgroup. Values of the nodes correspond to bootstrap values; only values > 50% are shown. Samples from this study are highlighted in bold (**PP859224**: IPMB-I 01.00188; **PP859225**: IPMB-I 01.00187; **PP859226 & PP859231**: IPMB-I 01.00190; **PP859227**: IPMB-I 01.00189; **PP859228**: IPMB-I 01.00185; **PP859229 & PP859230**: IPMB-I 01.00191; **PP859232**: IPMB-I 12.00314; **PP859233**: IPMB-I 12.00315; **PP859234**: IPMB-I 01.00186)

## DISCUSSION

### Morphological observations

The morphological examination of the observed specimens of *H. barbouri* and *H. comes* revealed insights on the size variations within and between species. Among the collected specimens of *H. barbouri*, males were observed to be shorter in total length compared to females from the same sites. Although the current finding contradicted with (Faleiro & Narciso, 2011) and (Shapawi et al., 2015) in every seahorse species, further investigation is required for confirmation as only a few samples were obtained throughout our study in comparison to the previous studies that had a more robust sample size. However, in this study, apart from the key characteristics of each species, the most notable morphological difference observed is that *H. comes* is longer in total length compared to *H. barbouri*.

As for the colouration, among the collected specimens, the body colour of each individual varied from pale yellow to brown. This could be due to the environment they inhabit. During the field surveys, most of the *H. barbouri* were collected from sandy–seagrass patches. In order to camouflage itself to the sand flats, it turns to a yellowish-brown colour, which can account for the variation of colours in the collected specimens (Curtis & Vincent, 2005). Besides that, the *H. comes* individual collected during the field survey at Tampi-Tampi Island had skin discolouration compared to the individual that was collected from Gaya Island and ODEC, UMS. This could be due to the camouflage effects that all syngnathids possess. Syngnathids mimic vegetation in colour, shape and behaviour (Curtis & Vincent, 2005) which likely reduces their visibility to both predators and prey. The dark colours of *H. comes* collected could be due to camouflaging to the same colours as artificial structures.

### Phylogenetic analysis

Based on the results of phylogenetic analysis of the present study, *H. barbouri* is a sister species to *H. comes* and *H. histrix*. Morphologically, *H. barbouri* is often misidentified with *H. histrix* but is distinguished by the length of the snout, number of fin rays, sharpness of spines, and number of cheek spines (Kuiter, 2000). Eventhough *H. barbouri* and *H. histrix* may look similar t morphologically, the molecular evidence indicates that it is more genetically related to *H. comes*. The overlapping distribution and shared habitats between *H. comes* and *H. barbouri* may have facilitated more genetic exchange and closer evolutionary ties between these two species compared to the more geographically distant *H. histrix* (Knowles, 2009). A study by Nurilmala et al. (2019), that investigated a different marker, 16S, also found a close genetic relationship between *H. comes* and *H. barbouri*. Other global studies with larger datasets and more genetic markers have resulted in robust conclusions (Thangaraj & Lipton, 2011; Zhang et al., 2014). It is possible that estimation of genetic similarity in smaller datasets and using only single genetic markers, may lead to underestimation and affect the assessments of species relationships, which is a limitation of this study and should be addressed in future research.

### Taxonomic implications on genetic aspects

Morphologically, *H. barbouri* and *H. comes* are clearly different between each other. Although morphological identification can be sufficient for identifying seahorse species in Malaysia with the available taxonomic keys, the potential presence of cryptic species means that relying solely on external features may not always be reliable (Aylesworth et al., 2017; Woodall et al., 2018).

However, in Malaysia, seahorse surveys and monitoring programmes have traditionally relied on morphological identification techniques (Choo & Liew, 2003; Lim et al., 2011; Shapawi et al., 2015). This suggests the possibility of overlooking any presence of cryptic seahorses. Therefore, in our study, we incorporated molecular analyses as a crucial step to enhance the accuracy of species identification and address potential challenges in distinguishing morphologically similar seahorse species. Although our results did not uncover any cryptic species, the integration of molecular data has expanded our understanding of seahorse diversity and provided a valuable addition to traditional taxonomic classifications based on morphology alone.

**Limitations and future directions**

The outcomes of this study provide significant updates to the biodiversity records of *Hippocampus* species in Sabah, Malaysia. Out of 11 species historically reported in this region, only *H. barbouri* and *H. comes* were recorded based on morphology and molecular methods during this survey, which are in line with recent standard practice of using integrative methods to minimise misidentification in seahorses (Casey et al., 2004; Sanaye et al., 2020) Ongoing anthropogenic activities in Sabah such as excessive fishing pressure, fish bombing, and coastal development in the surveyed areas can potentially be destructive to seahorse populations (Wood & Ng, 2016). Although at a small scale, this study provides updated distribution and molecular data that may help refine species classifications, document range extensions, and highlight genetic linkages in Southeast Asian populations, which in turn is important for supporting conservation strategies.

One possible reason that this study recorded only 2 out of the 11 species previously recorded by (Lim et al., 2011) may have been because of the different sampling methods used, in particular, this study did not use trawl fishing, which has been banned and strictly enforced since 2013 (Nuruddin & Isa, 2013). In addition, the study may have overlooked significant populations of seahorses due to limited sampling, which focused on specific locations as indicated by previous studies (Lim et al., 2011; Shapawi et al., 2015). The reliance on visual surveys and manual capture techniques could introduce observer bias, as less conspicuous or cryptically coloured seahorses may have been overlooked (Brauwer et al., 2020).

Future research may include expanding the scope of surveys to include under-represented habitats (Hao et al., 2025), using multi-locus genetic analyses on larger sample sizes and broader geographical representation to strengthen the robustness of conclusions (Panithanarak, 2020), application of environmental DNA for more comprehensive surveys (Thomsen & Willerslev, 2014), promoting carefully designed aquaculture efforts to reduce the pressure on wild populations (Kumaravel et al., 2012), ensuring long-term monitoring and improved understanding of their population genetics. Regular monitoring of *Hippocampus* populations across a wider range of habitats in under-sampled regions (Cohen et al., 2017) should be established and addressing population decline should be prioritised to safeguard potential loss of seahorse diversity in Sabah waters and to ensure long term sustainability of seahorse biodiversity in the region.

## CONCLUSIONS

This study has successfully identified and characterized two seahorse species, *H. barbouri* and *H. comes* in Sabah waters through both morphological and genetic analyses. Detailed morphological

descriptions and genetic characterization using CO1 gene sequences provide a comprehensive taxonomic foundation for these species in the region. The phylogenetic analysis revealed a close genetic relationship between *H. barbouri* and *H. comes*, despite some morphological differences, highlighting the importance of integrating both morphological and molecular approaches in seahorse taxonomy and identification. The findings may not accurately represent a comprehensive updated database for the seahorse checklist in Sabah due to the limitations of this study. However, this study can be a reference to future researchers to seek alternative ways to monitor the status of seahorse population by using non-destructive methods such as environmental DNA (eDNA).

## ACKNOWLEDGEMENTS

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## DECLARATIONS

**Research permits.** This study was conducted with the approval of the Sabah Biodiversity Council Access License Ref. - JKM/MBS.1000-2/2 JLD.16 (39).
**Ethical approval/statement.** This study was conducted with the approval from Animal Ethics Committee UMS (Ref no: AEC0008/2023).
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Seasonal Abundance of Common Honey Bees and Floral Resources in Mixed Agriculture and Grassland Habitats

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## ABSTRACT

Beekeeping is a traditional practice that has played a significant role in the sustainable development of rural and tribal communities. The diversity and abundance of honey bees depend on the floral resources available and can be influenced by seasonal environmental changes. In the present study, the seasonal abundance of colonies of three common honey bees *Apis cerana*, *Apis florea* and *Apis dorsata* in a draught-prone tropical area in Karjat, Maharashtra, India was monitored for three consecutive years. Also, the floral resources of flowering plants and their seasonal composition were studied. The study was conducted in mixed agriculture and grassland habitats at 11 locations. A total of 4408 colonies of three honey bee species were observed. The study revealed that *A. florea* (57.42%) is the most abundant species followed by *A. cerana* (25.88%) and *A. dorsata* (16.70%), and their proportion remains similar in different seasons. A total of 72 plant species were recorded with nectar and pollen as floral resources. It is found that the proportion of agricultural crop plants is high in the monsoon season whereas the composition of flowering plants varies in different seasons. The results of the present study suggest that mixed agriculture and grassland ecosystems support the abundance of *A. florea* species, i.e., the change in the seasonal floral resources influences bee species abundance and non-crop plants are important in maintaining the honey bee populations. The results of the present study will be helpful as baseline information for the sustainable development of apiculture in mixed agriculture and grassland habitats and to understand the role of tropical grassland flora in maintaining the diversity of bees.

**Keywords:** *Apis florea*; abundance; floral resources; seasonal variation; correspondence analysis.

## INTRODUCTION

Honey bees are ecologically and economically important insects as they are involved in plant pollination and the production of nutritional and medicinal products (Narang et al., 2022; Requier et al., 2019; Sharma et al., 2016). Their diversity and abundance are dependent on the availability of floral resources (Blaauw & Isaacs, 2014; Jha & Kremen, 2013; Kaluza et al., 2017). The availability of floral resources can be influenced by seasonal environmental changes (Coffey & Breen, 1997). As honey bees are dependent on floral resources, their diversity and abundance can also be the function of seasonal variations in environmental factors (Bänsch et al., 2020; Danner et al., 2016; Guezen & Forrest, 2021; Mensah et al., 2017). Also, temperature is a crucial factor responsible for the foraging of bees (Kamaraj & Rasappan, 2024). Therefore, seasonal dynamics in honey bee abundance and vegetation resources need to be investigated to understand the influence of fluctuating environmental conditions in the era of global warming.

Grassland habitats are more prone to seasonal changes as the life cycle of grass is short and can be influenced by environmental factors such as rain and temperature. Moreover, grasslands play a significant role in the ecosystem as they can support pollinator bees. Therefore, fluctuations in honey bee abundance in grassland ecosystems need to be investigated.

Further, agricultural habitats may have a significant role in determining bee diversity and abundance (Decourtye et al., 2010; Fisher et al., 2017). Several studies have shown that the increase in flowering plant diversity in agricultural ecosystem providing nutritional resources promote bee diversity and crop pollination (Kaluza et al., 2017; Sutter et al., 2017; Williams et al., 2015). Honey bee diversity studies in mixed agriculture and natural habitats could provide insight into the role of agricultural land in maintaining natural bee population and their role in crop pollination and production (Rogers et al., 2014). India is one of the leading agricultural countries covering a major portion of its land for crop production. The Indian agriculture system comprises seasonal and perennial crops as well. Since honey bees are important pollinators, studies on sustaining honey bee population in agro-ecosystems help in improving livelihoods of rural farmers and tribal communities (Dalwai, 2012).

Honey harvesting along with agriculture has long been practiced in human cultures and has been a supportive source for human livelihood (Abrol, 2023; Basu & Purkait, 2023). Although traditional beekeeping has been practiced in rural India, commercial honey production is also getting popular among farmers cultivating horticultural and agricultural crops (Abrol, 2023; Jamwal et al., 2021). Information on the bee diversity and abundance in agro-ecosystems can be helpful for the sustainable development of apiculture (Jamwal et al., 2021). Moreover, the concurrent studies of diversity of agricultural crops could help in evaluating their significance for the development of bee keeping practices (Al-Ghamdi & Al-Sagheer, 2023; Coffey & Breen, 1997; Waykar & Baviskar, 2015). Considering the need of bee diversity studies in mixed natural and agro-ecosystems, the abundance of three common honey bee species, Indian honey bee (*Apis cerana*), Dwarf honey bee (*Apis florea*) and Giant honey bee (*Apis dorsata*) was investigated in the present study. *Apis cerana* is domesticated in India and considered as ideal for beekeeping. *Apis florea* is an important pollinator and *A. dorsata* is known for production of enormous honey and wax. It builds its nest in dense foliage hanging to small branches nearly 0.2 to 8.2 m above the ground level. *Apis cerana* is a medium sized honey bee built in multiple combs in dark places parallel to each other keeping uniform distance while *A. dorsata* build single or aggregately multiple nests on the high tree branches or overhanging rocks about 30-50 m (Mishra, 2013). These species of honey bees play significant role in the pollination of several agricultural crops and natural plants. Additionally, combs of these

common honey bee species are harvested for honey and made into several other products supporting the local community (Mishra, 2013).

The abundance of active bee colonies of these three species was studied at eleven locations in Ahmednagar district of central Maharashtra, India. Additionally, the diversity of agricultural and surrounding non-agricultural plants providing floral resources in mixed agriculture and dry grassland habitats, was studied. All the locations in the present study are situated in the Karjat block of Ahmednagar district, which is a drought prone area mainly comprising grassland habitats and agricultural land that are mostly used for seasonal crop cultivation (Malaviya et al., 2018). The present study was undertaken to investigate the role of mixed grassland and agriculture habitats in a tropical region on the seasonal abundance of common honey bees. This study aims to (1) assess seasonal variations in the abundance of three honey bee species (*A. cerana, A. florea,* and *A. dorsata*) in a drought-prone region, and (2) investigate the role of agricultural and non-crop floral resources in honey bee populations across different seasons.

## MATERIAL AND METHODS

A total of 11 locations in Karjat block, Ahmednagar district, Maharashtra, India were selected (Fig. 1). The study locations consist of agriculture, grasslands and deciduous forest patches near rural settlements. All the sites were surveyed in different seasons for three consecutive years (2021–2023). The selected sites were visited each month to record plants with floral resources. Two consecutive visits were made in each season (summer, monsoon, and winter) to record the number of colonies per species. During field visits, *Apis* bee colonies were actively searched with the help of field assistants. Colonies inhabited by bees were considered active and these were included in the study. The number of bee hives observed were recorded and to confirm the species, 4–5 bees were collected by the net sweeping method. Collected honey bee specimens were preserved in 70% ethanol and brought to the laboratory. Species identification was done following the standard references (Mishra, 2013). Foraging activity of bees around flowering plants was recorded on field. The data of flowering plants was recorded along with the floral value (nectar and/or pollen) for honey bees. Plant species recorded in the present study are agricultural crops and common plants in the study area. In ambiguous situations, plant identity was determined after referring to the Flora of Ahmednagar district (Singh & Pradhan,1999) or by consulting with experts. Floral calendars were prepared for each flowering plant. Collected data of the plant species with floral resources was grouped into the seasons and data of seasonal distribution of plants with floral resources was analysed using Chi-square $\chi^2$ tests of contingency analysis and to show the relationship between seasons and the presence of the plant species. Correspondence analysis was performed in PAST Version 4.10 (Hammer & Harper, 2001).

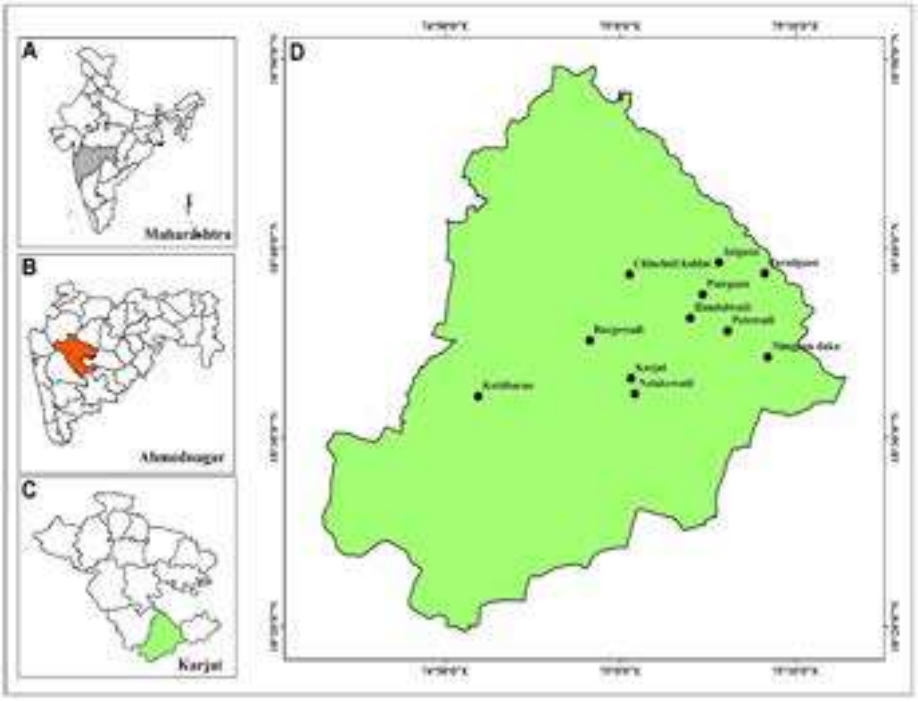


**Figure 1:** Study sites of common honey bee colonies investigated in Karjat, Ahmednagar, Maharashtra, India in 2021–2023. **A.** Map of India, with Maharashtra state shaded in gray. **B.** Map of Maharashtra state, with Ahmednagar district shaded in red. **C.** Ahmednagar district, with Karjat block shaded in green. **D.** Study sites.

## RESULTS

During different seasons, 4408 colonies of three species were recorded throughout the study. Colonies of *A. florea* were the most common (57.42%) in the study area, followed by *A. cerana* and *A. dorsata* (Fig. 2). The highest number of bee colonies were observed at Kuldharan site and the lowest bee colonies were recorded at Karjat site (Table 1). The highest number of *A. cerana*, *A. florea,* and *A. dorsata* colonies were observed at Taradgaon, Nimgaon daku and Netakewadi, respectively. The lowest number of *A. cerana*, *A. florea* and *A. dorsata* colonies were observed at Chincholi kaldat, Netakewadi and Bargewadi respectively (Table 1).

Colonies of *A. florea* were the most common in all seasons followed by *A. cerana* and *A. dorsata* (Fig. 3). In the summer season, the highest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Handalwadi, Kuldharan, and Kuldharan, respectively while the lowest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Karjat town, Netakewadi, and Bargewadi, respectively. In the monsoon season, the highest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Taradgaon, Nimgaon Daku, and Chincholi Kaldat, respectively while the lowest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Karjat town and Bargewadi, respectively. In the winter season, the highest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Bargewadi, Bargewadi, and Chincholi Kaldat, respectively while the lowest number of *A. cerana*, *A. florea*, and *A. dorsata* colonies were recorded at Chincholi kaldat, Netakewadi, and Pategaon, respectively. Details of the seasonal abundance of colonies of different species are described in Table 2.

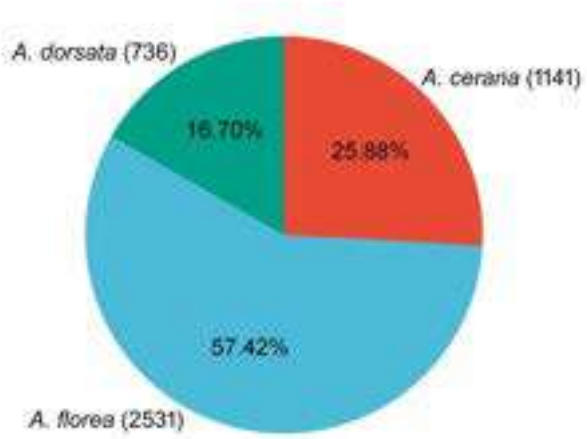


**Figure 2:** Proportion of all *Apis* honey bee colonies recorded across different seasons in Karjat, Ahmednagar, Maharashtra, in 2021–2023. Numbers in parenthesis are the number of colonies recorded for respective species.

**Table 1:** Percentage of *Apis cerana*, *A. florea*, and *A. dorsata* colonies recorded across sites in Karjat, Ahmednagar, Maharashtra, India in 2021–2023. Values in parentheses represent number of colonies recorded.

| **Study sites** | ***A. cerana*** | ***A. florea*** | ***A. dorsata*** |
|---|---|---|---|
| Karjat town | 20.20 (59) | 62.67 (183) | 17.12 (50) |
| Netakewadi | 22.74 (78) | 51.02 (175) | 26.23 (90) |
| Chincholi Kaldat | 19.78 (75) | 55.14 (209) | 25.06 (95) |
| Jalgaon | 29.33 (115) | 52.29 (205) | 18.36 (72) |
| Patewadi | 28.41 (106) | 54.42 (203) | 17.15 (64) |
| Handalwadi | 29.09 (112) | 58.44 (225) | 12.46 (48) |
| Pategaon | 30.06 (135) | 57.68 (259) | 12.24 (55) |
| Taradgaon | 31.65 (138) | 53.44 (233) | 14.90 (65) |
| Nimgaon Daku | 22.63 (98) | 65.12 (282) | 12.24 (53) |
| Bargewadi | 25.57 (112) | 63.01 (276) | 11.41 (50) |
| Kuldharan | 23.15 (113) | 57.58 (281) | 19.26 (94) |

A total of 72 species of plants belonging to 30 families were recorded (Table 3 and 4). Plants belonging to the family Fabaceae (15.2%) were predominant followed by Cucurbitaceae (11.1%) and Asteraceae (8.3%; Table 4). A total of 39 (54.16%) agricultural crop plants and 33 (45.83%) wild plants were recorded in the study area (Table 4). More than 50% of the plant species were found to be sources of nectar and pollen (Table 3). 26.38% plants were providing nectar source while 11.11% plants were providing pollen source. Five species of plants (*Coriandrum sativum*, *Moringa oeifera*, *Tridax procumbeans*, *Ocimum forskoelei*, and *Adhatoda vasica*) serve as a floral source for the entire year as these floweri throughout the year. Flowering plants along with the floral calendar are described in detail in Table 4.

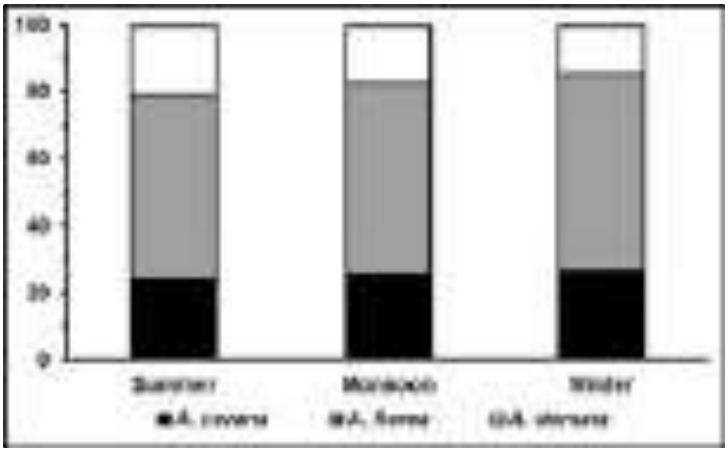

**Figure 3:** Season-wise proportion of colonies of *Apis cerana*, *A. florea*, and *A.dorsata* recorded in Karjat, Ahmednagar, Maharashtra, India in 2021–2023.

**Table 2:** Number of *A. cerana*, *A. florea*, and *A. dorsata* colonies at selected locations in Karjat, Ahmednagar, Maharashtra, India across seasons in 2021–2023.

| Study sites | Summer | | | Monsoon | | | Winter | | |
|---|---|---|---|---|---|---|---|---|---|
| | ***A. cerana*** | ***A. florea*** | ***A. dorsata*** | ***A. cerana*** | ***A. florea*** | ***A. dorsata*** | ***A. cerana*** | ***A. florea*** | ***A. dorsata*** |
| Karjat Town | 11 | 58 | 12 | 7 | 23 | 9 | 41 | 102 | 29 |
| Netakewadi | 14 | 43 | 36 | 11 | 34 | 17 | 53 | 98 | 37 |
| Chincholi Kaldat | 17 | 57 | 29 | 19 | 39 | 19 | 39 | 113 | 47 |
| Jalgaon | 31 | 47 | 24 | 23 | 37 | 16 | 61 | 121 | 32 |
| Patewadi | 29 | 49 | 21 | 21 | 38 | 16 | 56 | 116 | 27 |
| Handalwadi | 38 | 53 | 14 | 31 | 43 | 9 | 43 | 129 | 25 |
| Pategaon | 34 | 63 | 18 | 29 | 53 | 13 | 72 | 143 | 24 |
| Taradgaon | 37 | 59 | 21 | 33 | 50 | 18 | 68 | 124 | 26 |
| Nimgaon Daku | 19 | 71 | 13 | 17 | 64 | 11 | 62 | 147 | 29 |
| Bargewadi | 23 | 67 | 12 | 15 | 57 | 8 | 74 | 152 | 30 |
| Kuldharan | 24 | 72 | 37 | 20 | 61 | 18 | 69 | 148 | 39 |

**Table 3:** Number of plants and representing families recorded with floral value for honey bees in Karjat, Ahmednagar, Maharashtra, India. Numbers in parentheses are percentages.

| | Total | Nectar | Pollen | Nectar + Pollen |
|---|---|---|---|---|
| Number of plants | 72 | 18 (25.00) | 10 (13.88) | 44 (61.11) |
| Number of families | 30 | 11 (36.66) | 6 (20.00) | 13 (43.33) |

**Table 4:** List of the plants recorded in in Karjat, Ahmednagar, Maharashtra, India with flowering season and floral value for honey bees. Source: N = Nectar; P = Pollen; NP = Nectar + pollen.

| Family | Botanical name | Common name | Source (Nectar/ Pollen) | Flowering season |
|---|---|---|---|---|
| **Agricultural flora** | | | | |
| Anacardiaceae | *Mangifera indica* | Mango | NP | March–April |
| Annonaceae | *Annona squamosa* | Custard apple | NP | Sept–Oct |
| Apiaceae | *Coriandrum sativum* | Coriander | NP | Jan– Dec |
| Brassicaceae | *Brassica rapa* | Mustard | N | Jan– April |
| Brassicaceae | *Raphanus sativus* | Radish | NP | Dec–March |
| Caricaceae | *Carica papaya* | Papaya | NP | Feb–March |
| Compositae | *Helianthus annuus* | Sunflower | NP | March–May |
| Cucurbitaceae | *Cucumis sativus* | Cucumber | NP | Aug–Nov |
| Cucurbitaceae | *Cucurbita pepo* | Pumpkin | NP | Aug–Oct |
| Cucurbitaceae | *Citrullus lanatus* | Watermelon | P | July–Sept |
| Cucurbitaceae | *Cucumis melo* | Muskmelon | P | Feb–May |
| Cucurbitaceae | *Luffa acutangula* | Silk gourd | NP | July–Oct |
| Cucurbitaceae | *Momordica charanta* | Bitter gourd | NP | June–Sept |
| Cucurbitaceae | *Lagenaria siceraria* | Bottle gourd | NP | Aug–Sept |
| Fabaceae | *Trigonella foenum* | Fenugreek | NP | Jan–April |
| Fabaceae | *Cicer arietinum* | Chickpea | P | Dec–April |
| Fabaceae | *Vigna radiata* | Green gram | N | Aug–Nov |
| Fabaceae | *Vigna mungo* | Black gram | N | Aug–Oct |
| Fabaceae | *Cajanus cajan* | Pigeon pea | N | July–Sept |
| Fabaceae | *Tamariandus indica* | Tamarind | N | July–Oct |
| Leguminosae | *Cyamopsis tetragonolobus* | Cluster bean | N | June–Aug |
| Liliaceae | *Allium sepa* | Onion | NP | June–Aug |
| Liliaceae | *Allium sativum* | Garlic | NP | Aug–Sept |
| Malvaceae | *Abelmoschus esculentus* | Ladyfinger | P | March–April,June–Aug |
| Moringaceae | *Moringa oeifera* | Drumstick | NP | Jan–Dec |
| Myrtaceae | *Psidium guajava* | Guava | NP | May–June |
| Myrtaceae | *Syzygium cumini* | Jambhul | NP | Feb–April |
| Myrtaceae | *Callistemon* spp. | Bottlebrush | N | March–Sept |
| Poaceae | *Zea mays* | Maize | P | Aug–Sept, Feb–March |
| Poaceae | *Triticum aestivum* | Wheat | N | Feb–April |
| Poaceae | *Sorghum vulgare* | Jawar | N | Sept–Nov |
| Poaceae | *Pennisetum typhoides* | Bajara | N | July–Sept |
| Punicaceae | *Punica grantum* | Pomegranate | NP | March–June |
| Rhamnaceae | *Ziziphus jujuba* | Zizipus | NP | July–Nov |
| Rhamnaceae | *Ziziphus mauritiana* | Ziziphus | NP | May–June |
| Rutaceae | *Murraya koenigii* | Curry leaves | N | March–May |
| Rutaceae | *Aegle marmelos* | Bel | NP | May–June |
| Rutaceae | *Citrus limon* | Lemon | NP | Oct–Jan, July–Sept |
| Solanaceae | *Solanum melongena* | Brinjal | P | Jan–March, June–July |
| **Wild flora** | | | | |
| Acanthaceae | *Justicia betonica* | Squirrel tail | NP | Jan–April, Oct–Dec |
| Acanthaceae | *Adhatoda vasica* | Adhulsa | N | Jan–Dec |
| Amaranthaceae | *Achyranthes aspera* | Devil horsewhip | P | March–May |
| Amaranthaceae | *Amaranthus viridis* | Slender amaranth | P | Sept–Nov |
| Amaranthaceae | *Alternanthera sessils* | Dwarf copperleaf | NP | Dec– April |
| Amaranthaceae | *Digera muricata* | False Amaranthus | N | Jan–March |
| Apocynaceae | *Calatropis procera* | Calatropis (Rui) | NP | Feb, March, Nov |
| Apocynaceae | *Thevetia peruviana* | Yellow Oleander | N | May–Aug |
| Asteraceae | *Bidens pilosa* | Beggar tick | NP | July–Dec |
| Asteraceae | *Calendula arvensis* | Field marigold | NP | Jan–April |
| Asteraceae | *Centaurea sinaica* | Blooming plant | NP | Jan–May and Dec |

| | | | | |
|---|---|---|---|---|
| Asteraceae | *Flaveria trinervia* | Clustered yellowtop | NP | April–July, Jan, Dec |
| Asteraceae | *Parthenium hysterophorus* | Congress | N | Aug–Dec |
| Asteraceae | *Launaea nudicaulis* | Bold leaf launaeae | NP | Aug–Dec |
| Cactaceae | *Opuntia ficus indica* | Opuntia | NP | May, June |
| Compositae | *Tridax procumbeans* | Tridax daisy | NP | Jan–Dec |
| Convolvulaceae | *Convolvulus arvensis* | Shankhapushpi | NP | Oct–Dec |
| Cucurbitaceae | *Citrullus colocynthis* | Indrayan | P | Jan, Feb, June, Oct |
| Euphorbiaceae | *Phyllanthus emblica* | Amla | NP | March–May |
| Euphorbiaceae | *Ricinus communis* | Castor | NP | Nov–Feb |
| Fabaceace | *Delonix regia* | Gul mohar | NP | March–May |
| Fabaceae | *Acacia arabica* | Acacia | N | July–Dec |
| Fabaceae | *Tephrosia purpurea* | Unhali | N | Nov, Dec |
| Fabaceae | *Trifolium arevense* | Rabbit foot clover | NP | March, April, May |
| Fabaceae | *Senna alexandrina* | Tarvad | P | April, May, Nov |
| Lamiaceae | *Ocimum forskoelei* | Tulsi | NP | Jan–Dec |
| Malvaceae | *Abutilon theophrasti* | Velvet leaf plant | NP | July–Sept |
| Malvaceae | *Gossypium* spp. | Cotton | NP | Sept–Dec |
| Meliaceae | *Azadiracta indica* | Neem | NP | April–May |
| Mimosaceae | *Acacia catechu* | Khair | NP | Sept–Dec |
| Myrtaceae | *Eucalyptus* spp. | Nilgiri | NP | Nov–March |
| Papaveraceae | *Argemone mexicana* | Styanashi | NP | Feb–April |
| Verbenaceae | *Lantana camara* | Tantani | N | Jan–May, July–Sept |

The highest number of plants were available during the monsoon season (Fig. 4). The proportion of crop plants was high during the monsoon season and low in summer and winter. Conversely, non-crop plant proportion was higher during summer and winter compared to the monsoon season (Fig. 4).

Correspondence analysis revealed similarities in the presence of flowering plants in different seasons. First two axes highlighted 54.25% similarity in flowering plants in different seasons (Table 5). Scatter plot clearly shows seasonal groups based on plants with floral resources (Fig. 5).

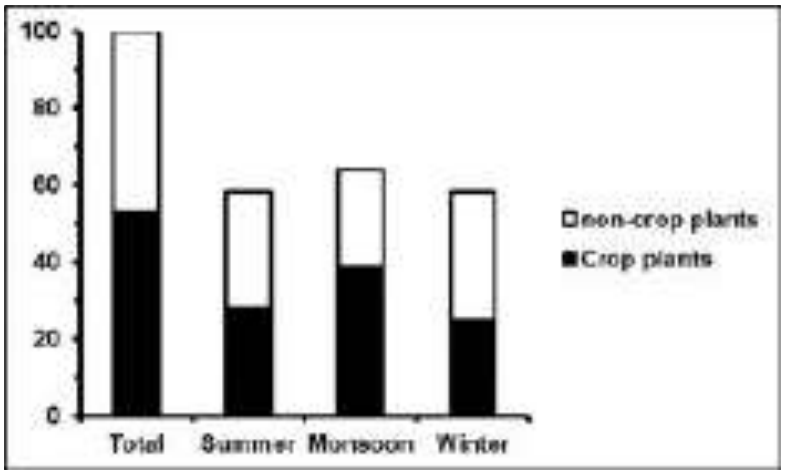


**Figure 4:** Seasonal distribution of all crop and non-crop plant sources available for honey bees from eleven study sites in Karjat, Ahmednagar, Maharashtra, India in 2021–2023.

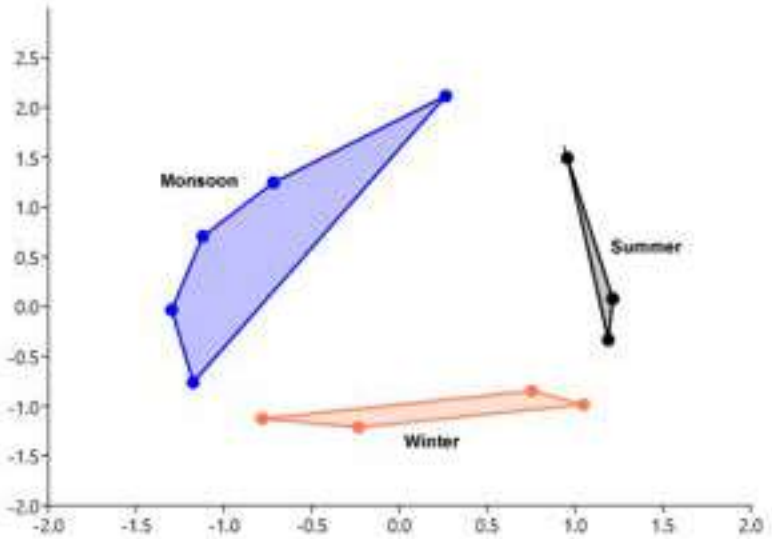


**Figure 5:** Correspondence analysis ordination biplot showing relationship of composition of plants with floral resources for honey bees across different seasons in Karjat, Ahmednagar, Maharashtra, India in 2021–2023. (Note- Biplot represents the plotting of Chi-squared distances between months and plant species for axis 1 (X axis) and axis 2 (Y axis). Dots represent the month of the particular season).

**Table 5:** Eigenvalues and percentage of variance explained on different axes after Correspondence analysis of floral resources available for honey bees across different seasons.

| Axis | Eigenvalue | % of variance |
|---|---|---|
| 1 | 0.56 | 32.20 |
| 2 | 0.38 | 22.05 |
| 3 | 0.24 | 14.01 |
| 4 | 0.20 | 11.66 |
| 5 | 0.11 | 6.13 |
| 6 | 0.07 | 3.94 |
| 7 | 0.06 | 3.25 |
| 8 | 0.04 | 2.53 |
| 9 | 0.03 | 1.66 |
| 10 | 0.02 | 1.39 |
| 11 | 0.02 | 1.17 |

## DISCUSSION

Beekeeping, a traditional practice, has played a significant role in sustainable development of tribal and rural communities (Abrol, 2023; Basu & Purkait, 2023). Understanding the honey bee species community of a particular area along with the data on floral resources is a preliminary step toward sustainable apiculture practices (Al-Ghamdi & Al-Sagheer, 2023; Waykar & Baviskar, 2015). In the present study, we evaluated the abundance of colonies of three important honey bee species over three years along with the plants providing floral resources from mixed dry grassland and agricultural area from rural Maharashtra. The results revealed that *A. florea* is the most predominant honey bee species in the study area (Fig. 2). Previously, *A. florea* was reported as a dominant honey bee in some Indian habitats including the agro-ecosystem, coastal regions, and mountain grasslands (Goyal, 1974; Indhu et al., 2022; Kamaraj & Rasappan, 2024). Contrastingly, other honey bees including *A. dorsata* and *A.*

*mellifera* were also reported to be predominantly present in different parts of the country (Abrol, 2020; Balachandran et al., 2014; Goyal, 1974; Rathee et al., 2023). These observations highlight differences in the abundance of honey bees belonging to various species in different geographical locations (Parveen et al., 2022; Potts et al., 2003; Sen et al., 2023). Moreover, several biotic and abiotic factors influence the diversity of honey bees in a particular habitat (Horn et al., 2021; Neov et al., 2019; Smart et al., 2016). The abundance of different species of honey bees is mainly dependent on the floral resources available in the particular habitat (Fisher et al., 2017; Rollin et al., 2013; Smart et al., 2016).

The present study revealed that the proportion of colonies of different species was similar throughout the year. However, the composition of the plants providing floral resources varied in different seasons. These observations suggest that irrespective of the changes in the vegetation in selected sites, the colony proportions remains similar. The stability of colonies proportions across seasons may indicate that honey bees are capable of utilizing a diverse range of floral resources (Abrol, 2020; Chauhan et al., 2017; Lazar et al., 2024). Moreover, other biological factors including predation, competition, etc. can also be responsible for the maintenance of their proportion (Monceau et al., 2013; Roubik & Wolda, 2001). Flowering plant communities vary in different seasons mainly due to the increase in agricultural crops during the monsoon season. However, the proportion of non-agricultural plants remained similar in different seasons. As the proportion of different species of honey bees is similar in different seasons, it seems that non-agricultural plants are playing a crucial role in supporting the populations of the bees included in the present study. Previous studies also have shown the role of non-crop plants in maintaining bee diversity in agricultural fields (Nicholls & Altieri, 2013; Sutter et al., 2017; Williams et al., 2015).

The selected study region is a drought prone dry grassland which flourishes during the monsoon season only. Also, the area included in the present study mainly consistsof agricultural land along with semi-urban patches, grasslands with small bushy forests, and some dense forest patches. Therefore, diverse flora in the habitat provide rich floral resources throughout the year. More than 50% agricultural crop plants were recorded in the study area. These observations suggested that consistent availability of agricultural crops throughout the year support *A. cerana*, *A. florea* and *A. dorsata* bee population. Previous studies also demonstrated that the plant diversity in agricultural land enhances and supports bee diversity (Nicholls & Altieri, 2013; Rivers-Moore et al., 2020; Sutter et al., 2017). The present study revealed the abundance of *A. florea* in mixed agricultural and natural grassland habitats suggesting this species is suitable for bee keeping practices (Kishan et al., 2017).

In the present study, we recorded the interaction of honey bees with fruit crops, suggesting that honey bees species monitored in the present study are important for sustainable agriculture in the study area. Furthermore, bee pollination of many wild plants is extremely important for their survival and ecosystem function (Kumar et al., 2024; Lazar et al., 2024), implying that the natural flora supporting bee diversity is crucial for the conservation of the grassland ecosystem, sustainable agriculture and apiculture practices. Importantly, the presence of natural flora around the agriculture land is also important for maintaining honey bee populations in different seasons of the year (Nicholls & Altieri, 2013). The data on the floral resource that provide plants could serve as a baseline for sustainable apiculture practices in tropical mixed habitats (agriculture and grassland). Future comparative studies of the abundance of the honey bee colonies in agriculture land, grassland, and mixed agriculture-grassland habitats need to be undertaken to find out the requirements for the sustainable apiculture and agriculture practices in the grassland ecosystem.

In the present study, seasonal honey bee comb abundance in mixed grassland and agriculture habitats was observed. Environmental parameters including rainfall and temperature were not monitored during the present study. Investigation of the influence of these environmental parameters on honey bee abundance is one of the major limitations of the present study. Moreover, the present study in an observational study of mixed grassland and agriculture habitat. Further studies can be conducted by considering environmental parameters and comparative approach to understand the role of grasslands and agriculture in maintaining honey bee diversity.

## CONCLUSION

It is concluded that the proportion and abundance of *A. cerana*, *A. florea* and *A. dorsata* is stable throughout the year in mixed agriculture and dry grassland habitats of central Maharashtra. Although plant community providing floral resources vary in different seasons, it does not influence hive diversity and abundance. It is found that *Apis florea* was predominantly found in the study area and therefore can be used for apiculture practice in the studied area. Further comparative studies on the influence of grassland and agriculture habitats considering environmental factors could help in understanding their role in maintaining honey bee diversity and abundance.

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## DECLARATIONS

**Research permit(s).** Not applicable.
**Ethical approval/statement**. The permission to conduct the study and collect required bee samples was obtained from Maharashtra State Biodiversity Board (MSBB/Desk-5/Research/898/2022-23).
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Rural Tourism in Kiulu, Sabah, Malaysia: A Critical Examination through The Lens of The Host Community

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## ABSTRACT

Local communities often face a number of challenges when seeking to participate in rural tourism including participation in decision making, the level of empowerment the community enjoys and other factors including knowledge of tourists and local culture and the role governments play in supporting rural tourism development. To investigate these issues, a mixed methods approach was used to collect data from 118 residents of Kiulu, a rural destination in Sabah, Malaysia through questionnaires and in-depth interviews. The results show that successful rural tourism in Kiulu is driven by strategies that promote local participation in decision-making, community empowerment and better knowledge of the tourism industry. However, the study also shows that there is a lack of positive correlation between rural tourism growth and knowledge sharing, a challenge that appears to be rooted in strained relationships between tourism stakeholders. Other barriers include financial constraints, inadequate road access and the need for specialised training in tourism services. Addressing these barriers through targeted government interventions such as financial support and capacity building programmes may help distribute the benefits of rural tourism more equitably and ultimately promote sustainable development within the community.

**Keywords**: rural tourism; community involvement; decision-making; empowerment; community knowledge; role of women.

## INTRODUCTION

Effective rural tourism development benefits local communities by generating job opportunities and local investment, enhancing local prosperity, safeguarding the environment and conservation of cultural resources (Li et al., 2024). However, achieving these benefits requires relevant community stakeholders to have a strong understanding of tourism as a service business, the opportunity to actively participate in and benefit from tourism as well as opportunities to manage the development and implementation of tourism activities (Mendoza-Ramos & Prideaux, 2014). Knowledge is also an important prerequisite for successful rural tourism through its ability to empower communities to participate in the benefits that tourism can provide (Riyanto et al., 2023). Karthik (2023) and Tong et al. (2024) observed that communities in rural areas with a deep understanding of visitors and of their own local culture had the most success with tourism. Nevertheless, challenges persist, such as inadequate knowledge about how to operate a tourism business and an understanding of the tools required to adequately engage locals in tourism projects (Dogra & Gupta, 2012; Tong et al., 2024).

Local communities often face a number of other challenges when seeking to participate in rural tourism including racism, paternalism, and patronage (Fong & Lo, 2015; Arismayanti & Suwena, 2022). Dogra and Gupta (2012) and Sood et al. (2017) both note that a lack of knowledge and resources are other indicators of ineffective participation. As Rasoolimanesh et al. (2018) observed, it is important to involve the community in decision-making and encourage their active involvement. Using local knowledge and expertise also enables stakeholders to develop unique tourism experiences (Carneiro et al., 2015). The aim of this research was to evaluate the attitudes and the extent of involvement of the local community in Kiulu, Sabah, towards rural tourism development. By examining how community members perceive tourism and participate in related activities, the study seeks to generate insights that can inform policy decisions aimed at enhancing community engagement, fostering inclusive tourism planning, and supporting the long-term sustainability of rural tourism in Sabah. Specifically, this study aimed to assess the attitudes of the Kiulu community towards rural tourism and to determine the level and forms of their involvement in tourism-related activities. It also sought to identify the key factors that influence community participation in rural tourism initiatives. In doing so, the research intended to offer policy-relevant recommendations that could help strengthen local engagement, empower communities, and contribute to the formulation of more effective and sustainable rural tourism strategies in Sabah.

## METHODOLOGY

### Study area

Kiulu is a municipality of 17,565 residents (Department of Statistics Malaysia, 2022) located in Sabah's Tuaran District that is 47 kilometres from Kota Kinabalu, the state capital of Sabah (Fig. 1). In 2019, prior to COVID-19, the district generated about RM5.45 million in income, welcomed around 54,000 visitors and supported 300 tourism related jobs (Chuah, 2022). Initial development of the region's tourism industry in the first decade of the 21st century focused on homestays and was supported by the Sabah Tourism Board. Key tourism products include white water rafting, wildlife tourism and homestays. Kiulu has a dedicated tourism management body known as the Kiulu Tourism Association (KTA) which was established in 2014. At that time, a number of outside tour operators were actively involved in Kiulu.

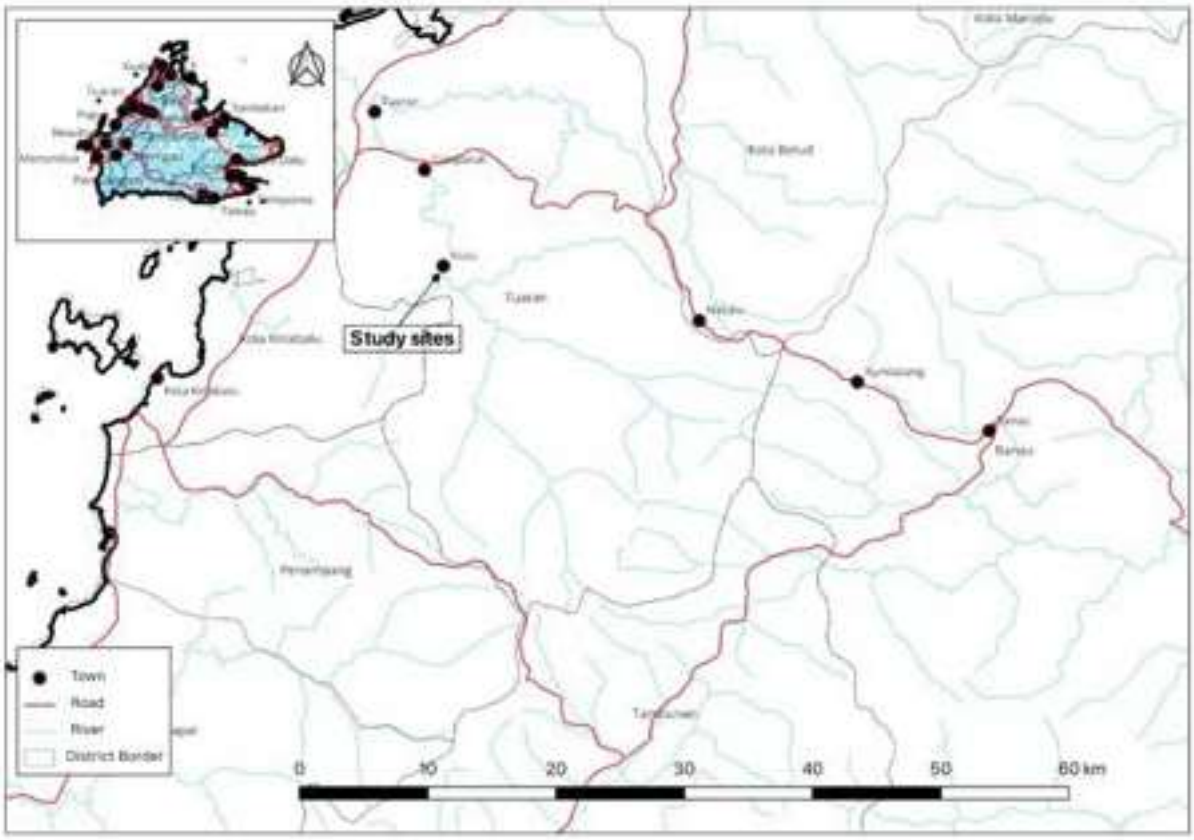


**Figure 1:** Map indicating the location of Kiulu, Sabah, Malaysia where questionnaires regarding rural tourism development were distributed in the local community. Inset shows a map of Sabah.

Establishment of the KTA has encouraged greater participation by members of the local community. The KTA (https://www.facebook.com/KiuluTourismAssociation/) actively promotes local tourism products and initiatives under its banner. As of 2024, the KTA had 78 members with 90% being members of the Kiulu community involved in tourism. Non-members of the KTA can express their views but are not able to vote on issues related to decisions made by the KTA board. The association is supported by the Sabah Tourism Board (STB) which aims to position Kiulu as a world-class rural tourism destination.

Kiulu's popularity as a rural tourism destination has led to ongoing year-on-year growth in tourism arrivals. While the significant economic impact of tourism is readily observable, the impact that increasing tourist arrivals is having on the Kiulu community is less observable. Some concerns have been raised about the impact of fast tourism growth on the local community and on the district's long-term environmental sustainability. Therefore, it is important to understand how the community is involved in Kiulu's rural tourism development and management, and how local issues related to tourism development are dealt with. Ensuring that the economic benefits of tourism do not negatively impact the community and its environment is important, and this issue is investigated in this paper. Given the rapid growth of interest in rural tourism not only in Kiulu, but also in other parts of Sabah and in other countries, there is a pressing need to understand how the impact of tourism affects community beliefs and attitudes and how these impacts are able to be balanced against the economic advantages generated by tourism (Harrill, 2004; Wang et al., 2010).

As the aim of this research was to investigate the attitude and level of involvement of the local Kiulu community in rural tourism development, we adopted stakeholder theory as the theoretical framework that could best assist the research team to identify positive and negative views of participants. Wondirad and Ewnetu (2019) described stakeholder analysis as a tool for understanding 'the diverse relationships amongst all relevant parties who have a stake in tourism development and their respective interest on the stake at hand' and on this basis is a useful tool for assessing if the community supports development. In a tourism context, achieving a successful and sustainable tourism sector requires an equitable balance of power between all stakeholders to ensure that social equality, economic balance and ecological integrity are achieved (Wondirad & Ewnetu, 2019). Other scholars have supported this view. For example, Lepp (2008) argued that genuine community participation is required to ensure local people are able to participate in the benefits of development as well as have the ability to exercise some level of control over decision making and management. Adoption of stakeholder theory as the theoretical framework for this research enabled the research team to develop a survey instrument that was able to evaluate the four indicators of community engagement suggested by Utama et al. (2021). These were community involvement in decision-making, empowerment, dissemination of information and community awareness of rural tourism.

**Three-stage data collection**

A three-stage data collection and analysis approach were adopted for this study. In stage one, locals were asked to complete a 30-item survey followed by an in-depth interview to develop an understanding of their views on rural tourism and to discover if they were involved with the management of tourism in their community. Prior to undertaking data collection, the Sabah Tourism Board Research Ethics Committee granted ethical approval for the research (Ethical Code: STG-RT Kiulu). All participants were asked to provide their informed consent after the study's goals, methods and risks were explained to them.

Simple random sampling was employed to ensure that every individual in the Kiulu community had an equal chance of being selected for the study. The population consisted of all residents in the Kiulu community, totaling 17,565 people according to the 2022 household census report (Department of Statistics Malaysia, 2022). Using Krejcie and Morgan's (1970) sample size determination table, a target sample size of 375 was established. A comprehensive list of community members was prepared with the assistance of local leaders and administrative records. Each individual on the list was assigned a unique identification number, and a random number generator was used to select participants. While this method was designed to produce a representative sample and ensure objectivity in selection, unforeseen challenges—such as heavy rainfall and landslides that cut off access to certain areas of the village during the sampling process—resulted in the final compilation of only 195 participants for the study.

The questionnaire was structured into six sections to comprehensively assess various aspects of the study. The first section focused on the demographic profile of respondents, followed by section two that focused on community participation in decision-making, section three that focused on community participation in sharing knowledge, and community participation in empowerment, section four that explored community knowledge about rural tourism and the final section that concluded with a section on perspectives regarding rural tourism in Kiulu.

The questionnaire items were developed through consultation with experts from the Sabah Tourism Board and a review of relevant literature (Paimin et al., 2014; Rasoolimanesh et al., 2018; Dasan et al., 2022). To ensure the clarity, relevance, and comprehensibility of the items, the survey instrument was pilot-tested with 15 respondents. Feedback from the pilot test led to several minor adjustments, improving the overall quality and usability of the questionnaire.

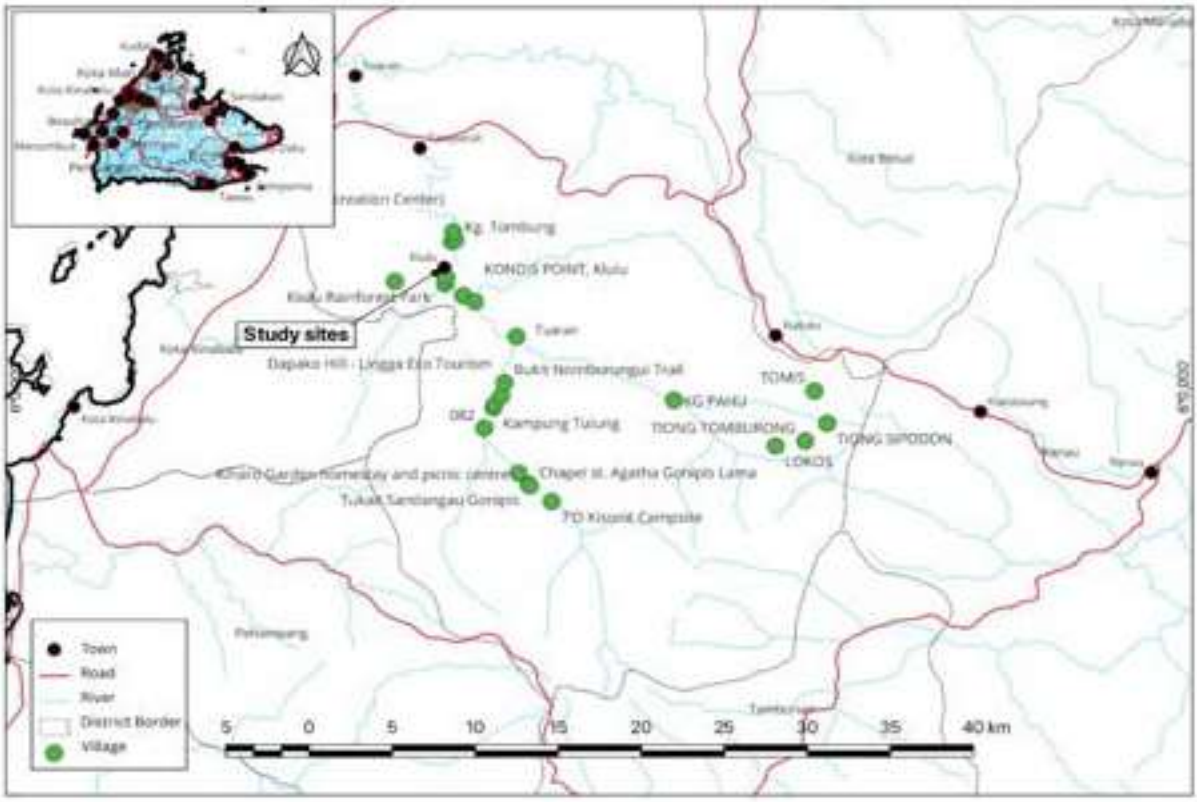


**Figure 2:** Villages in Kiulu, Sabah, Malaysia where questionnaires regarding rural tourism development were distributed in the local community. Inset shows a map of Sabah.

To ensure consistent and trustworthy data collection, the questionnaires were distributed by qualified research specialists. A key element of the research was to first establish a good relationship with the community which we achieved through direct engagement. A mixed-methods approach was adopted, combining both quantitative and qualitative data collection techniques. The survey and semi-structured interviews were conducted concurrently with 195 individuals invited to participate. Respondents were first asked to complete the six-section structured questionnaire, which was designed to gather quantitative data on the study's key themes. Following the completion of the questionnaire, semi-structured interviews were conducted using a pre-determined interview guide. These interviews allowed research staff to explore participants' perspectives in greater depth and capture qualitative insights to complement the survey data. To accurately capture detailed responses, all interviews were tape recorded with the respondents' permission. To ensure all of the communities in the Kiulu were able to participate in the survey and ensure a variety of viewpoints and experiences pertaining to the growth of rural tourism were captured, 22 locations in the study region were selected as interview sites. These are indicated in Fig. 2.

In Stage 2, qualitative data collection based on researcher observations of visitors, residents, and the environmental dynamics within Kiulu's rural tourism sites was employed to understand visitor behaviour, interactions, and dynamics. Observations were recorded in note books by survey staff. This choice of data collection allowed for the capture of events as they happened and the identification of subtle patterns that other data collection approaches may have missed.

In Stage 3, we systematically assessed the quantitative data collected via surveys and qualitative data gained from interviews. Thematic analysis was used to find patterns and themes

in the qualitative data collected from interviews and observations. To discover commonalities or patterns among respondents' opinions, views, and preferences about various aspects of rural tourism in Kiulu, statistical analysis was performed on quantitative data collected from surveys that contained Likert scale items.

A Likert scale of 1 to 5 was employed to measure variables. The opinions, attitudes, and perceptions of individuals or groups on a social issue can be evaluated using the Likert scale, as stated by Sugiyono (2016). To calculate the position on the Likert scale, each respondent was asked to answer a series of questions. Each question had a weighted value from 1 to 5, and the response options ranged from "strongly agree" to "strongly disagree" (Sugiyono, 2016) (Table 1). Descriptive statistics was used to describe the data without drawing any broad conclusions. To establish the size of the interval of the survey's findings, the following formula was used:

**Table 1:** Likert-scale used in questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

| Criteria | Positive | Negative |
|---|---|---|
| Very Low | 5 | 1 |
| Low | 4 | 2 |
| Medium | 3 | 3 |
| High | 2 | 4 |
| Very High | 1 | 5 |

Based on Table 1, the ideal score scale for each answer can be obtained as follows:

**Highest Score** = Highest Weight × Number of Respondents
= 5 × 118
= 590

**Lowest Score** = Lowest Weight × Number of Respondents
= 1 × 118
= 118

After the highest and lowest scores are obtained, the scale range (RS) is calculated based on Likert (1932) and Jamshed (2014):

$$\mathbf{RS = n(m-1)/m}$$

Information:
RS = Scale Range
N = Number of Respondents
M = Number of Alternative Answers

**RS** = (118(5−1))/5
= (118(4))/5
= 472/5
= 94.4

To determine the intervals, the calculation starts with the lowest value, 118. The interval size is 94.4, which is added to 118 to determine the upper limit of the first category. Beginning with

the Very Low criterion, the calculation adds 94.4 to 118, resulting in an upper limit of 212.4 for this category (Table 2).

**Table 2:** Answer criteria scale range for questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

| No. | Criteria Classification | Intervals |
|---|---|---|
| 1 | Very Low | 118.0–212.4 |
| 2 | Low | 212.5–306.9 |
| 3 | Medium | 307.0–401.4 |
| 4 | High | 401.5–495.9 |
| 5 | Very High | 496.0–590.4 |

To calculate the classification score as a percent of respondents, it is necessary to find the minimum score, maximum score and interval obtained from the quotient of maximum score – minimum score divided by the number of choice weights, which can be described as follows:

**Minimum Score** = Number of Items × Lowest Weight
= 25 × 1
= 25

**Maximum Score** = Number of Items × Highest Weight
= 25 × 5
= 125

**Intervals** $= \frac{\text{Max Score} - \text{Min Score}}{\text{Scale}}$
= (125 – 25) / 5
= 20

To convert it into a percentage, the upper limit of the lowest scale range (45) is divided by the maximum score (125) multiplied by 100 for each classification (Table 3).

**Table 3:** Score category classification based on percentage for respondents to questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

| No. | Criteria Classification | Intervals | Percentage |
|---|---|---|---|
| 1 | Very Low | 25 – 45 | 0%–36% |
| 2 | Low | 46 – 66 | 37%–53% |
| 3 | Medium | 67 – 87 | 54%–70% |
| 4 | High | 88 – 108 | 71%–87% |
| 5 | Very High | 109 – 125 | 88%–100% |

An Importance-Performance Analysis (IPA) was conducted in this study based on the framework of Martilla & James' (1977) original design and its subsequent use in leisure and tourism research (Boley et al., 2017). Thirty attributes that relate to rural tourism in Kiulu, encompassing several forms of rural tourism, were presented in the survey instrument, namely farm visit, nature uniqueness, traditional cuisines, traditional culture and traditions, community history, landscape and scenery, religious heritage, peaceful and serene, meeting with new people, adventure activities, personal safety and security, destination can be reached, clear road

signs and maps, easy transportation access, cultural sights and offers, Internet connection, entertainment offers, recreational activities, local festivals, accommodation quality, tour guide service, access to public infrastructure, size of destination, quality of destination, cleanliness of destination, quality of infrastructure, involvement of local community, variety and linkage of ecotourism destinations, management of tourism destination, and total travel cost. The importance of each attribute was measured using a five-point Likert scale from one "not at all important" to five "extremely important". Performance was measured using a five-point Likert scale where one is "poor" to five "excellent", with a 'not applicable' (n/a) response option for attributes that were not present in each of the rural tourism attributes in Kiulu. These 'n/a' responses on the performance scale were recorded as poor performance. For example, if a road was considered extremely important by ecotourists, but a village did not have a proper road, the 'N/A' responses on the performance scale were coded as one "poor", resulting in the attribute falling in the 'concentrate here' quadrant.

For this study, thematic exploration of the interview transcripts was conducted using Leximancer, a text analytics tool capable of detecting patterns and conceptual linkages within qualitative data. Rather than relying on manual coding alone, the software analysed the transcripts by identifying frequently co-occurring words and clustering them into emergent themes (Angus et al., 2013). These themes were presented visually on a concept map, where each was represented by a coloured node or "bubble." The colour scheme followed a gradient system, warmer tones (e.g., red and orange) highlighted themes that appeared more frequently or held greater centrality in the discourse, whereas cooler tones (e.g., green and blue) pointed to those with less emphasis but still meaningful presence. Additionally, the relative size of each bubble reflected the prominence or connectivity of the theme across the data. By allowing themes to surface directly from the language used by participants, this method helped preserve the authenticity of their responses and provided a more organic view of the key issues raised (Harwood et al., 2015).

## RESULTS

### Demography profile of respondents

The response rate for the survey was 61% (118 out of 195). Several factors affected the level of participation. In the first instance, heavy rain during the period of the survey reduced the number of participants agreeing to take part in the survey. Second, many women declined to participate in the survey. This reluctance can be attributed to a number of factors. Firstly, time constraints played a significant role, as women in rural areas often shoulder primary responsibility for household chores and caregiving duties. These responsibilities can consume a substantial portion of their time, leaving little opportunity to engage in external activities such as surveys. Secondly, cultural norms rooted in traditional gender roles may reinforce the prioritization of domestic responsibilities over external engagements. Women may feel obligated to uphold these roles, limiting their willingness or ability to participate in surveys or interviews (Hirschman, 2016). Additionally, a lack of awareness about the purpose or benefits of the survey may have contributed to the reluctance of some rural women to participate. Limited access to information or communication channels, coupled with language barriers, may also have hindered their understanding of the survey's relevance or potential impact. Importantly, while these factors may influence women's participation rates, it is essential to note that the survey questions themselves did not directly relate to gender, thereby minimizing the potential for bias in survey responses.

Fig. 3 illustrates the demographic profile of the respondents, highlighting the diverse characteristics of the community. The majority of respondents were male (71%), while females made up 29% of the sample. Age distribution showed that most respondents were 60 years and above, followed by those aged 36–40 and 31–35 years. Educational attainment was largely at the secondary level (50%), with fewer respondents having completed primary or tertiary education, and only a small proportion reporting no formal education. In terms of occupation, a notable number of respondents were unemployed (13%), followed by those working in the private sector, tourism, and self-employment. Income classification revealed that most respondents fell within the B40 income group (low-income households), with minimal representation in the M40 and T20 income brackets. These income brackets are based solely on the household income level with B40 to RM4,850, M40 from RM4,851 to RM10,970 and T20 more than RM10,971 (Ministry of Finance Malaysia, 2024). Residency duration varied, with the largest group having lived in Kiulu for more than 50 years, while smaller groups reported shorter periods of residence.

**Community participation in Kiulu's rural tourism industry**

The participation level of the Kiulu community in tourism development is high, as shown in Table 4. The research data collected indicates that all 118 respondents completed the questionnaire, which consisted of 25 statement items. This resulted in a total of 2,950 responses, representing a 100% response rate for the questionnaire items. The data was further analyzed by calculating the percentage value answer, yielding a value level of 75.36%, categorized as "High." This percentage was derived by comparing the total score obtained with the total ideal score. The total score recorded was 11,115 points, while the ideal score was 14,750 points. The detailed calculation to determine the total score, ideal score and percentage value answer is presented below:

Variable 1: $Item_1 = n_5(5) + n_4(4) + n_3(3) + n_2(2) + n_1(1)$
$= 76(5) + 28(4) + 8(3) + 4(2) + 2(1)$
$= 380 + 112 + 24 + 8 + 2$
$= 526$

*Where $n_x$ is the number of respondents based on Likert scale.

Total Score for Variable 1 ($TSV_1$) $= Item_1 + Item_2 + Item_3 + Item_4 + Item_5$
$= 526 + 545 + \ldots + \ldots$
$= 2615$

**Total Score** $= TSV_1 + TSV_2 + TSV_3 + TSV_4$
$= 2615 + 1498 + 2522 + 4480$
$= 11,115$

Ideal Score for Variable 1 ($ISV_1$) $=$ ntotal × 5-point Likert scale × statement items
$= 118 \times 5 \times 5$
$= 2950$

**Ideal Score** $= ISV_1 + ISV_2 + ISV_3 + ISV_4$
$= 2950 + 2950 + 2950 + 5900$
$= 14,750$

**Percentage Value Answer** $=$ Total Score/ Ideal Score × 100%
$= (11,115 / 14,750) \times 100\%$
$= 75.36\%$

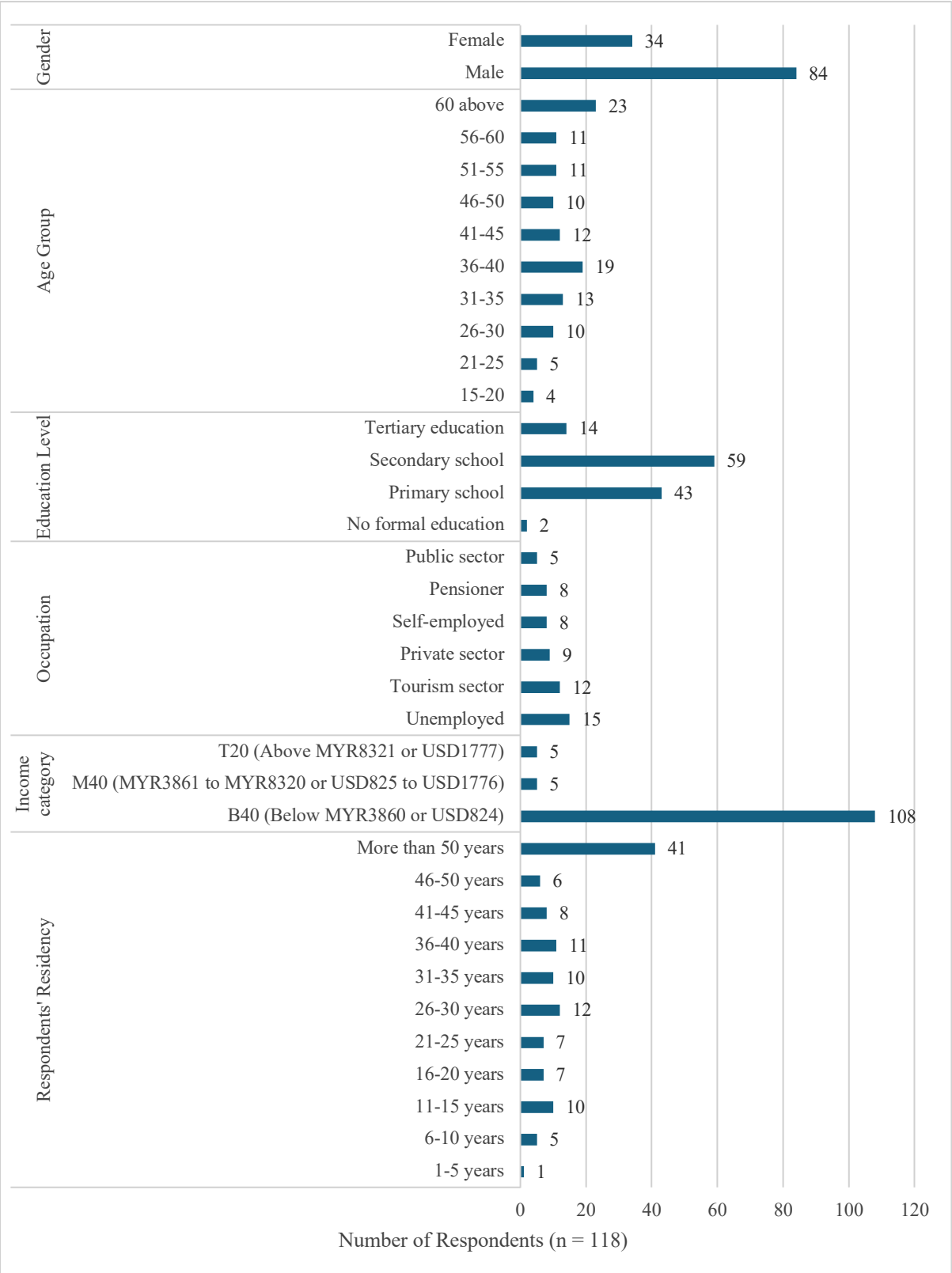


**Figure 3:** Demography profile of respondents to questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

**Table 4:** Community participation in rural tourism based on questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

| Variable | Item | Indicator | Total Score | Ideal Score | Percentage Value Answer | Classification |
|---|---|---|---|---|---|---|
| Community participation in decision-making. | 1 | Tourism policy | 2615 | 2950 | 88.64% | Very High |
| | 2 | Have voice | | | | |
| | 3 | Opinion asked | | | | |
| | 4 | Consulted issues | | | | |
| | 5 | Active involvement | | | | |
| Community participation in sharing knowledge. | 6 | Happy to share | 1498 | 2950 | 50.78% | Low |
| | 7 | Share with stakeholders | | | | |
| | 8 | Share new lesson | | | | |
| | 9 | Share information | | | | |
| | 10 | Share skills | | | | |
| Community participation in empowerment. | 11 | Proud resident | 2522 | 2950 | 85.49% | High |
| | 12 | Feel connected | | | | |
| | 13 | Have voice | | | | |
| | 14 | Access decision making | | | | |
| | 15 | Voice makes different | | | | |
| Community knowledge about rural tourism. | 16 | Farm visit | 4480 | 5900 | 75.93% | High |
| | 17 | Traditional Cultures and Heritage | | | | |
| | 18 | Nature's beauty and serenity | | | | |
| | 19 | Community history | | | | |
| | 20 | Adventure excursion | | | | |
| | 21 | Traditional cuisines | | | | |
| | 22 | Religious heritage | | | | |
| | 23 | Meeting new people | | | | |
| | 24 | Rewarding jobs | | | | |
| | 25 | Rural settings | | | | |
| **TOTAL** | | **25 items** | **11,115** | **14,750** | **75.36%** | **High** |

Based on Table 4, the Kiulu community's participation in rural tourism development shows notable variations across different aspects. Their involvement in decision-making is exceptionally high (88.64%), demonstrating active engagement in shaping tourism initiatives and advocating for sustainable practices that align with community values. Similarly, their participation in empowerment is also significant (85.49%), indicating a strong sense of involvement and influence in tourism-related activities.

In contrast, participation in sharing knowledge is notably low (50.78%), highlighting limited opportunities or platforms for the community to contribute their expertise to tourism stakeholders. On the other hand, community knowledge about rural tourism is relatively high (75.93%), reflecting an informed awareness of potential of tourism and related practices. Overall, the community's strong role in decision-making underscores a collaborative and inclusive approach to rural tourism development in Kiulu, emphasizing the value of local perspectives while fostering empowerment and active participation.

**The Importance-Performance Analysis (IPA) of respondents' perspectives on rural tourism in Kiulu**

Table 5 shows the results of the IPA classification which reveal that the majority of attributes assessed by respondents fall into Quadrant 1: *Keep Up the Good Work*, indicating high importance and high performance. These attributes include farm visits, nature uniqueness, traditional cuisines, traditional culture and traditions, community history, landscape and scenery, religious heritage, peaceful and serene environment, meeting new people, adventure activities, personal safety and security, accessibility of the destination, clear road signs and maps, cultural sights and offers, recreational activities, local festivals, accommodation quality, tour guide services, access to public infrastructure, size and quality of the destination, cleanliness, quality of infrastructure, involvement of the local community, variety and linkage of ecotourism destinations, tourism destination management, and total travel cost.

Conversely, Quadrant 2: *Concentrate Here* highlights two attributes—easy transportation access and Internet connection—that require immediate attention due to their high importance but relatively low performance. Finally, Quadrant 3: *Low Priority* includes only one attribute, entertainment offers, which is perceived as less critical by respondents and performs at a satisfactory level. These findings emphasize the strengths and areas for improvement in the Kiulu tourism experience. There were no attributes in Quadrant 4.

**Table 5**: Importance Performance Analysis (IPA) classification of respondents' attributes based on questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

| Quadrant | Indicator | Variables |
|---|---|---|
| Q1 | Keep up the good work | V1 (Farm visits)<br>V2 (Nature uniqueness)<br>V3 (Traditional cuisines)<br>V4 (Traditional culture and traditions)<br>V5 (Community history)<br>V6 (Landscape and scenery)<br>V7 (Religious heritage)<br>V8 (Peaceful and serene)<br>V9 (Meeting with new people)<br>V10 (Adventure activities)<br>V11 (Personal safety and security)<br>V12 (Destination can be reached)<br>V13 (Clear road signs and maps)<br>V15 (Cultural sights and offers)<br>V18 (Recreational activities)<br>V19 (Local festivals)<br>V20 (Accommodation quality)<br>V21 (Tour guide service)<br>V22 (Access to public infrastructure)<br>V23 (Size of destination)<br>V24 (Quality of destination)<br>V25 (Cleanliness of destination)<br>V26 (Quality of infrastructure)<br>V27 (Involvement of local community)<br>V28 (Variety and linkage of ecotourism destinations)<br>V29 (Management of tourism destination<br>V30 (Total travel cost) |
| Q2 | Concentrate here | V14 (Easy transportation access)<br>V16 (Internet connection) |

| | | |
|---|---|---|
| Q3 | Low priority | V17 (Entertainment offers) |
| Q4 | Possible overkill | None |

The majority of Kiulu rural tourism attributes, however, were positioned above the iso-priority diagonal line, meaning the importance of these attributes currently exceeds their performance. Of the 30 attributes, only seven (V2 nature uniqueness, V3 traditional cuisines, V8 peaceful and serene, V9 meeting with new people, V11 personal safety and security, V2 quality of destination and V25 cleanliness of destination) were positioned below the iso-priority diagonal, meaning they were the only seven attributes whose performance exceeded the importance respondents expressed concerning them (Fig. 4).

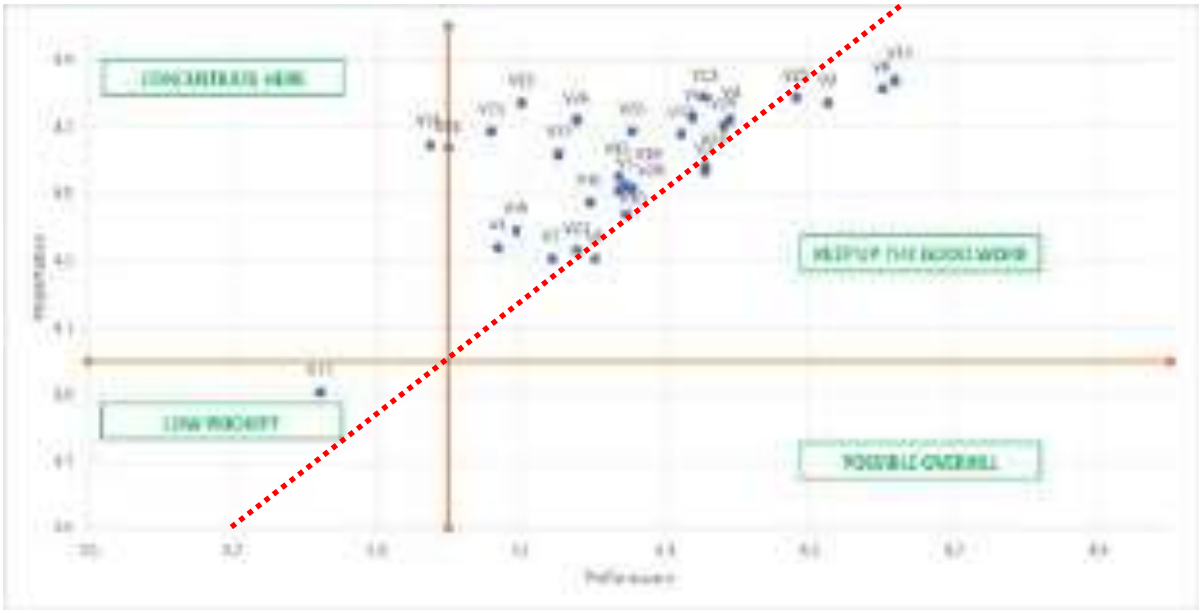

**Figure 4:** Importance-Performance Analysis (IPA) quadrants based on questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia. V1: Farm visit, V2: Nature uniqueness, V3: Traditional cuisines, , V4: Traditional culture and traditions, V5: Community history, V6: Landscape and scenery, V7: Religious heritage, V8: Peaceful and serene, V9: Meeting with new people, V10: Adventure activities, V11: Personal safety and security, V12: Destination can be reached, V13: Clear road signs and maps, V14: Easy transportation access, V15: Cultural sights and offers, V16: Internet connection, V17: Entertainment offers, V18: Recreational activities, V19: Local festivals, V20: Accommodation quality, V21: Tour guide service, V22: Access to public infrastructure, V23: Size of destination, V24: Quality of destination, V25: Cleanliness of destination, V26: Quality of infrastructure, V27: Involvement of local community, V28: Variety and linkage of ecotourism destinations, V29: Management of tourism destination, V30: Total travel cost.

## Issues and challenges faced by the respondents

Fig. 5 illustrates problems and challenges faced by respondents in the Kiulu rural tourism industry. Respondents were presented with a set of 18 items related to issues and challenges encountered in operating tourism in the Kiulu region, and were asked to indicate their views using a five-point Likert scale ranging from "strongly disagree" to "strongly agree." The spider-web figuration shows three major problems and challenges were identified by respondents. Financial problems were the most significant problem (45 respondents), followed by problems with road access (11 respondents) and the need for tourism services and management training (11 respondents).

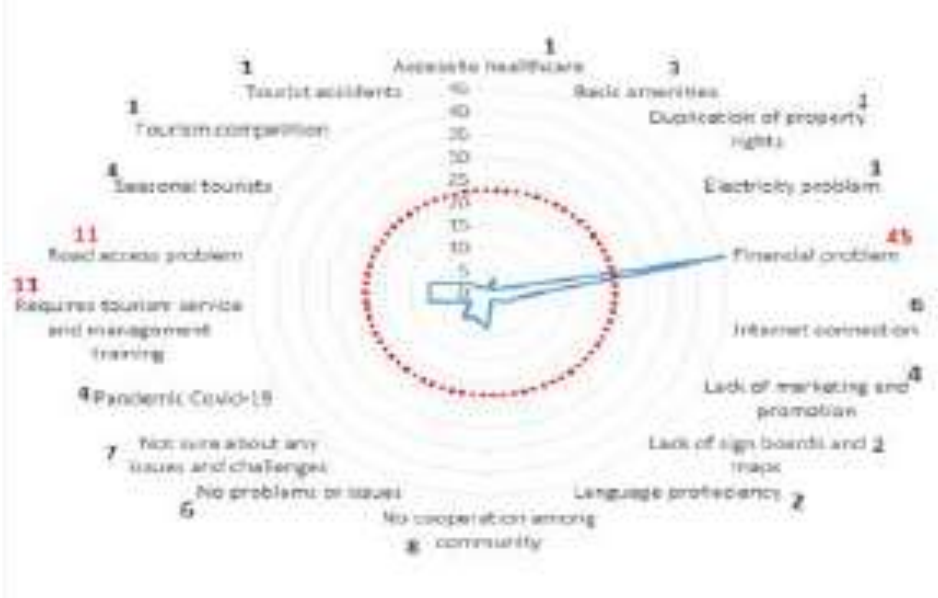


**Figure 5:** Issues and challenges faced by the respondents in the tourism industry based on questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

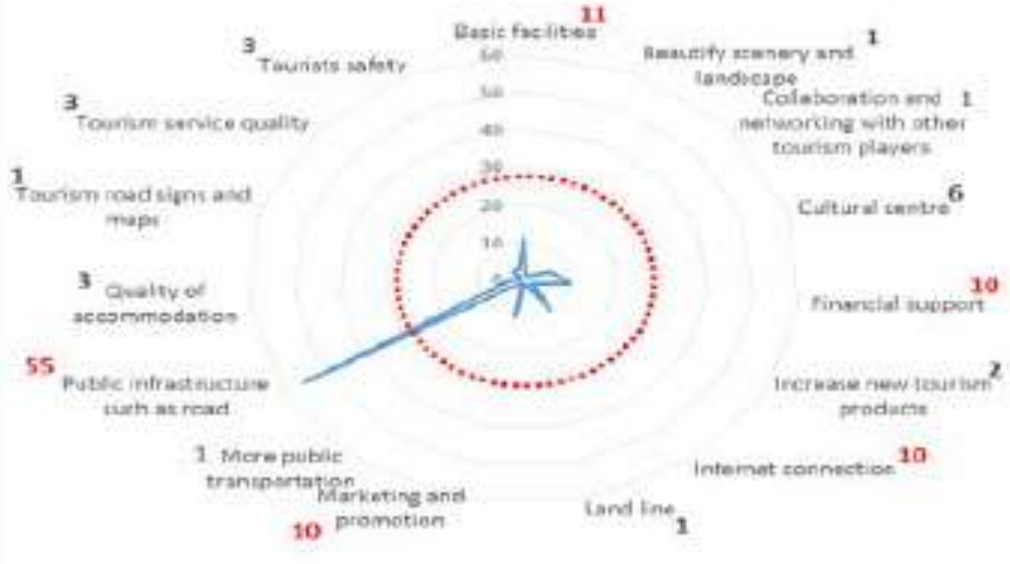


**Figure 6:** Improvement suggested and required by respondents based on questionnaires regarding rural tourism development that were distributed in the local community of Kiulu, Sabah, Malaysia.

## Recommendations suggested by the respondents as mitigation measures for the problems and challenges faced

The spider web configuration depicted in Fig. 6 illustrates the predominant sentiment among respondents—based on their responses to 15 specific items—regarding the crucial role of government agencies in bolstering the rural tourism industry in Kiulu. Respondents were asked

to indicate their views using a five-point Likert scale ranging from "strongly disagree" to "strongly agree." A majority of respondents (55 respondents) believed that additional government support was needed to enhance public infrastructure (particularly roads) to facilitate seamless access to rural tourism sites across Kiulu villages. Additionally, respondents identified various other essential improvements vital for the sustainability of the rural tourism industry in Kiulu. These encompassed the provision of basic amenities such as public toilets (11 respondents), intensified efforts in marketing and promotion (10 respondents), improved Internet connectivity (10 responses), and increased financial support (10 respondents).

**Respondents' perceptions about Kiulu as Sabah's rural tourism destination**

Fig. 7 presents the Leximancer concept map illustrating respondents' perceptions of Kiulu as a rural tourism destination in Sabah. This map includes 46 concepts (represented as small grey nodes) grouped into nine themes (depicted by larger coloured circles). The themes are colour-coded, with hot colours (red, orange) indicating the most relevant themes and cool colours (blue, green) indicating the least relevant. The themes and their corresponding connectivity rates are: "rural" (100%), "residents" (47%), "destination" (37%), "tourists" (20%), "quality" (18%), "economic" (5%), "model" (3%), "Malaysia" (2%), and "competitiveness" (1%).

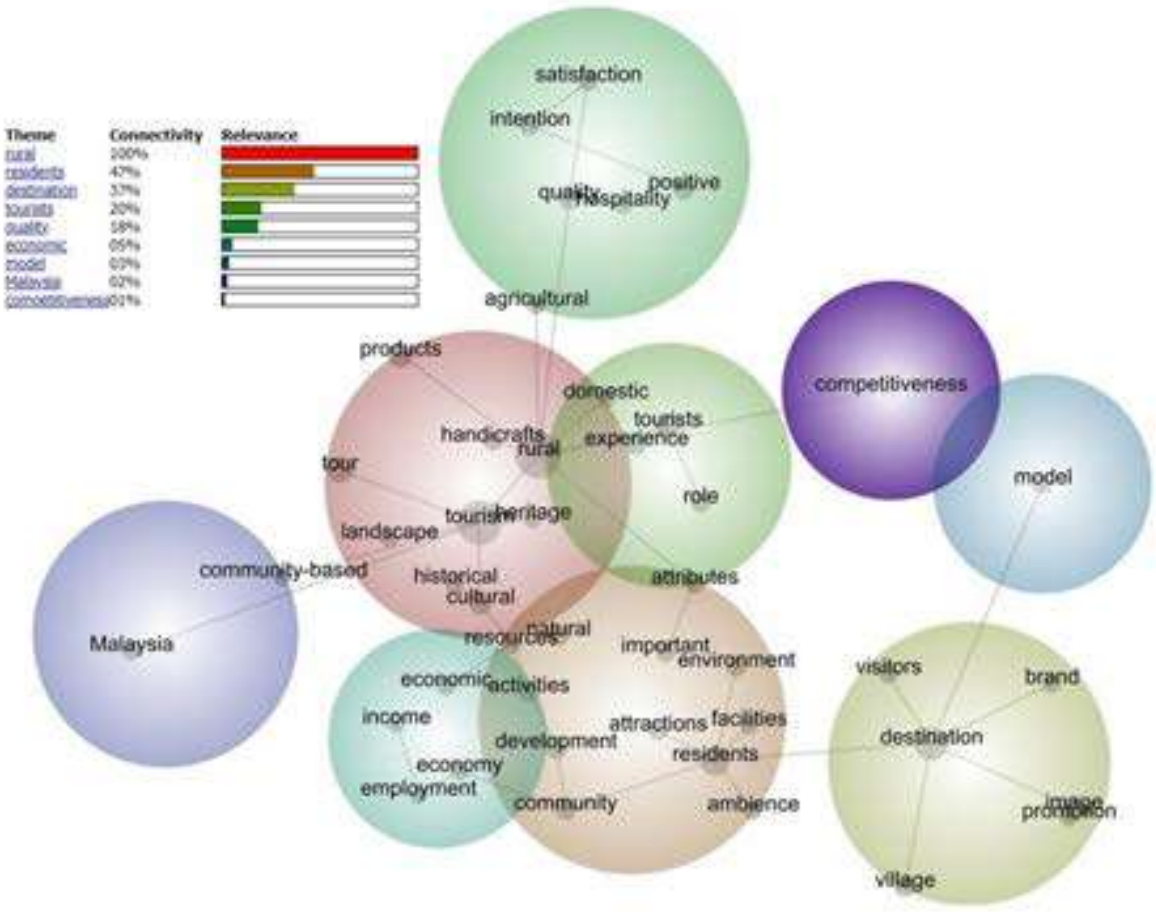


**Figure 7:** Concept map derived from Leximancer analysis about the local community's perception of the rural tourism industry in Sabah, Malaysia.

In the Leximancer analysis, connectivity rates indicate how often the concepts within a theme are mentioned together, reflecting the relative importance of each theme. The most essential theme is assigned a connectivity rate of 100%. These rates were calculated based on the connectedness of concepts within the themes, showcasing their relative importance within the dataset (Leximancer, 2025). The theme "rural" emerged as the most significant among respondents' perceptions of Kiulu, with the term 'rural' mentioned 475 times across the 46 concepts identified. This indicates strong community support for Kiulu as a rural tourism destination in Sabah. Supporting concepts included "tourism," "handicrafts," "products," "tour," "heritage," "landscape," "historical," "cultural," and "resources." The findings illustrated in the Leximancer analysis suggest that community attitudes towards rural tourism have significantly enhanced Kiulu's performance as a tourism destination.

The responses to the semi-structured survey indicated that from the respondents' perspective, there is consensus that Kiulu possesses all the essential components of an ideal rural tourism destination—an observation that strongly aligns with the dominant "rural" theme identified in the Leximancer analysis. This is illustrated by the following responses.

| | |
|---|---|
| Respondent 5 | *In Kiulu, examples of rural community-based tourism include traditional culture, customs, local cuisine, wildlife, locally grown produce, homestays, rural landscape, and others. We have whatever you need!* |
| Respondent 46 | *There are a lot of historical backgrounds in Kiulu that nobody knows. Therefore, it's a perfect product to be highlighted in tourism.* |
| Respondent 78 | *Kiulu is the best place for rural tourism destinations. We got the river, the hills, the food, culture, music, handicrafts from bamboo…* |

Fig. 7 also shows that the theme 'residents' has a significant connection with the theme 'tourism' with connectivity of 47%. This indicates that respondents believed that the Kiulu community is very supportive of the development of Kiulu as a rural tourism destination.

| | |
|---|---|
| Respondent 13 | *I am not a local here. I married a local and reside here after that. For almost 10 years living here, I can see that there is a potential for Kiulu to be a touristic destination. We have the attractions such as the river. I saw a lot of people coming here for the white-water rafting. The environment here is very peaceful and safe.* |
| Respondent 22 | *I have been living here for 25 years. There is potential for tourism. Facilities are there but need upgrades though. I love the ambience here. I guess I made the right choice moving here 25 years ago.* |
| Respondent 47 | *Kiulu is the best place for tourism! All the important tourism attributes are here. We have the river, the hills, the culture, aaaahhh there are a lot of activities you can do it here. I came from Tamparuli. Married a local here by the way. I decided to reside in Kiulu as I see potential for community development especially in tourism. Resources are there, we just need someone to guide us.* |

The theme 'destination' with 37% connectivity was also related to the concept of 'model' at 3%, indicating that the respondents agreed that Kiulu can be a model area for rural tourism development and industry. However, the theme 'destination' in the data sets refers to strategies that the villages in Kiulu need to focus on. Hence, the concepts "visitors", "image", "promotion" and "brand" emerged from the analysis.

| | |
|---|---|
| Respondent 27 | *I do agree that Kiulu has the potential but more works need to be done. Me, I need to be guided about the way how to manage a tourism destination especially if it concerns the visitors coming to Kiulu.* |
| Respondent 52 | *For important of all, the image… Kiulu needs to be developed as an image for rural tourism… Naaaaa… Then, people will come here!* |
| Respondent 69 | *Image is important. It's like promotional branding for Kiulu. If Kiulu is going towards rural tourism, works toward it. If we mix everything in one pot, nobody knows us. We need to showcase our strength in this tourism industry. Must champion one thing, to create that brand image.* |

Moreover, with support and proper training in rural tourism management, the majority of the respondents stated that Kiulu has the potential to become a model for rural tourism destinations in Sabah. This sentiment is reflected in the "model" (3%) theme identified in the Leximancer analysis, which—although less prominent—highlights the aspiration among community members to see Kiulu serve as an example for other rural tourism sites.

| | |
|---|---|
| Respondent 73 | *If guided well, Kiulu can be a model rural tourism village.* |
| Respondent 81 | *I agree that, if there's support from the government, especially in providing and upgrading the infrastructure here in Kiulu, Kiulu can be a role model for other rural tourism players.* |
| Respondent 101 | *Oh, yes! Kiulu should become a rural tourism destination model for others. We had successfully created a branding for Kiulu, thanks to the YB.* |

The theme "tourists" with 20% connectivity in the data sets show a direct connection to the theme "competitiveness" with 1% connectivity. This illustrates that the respondents do understand the important roles that they should play in terms of tourists' experiences when participating the domestic tourism in Kiulu, especially after Covid19.

| | |
|---|---|
| Respondent 4 | *I guess we must start all over again in this post-Covid19 situation. With all the new norms, SOPs, face masks… Well, what's important is that the tourists shouldn't be declined their rights to experience quality domestic tourism.* |
| Respondent 100 | *Our domestic tourism will open soon. I hope we can be prepared with all SOPs.* |
| Respondent 117 | *I don't know if I am prepared for tourists coming to Kiulu for domestic tourism purposes But I guess, we all know what to do by the time they come.* |

"Quality" with 18% connectivity shows the important attributes that the respondents thought could help boost the rural tourism industry in Kiulu. Based on the analysis, it shows that the concept of "hospitality", "positive", "intention" and "satisfaction" emerged from the data set. These concepts signified that the respondents think that to become a successful rural tourism destination, the most important attribute is that visitors have a high level of satisfaction when visiting Kiulu. These respondents believe that a satisfied tourist is always related to quality hospitality and thus provides a positive vibe and has the intention of revisiting the destination.

| | |
|---|---|
| Respondent 12 | *The most important thing about managing a tourism destination is of course tourists' satisfaction. We must ensure they are happy! When they are happy, we are also happy. Sometimes, they come again to us. If not them, their friends, or their families.* |
| Respondent 67 | *Hospitality is important. The way how we treat our visitors will determine their satisfaction.* |

| | |
|---|---|
| Respondent 89 | *A beautiful destination sometimes does not reflect a quality experience. A satisfied tourist will put a good word about you and the services you provide for them. So, being nice to people is important. What do you call that....? Aaaahhh… Hospitality…* |

Finally, the theme "Malaysia" with 2% connectivity is linked to the theme "tourism", indicating that rural tourism is perceived as another mechanism to support the country's economy.

| | |
|---|---|
| Respondent 9 | *Tourism has been an important pillar of the Malaysian economy. It was already shown to be a resilient sector despite economic and political uncertainty… Just look at us, during the Covid-19 pandemic. Once the MCO is uplifted, everyone just goes travelling.* |
| Respondent 30 | *If our government helps in facilitating rural tourism development by giving allocation of budget, let us be part of the policy and gives us guidance to manage this tourism, Kiulu can become a powerful rural tourism destination that can contribute to Malaysia's economy.* |
| Respondent 91 | *Tourism has always been an important pillar of the Malaysian economy. I guess, we should just take this opportunity.* |

## DISCUSSION

This study aimed to evaluate the attitudes and level of involvement of the Kiulu community in rural tourism development, focusing on four critical dimensions: participation in decision-making, empowerment, knowledge sharing, and rural tourism awareness. The research findings reveal a nuanced picture of a community that is actively engaged and optimistic about its tourism potential but is also constrained by infrastructural, financial, and knowledge-related challenges.

### High engagement in decision-making and empowerment

One of the key findings of this study is the very high level of community participation in decision-making (88.64%) and high level of empowerment (85.49%), indicating that the local community in Kiulu is not merely a passive recipient of tourism development but an active contributor to its direction and management. These findings reflect the theoretical underpinnings of stakeholder theory, which posits that successful and sustainable tourism outcomes depend on balanced and inclusive participation of all stakeholders (Wondirad & Ewnetu, 2019). In the context of rural tourism, community inclusion in decision-making not only fosters legitimacy and social acceptance but also ensures that tourism development aligns with local values, needs, and aspirations (Lepp, 2008).

The concept of empowerment has been widely recognized in tourism studies as a key element in achieving sustainable and inclusive development. As Rasoolimanesh et al. (2018) highlighted, community empowerment encompasses not only participation in decisions but also the ability to influence outcomes, develop self-confidence, and build the capacity to manage tourism initiatives. Empowerment in rural tourism goes beyond economic gain—it involves strengthening local voices, fostering pride in cultural identity, and ensuring equitable access to opportunities. In Kiulu, respondents expressed pride in being part of a community that is increasingly seen as a rural tourism hub, which suggests that psychological and social empowerment are as important as material benefits. Such empowerment also increases the community's resilience to external shocks and supports long-term stewardship of local resources.

**Knowledge awareness versus knowledge sharing**

Despite high levels of awareness and understanding of rural tourism concepts (75.93%), the findings highlight a notably low level of knowledge sharing (50.78%) among the Kiulu community. This discrepancy suggests that while community members may be informed about rural tourism practices, there may be limited mechanisms or opportunities to exchange this knowledge with peers, stakeholders, and institutional actors.

This finding supports the arguments made by Dogra & Gupta (2012) and Sood et al. (2017) that knowledge without effective platforms for sharing can undermine inclusive participation. In the case of Kiulu, the lack of formal channels for horizontal learning and collaboration—such as community forums, peer learning groups, or structured training programmes—may hinder the community's ability to co-create tourism strategies and innovate collectively. Closing this gap could significantly enhance the collective capacity of the community, particularly in areas like service quality, visitor experience design, and sustainable resource use.

**Infrastructure, training, and financial barriers**

The study also identified financial constraints, inadequate road access, and lack of training in tourism management as major challenges faced by the local community. These findings echo the broader literature on rural tourism development in Southeast Asia, where infrastructural deficits and limited access to capital are commonly cited as barriers to inclusive growth (Arismayanti & Suwena, 2022). In Kiulu's case, these issues are not only logistical in nature but are deeply intertwined with questions of equity and long-term sustainability.

Importantly, respondents emphasized the need for government support in addressing these challenges. This includes improvements in road infrastructure, digital connectivity, basic amenities, and the provision of training and financial assistance. These calls for intervention suggest a community that is ready and willing to participate but is in need of enabling conditions. If addressed effectively, these improvements could unlock a wider distribution of tourism benefits, prevent marginalization, and catalyze innovation in community-based tourism products.

**Strengths and opportunities identified through IPA**

The Importance-Performance Analysis (IPA) reinforced many of these findings. A majority of tourism attributes—such as traditional cuisine, cultural experiences, scenic landscapes, and farm visits—were found to be both highly important and performing well, falling into the "Keep Up the Good Work" quadrant. This suggests that the foundation for rural tourism excellence already exists in Kiulu, and that current efforts by the Kiulu Tourism Association (KTA) and other local actors have been effective in promoting these core experiences.

However, two critical attributes—transportation access and Internet connectivity—fell into the "Concentrate Here" quadrant, indicating urgent attention is required to improve these essential services. These issues not only affect visitor satisfaction but also limit local entrepreneurs' ability to market their products, communicate with tourists, and engage in digital tourism ecosystems.

**Community aspirations and identity**

The Leximancer concept map analysis revealed that the most dominant theme emerging from interview data was "rural" (connectivity rate: 100%), reflecting strong community identification with rural heritage, traditions, and landscapes. This supports the notion that rural

identity is not just a branding label but a lived experience and a shared value system among residents. Concepts like handicrafts, heritage, landscape, and resources further affirm the role of cultural and natural capital as core components of Kiulu's tourism appeal.

Themes such as "model", "destination", and "quality" provide deeper insights into community aspirations. Many respondents see Kiulu as a potential benchmark for rural tourism in Sabah, provided there is strategic guidance, targeted investment, and sustained collaboration with government agencies. The community's emphasis on visitor satisfaction, hospitality, and branding underscores a sophisticated understanding of the tourism value chain and the importance of creating memorable, high-quality experiences.
The "residents" theme (connectivity rate: 47%) also indicates that residents see themselves as active custodians and beneficiaries of tourism, which is a critical mindset for fostering sustainable tourism development. Meanwhile, the "Malaysia" theme, although less dominant, points to a broader awareness that rural tourism is part of the national economic strategy and that local communities play a vital role in realizing its potential.

**Post-pandemic readiness and sustainability**
An interesting sub-theme that emerged was the community's reflection on post-COVID-19 tourism and their readiness to adapt to new norms, especially with regard to SOPs, health protocols, and service expectations. This awareness illustrates resilience and adaptability—traits that are essential in the post-pandemic tourism landscape.

Importantly, the findings suggest that community-based tourism in Kiulu is both socially and environmentally sustainable, as long as growth is managed carefully and inclusively. The community's emphasis on preserving nature, heritage, and quality over mass commercialization aligns with global best practices in sustainable tourism and should be maintained as tourism in Kiulu continues to expand.

**Study limitations and future directions**
A key limitation of this study lies in the limited participation of women, which resulted in a gender imbalance in the dataset. This underrepresentation is likely due to traditional gender roles and time constraints, which restricted women's availability to participate in surveys and interviews. As women play an important role in rural livelihoods and cultural continuity, their voices are essential in shaping a comprehensive understanding of rural tourism development. The absence of these perspectives may have led to an incomplete portrayal of community dynamics, particularly in areas related to informal hospitality, household-based tourism, and cultural knowledge transmission.

To address this gap, future research must adopt more inclusive engagement strategies, such as targeted interviews, gender-sensitive facilitation, and flexible scheduling that accommodates women's domestic responsibilities. Incorporating Participatory Rural Appraisal (PRA) techniques or women-only focus group discussions could ensure a more balanced representation. In doing so, future studies can capture a fuller range of local experiences and enhance the validity of rural tourism research. Such approaches will also contribute to more equitable and gender-responsive tourism policies that empower all segments of rural communities.

## CONCLUSIONS

This study has demonstrated that the Kiulu community exhibits a high degree of involvement and a positive attitude toward rural tourism development, particularly in areas of decision-making and community empowerment. The findings confirm that local residents recognize the value of rural tourism not only as a driver of economic benefits, but also as a means to preserve cultural heritage and promote environmental stewardship. However, the study also revealed critical gaps, particularly in knowledge sharing, access to infrastructure, and capacity development, which require immediate policy attention to ensure the long-term sustainability and inclusiveness of rural tourism in Kiulu.

Despite limitations such as the underrepresentation of women and access challenges during data collection, the research has provided a grounded and community-driven understanding of the factors shaping rural tourism in Sabah. Moving forward, more inclusive engagement strategies, particularly to amplify marginalized voices, will be essential. Likewise, coordinated interventions by government agencies, tourism boards, and community-based organizations are needed to improve infrastructure, enhance digital connectivity, and expand training opportunities. With the right support, Kiulu holds the potential to serve as a model for community-based rural tourism in Malaysia, contributing meaningfully to national economic growth, cultural resilience, and sustainable development.

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## DECLARATIONS

**Research permit(s).** Not applicable.
**Ethical approval/statement**. This study was conducted with the approval from Sabah Tourism Board and questionnaires were checked and endorsed under the project code: STG-RT Kiulu.
**Generative AI use.** The author(s) declare that generative AI has been used in compliance with the JTBC policies, and that we have reviewed and edited the content after using this tool and we take full responsibility for the content of the publication.

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**Research Article**

# Modulation of Oxidative Stress by *Centella asiatica* (L.) Urb. Leaves Against Carbon Tetrachloride-Induced Hepatic Damage in Rats

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## ABSTRACT

Liver injury induced by oxidative stress remains a significant global health concern, necessitating the exploration of safe and effective hepatoprotective agents derived from natural sources. *Centella asiatica* (L.) Urb., frequently referred to as 'pegaga', is a plant renowned for its notable medicinal attributes. The primary aim of this study is to assess the antioxidant capacity and hepatoprotective activity of the ethanolic extract of *C. asiatica* (EECA). The assessments used to evaluate EECA for its antioxidant properties included total phenolic content (TPC), as well as 2,2-diphenyl-1-picrylhydrazyl (DPPH) and reducing power assays. Additionally, the study evaluated the ability of EECA to mitigate experimentally induced hepatic injury in Sprague-Dawley rats caused by carbon tetrachloride ($CCl_4$) at 100, 500, and 1,000 mg/kg b.wt. doses. Biochemical assays included measuring hepatic marker enzymes alanine transaminase (ALT) and aspartate transaminase (AST) in serum, as well as evaluating malondialdehyde (MDA) and reduced glutathione (GSH) levels in liver homogenates. The TPC of EECA was 106.55 ± 2.23 mg GAE/g. Its DPPH and reducing power assays exhibited moderately potent antioxidant attributes compared to standard references. Nonetheless, groups subjected to $CCl_4$ displayed notably elevated levels of ALT and AST, increased MDA, and decreased GSH. Pretreatment with EECA resulted in a marginal decrease in hepatic marker enzyme activity, with reductions in ALT (14% to 2-fold), AST (25% to 3-fold), and MDA (15–57%), along with an increase in GSH (3–23%). These findings imply that EECA contains active constituents capable of mitigating the hepatotoxic effects induced by $CCl_4$.

**Keywords**: Oxidative stress; hepatoprotection; ethanolic extract; *Centella asiatica*; antioxidants; carbon tetrachloride.

## INTRODUCTION

Oxidative stress-induced liver injuries represent a significant medical challenge, arising from an imbalance between the ability of the body to neutralise reactive oxygen species (ROS) and their production. This condition occurs when the liver is exposed to harmful chemicals, resulting in cellular damage and disruption of essential liver functions. Contributing factors include prolonged alcohol consumption, infections, specific medications, environmental pollutants, high-calorie diets, and exposure to toxins, ultraviolet radiation, or heavy metals. Oxidative imbalance plays a critical role in driving inflammation, necrosis, apoptosis, fibrosis, and malignant transformation, and is a major contributor to the pathogenesis of non-alcoholic fatty liver disease (Allameh et al., 2023). According to the WHO (2024), cirrhosis and hepatocellular carcinoma—the most prevalent form of liver cancer—account for the majority of the estimated 290,000 hepatitis C-related deaths. Synthetic chemicals and medications used to treat or model liver diseases, such as carbon tetrachloride ($CCl_4$) and acetaminophen, have been reported to aggravate liver damage (Hota et al., 2022). This has led to an increased reliance on herbal drugs, which are now widely utilised. Herbal medications have a long-standing history in the treatment of liver conditions and offer a holistic approach to promoting liver health (Abdel-Hamid et al., 2018).

*Centella asiatica* (L.) Urb., commonly referred to as 'pegaga', is a medicinal plant with a long-standing role in traditional medicine, indigenous to Southeast Asian countries, including Malaysia and Indonesia (Orhan, 2012). For centuries, this herb has been utilised for its pharmacological properties, particularly due to its triterpene content. Studies have identified triterpene and phenolic compounds as the active constituents in *C. asiatica*, highlighting their relevance to the biological effects of the plant (Gnanapragasam et al., 2004). *C. asiatica* contains various bioactive compounds, such as madecassoside, madecassic acid, asiaticoside, and asiatic acid, which significantly contribute to its antioxidant, anti-inflammatory, and wound-healing activities (Hashim et al., 2011; Su et al., 2015). The rich phytochemical composition of *C. asiatica*, particularly its abundance of phenolic compounds, is associated with a broad spectrum of pharmacological effects. The presence of phenolics, including flavonoids, has been linked to antioxidant activities that play a vital role in protecting against oxidative stress and lipid peroxidation (LPO) (Zainol et al., 2003). Additionally, the phenolic compounds in *C. asiatica* have demonstrated antioxidant and cytotoxic activities, reinforcing the association between these compounds and the pharmacological properties of the plant (Nazmi & Sarbon, 2020). This establishes the significance of *C. asiatica* as a valuable herbal remedy in both traditional and modern medicine (Niamnuy et al., 2013; Ratz-Lyko et al., 2016).

Despite existing literature highlighting the potential of *C. asiatica* in liver protection, scientific confirmation remains limited, partly due to the narrow focus of studies relying predominantly on $CCl_4$-induced models, which may not fully reflect the complexity of hepatic injury in clinical settings. This limitation also stems from the absence of parameters involving antioxidative enzymes in the proposed mechanisms. Therefore, the present study aims to assess the antioxidant and hepatoprotective effects of the ethanolic extract of *C. asiatica* (EECA), with a focus on its potential to mitigate hepatic dysfunction and oxidative stress induced by $CCl_4$ in a rat model.

## METHODOLOGY

### Preparation of EECA

Whole *C. asiatica* plants were obtained from Papar, Sabah, Malaysia, verified by an ethnobotanist, and stored at the Biotechnology Research Institute, Universiti Malaysia Sabah, with the voucher number MI 001. Following rinsing with distilled water, the leaves were dried at 37 °C for 72 h until a constant weight was achieved, and then finely ground into powder. A total of 100 g of dried powder was extracted with 400 mL of 80% ethanol by shaking in a water bath at 40 °C for 4 h. The mixture was centrifuged at 3,000 rpm for 10 min and filtered through Whatman No. 1 filter paper. Ethanol was removed using a rotary evaporator (BÜCHI Labortechnik AG, Flawil, Switzerland) at 40 °C for 30 min under reduced pressure. The sample was frozen overnight at −80 °C, lyophilised, and the resulting EECA powder was stored at −80 °C for further analysis.

### Chemical antioxidant assays

The total phenolic content (TPC) was analysed using a modified Folin–Ciocalteu procedure reported by Awang et al. (2023). Absorbance was measured at 720 nm using a spectrophotometer (PerkinElmer, Waltham, MA, USA), and results were expressed as mg GAE/g of extract. The free radical scavenging activity of EECA was evaluated using a slightly modified 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay based on the method of Jinoni et al. (2024). Absorbance was measured at 517 nm following vortexing and 60 min of incubation at room temperature, with results expressed as percentage inhibition. The reducing power assay followed the method of Bhalodia et al. (2013), with absorbance measured at 700 nm against a blank, where higher absorbance indicated greater reducing capacity. Ascorbic acid (AA) served as the positive control in both DPPH and reducing power assays.

### Experimental design

The animal experiment complied with the ethical guidelines of the university and federal laws governing animal experimentation. Approval was obtained from the Animal Ethics Committee under protocol number UMS/IP7.5/M3/4-2012. Male Sprague-Dawley rats (7–8 weeks old, 200–250 g) were acclimated prior to the study and housed under controlled conditions at the Biotechnology Research Institute, Universiti Malaysia Sabah, with free access to tap water and standard rodent chow. Rats were randomly divided into five groups (n = 6 per group) based on treatment and hepatotoxicity protocols, as shown in Fig. 1. All animals received treatments by oral gavage once daily for 14 consecutive days. On days 13 and 14, rats (except those in the normal group) received $CCl_4$ (Sigma-Aldrich, St. Louis, MO, USA) diluted 1:1 in corn oil, administered orally via gavage at a dose of 1 mL/kg b.wt. to induce hepatotoxicity. The selection of experimental groups and the $CCl_4$ dosing regimen were based on established hepatotoxicity models in rodents, as described by Vun-Sang et al. (2024).

Each treated animal was euthanised 24 h after the final administration of $CCl_4$. Following anaesthesia induction with mild ether, the animal was decapitated, and blood was collected via cardiac puncture into tubes coated with lithium heparin to obtain plasma samples. The liver was excised, connective tissue removed, and the organ rinsed with saline to eliminate potential blood contamination. Liver tissue was then stored at –80 °C in a freezer (Thermo Fisher Scientific, Waltham, MA, USA) for biochemical analyses to assess the activity of hepatic antioxidant enzymes.

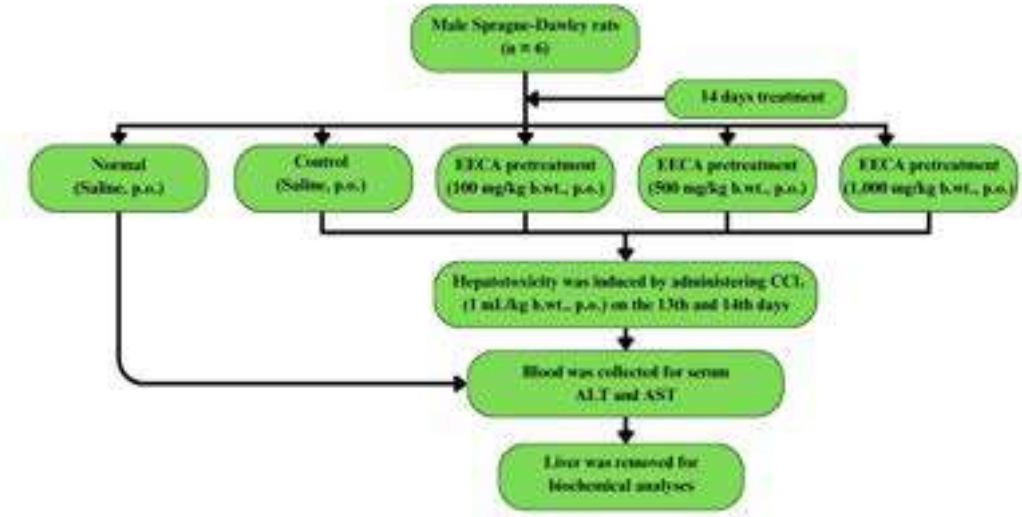


**Figure 1:** Summary of experimental design.

## Preparation of post-mitochondrial supernatant

Post-mitochondrial supernatant was prepared from liver tissue using a standard procedure adapted from Iqbal et al. (1999). The tissue was homogenised using a homogeniser (Kinematica AG, Malters, Switzerland) in cold phosphate buffer (0.1 M, pH 7.4) containing 1.17% (w/v) potassium chloride. The homogenate was centrifuged at 2,000 rpm for 10 min at 4 °C to remove nuclei and cell debris. The resulting supernatant was further centrifuged at 10,000 rpm for 30 min at 4 °C to obtain the post-mitochondrial fraction, which was used for the assessment of endogenous antioxidant enzymes. Protein concentration was determined prior to enzyme assays using the Bradford method, with bovine serum albumin as the standard.

## In vitro antioxidant and oxidative stress biomarkers

Serum obtained by centrifuging blood at 1,500 rpm for 15 min and stored at –20 °C was used to analyse alanine transaminase (ALT) and aspartate transaminase (AST) through separate enzymatic assays. According to Reitman and Frankel (1957), colour development in the reaction mixtures was measured at 510 nm after 30 min. LPO was evaluated by determining malondialdehyde (MDA) levels using the thiobarbituric acid reactive substances (TBARS) method described by Buege and Aust (1978), with absorbance measured at 535 nm. MDA levels were calculated using a molar absorptivity of $1.56 \times 10^5$ $M^{-1}$ $cm^{-1}$ and expressed as nmol MDA formed/g tissue. Reduced glutathione (GSH) levels were determined using the method described by Jollow et al. (1974), with absorbance measured at 412 nm and results reported as µmol reduced GSH/g tissue.

## Statistical analysis

Data are expressed as mean ± standard deviation. Statistical comparisons between groups were conducted using one-way analysis of variance (ANOVA), followed by Tukey's Honestly Significant Difference (HSD) post hoc test. Statistical evaluation was performed using GraphPad Prism (version 10), with $p < 0.05$ considered significant.

## RESULTS

### Effect of EECA on antioxidant activity

Leaves and other plant components are rich in phenolic compounds, which contribute to stable antioxidant enzyme activity. The TPC of EECA was 106.55 ± 2.23 mg GAE/g, indicating high phenolic content. As shown in Fig. 2, EECA exhibited concentration-dependent DPPH scavenging activity, with the highest inhibition recorded at 80.00 ± 0.01% at 600 µg/mL. Similarly, Fig. 3 shows a concentration-dependent increase in reducing power by EECA (10–200 µg/mL), compared to AA. Although EECA demonstrated significant antioxidant potential, its reducing capacity was markedly lower than that of AA.

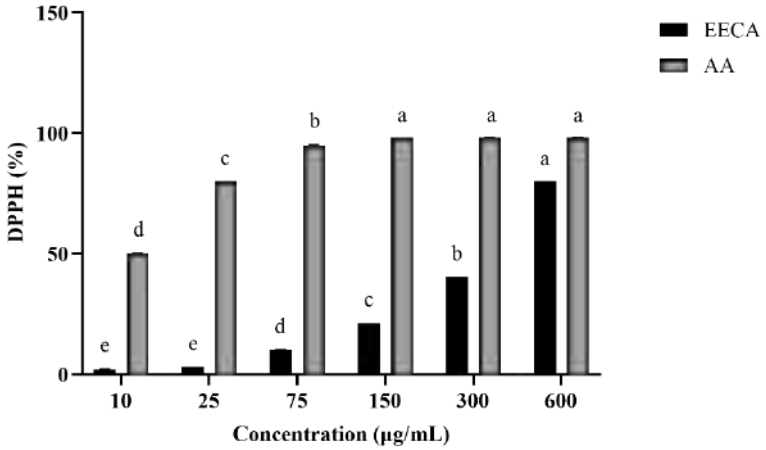


**Figure 2:** Effects of EECA on DPPH radical scavenging activity across various concentrations (10–600 µg/mL). Data are presented as mean ± standard deviation (n = 3). Different letters indicate significant differences between groups (one-way ANOVA, Tukey's HSD, $p < 0.05$).

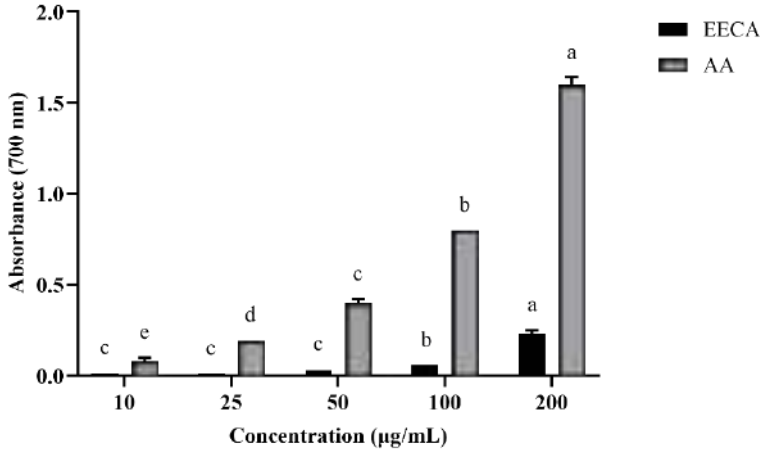


**Figure 3:** Effects of EECA on reducing power across various concentrations (10–200 µg/mL). Data are presented as mean ± standard deviation (n = 3). Different letters indicate significant differences between groups (one-way ANOVA, Tukey's HSD, $p < 0.05$).

**Effects of EECA on serum ALT and AST**

Assessment of liver structural integrity relies on evaluating aminotransferase activities, namely ALT and AST. Serum ALT and AST levels in the control group increased significantly ($p < 0.05$) by 99% and 97%, respectively, compared to the normal group, as shown in Fig. 4. EECA pretreatment at 100 and 500 mg/kg b.wt. significantly ($p < 0.05$) reduced the $CCl_4$-induced increase in serum ALT by 14% and 35%, and AST by 25% and 91%, respectively. Notably, EECA at 1,000 mg/kg b.wt. demonstrated greater efficacy ($p < 0.05$), reducing ALT and AST levels by approximately 2- and 3-fold, respectively, in a dose-dependent manner compared to the lower doses.

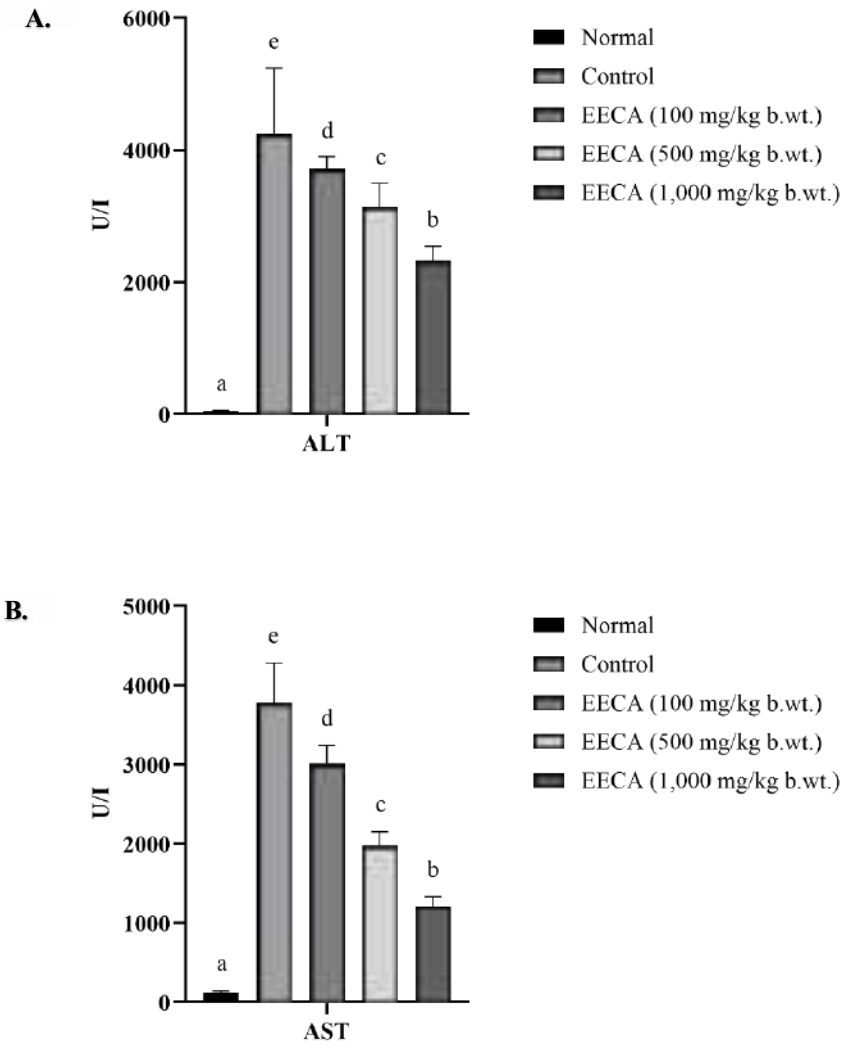


**Figure 4:** Effects of EECA on serum **A.** ALT and **B.** AST levels across various groups. Data are presented as mean ± standard deviation (n = 6). Different letters indicate significant differences between groups (one-way ANOVA, Tukey's HSD, $p < 0.05$).

**Effects of EECA on MDA activity**

Hepatic TBARS levels were assessed by measuring MDA formation through its reactivity with TBA, producing a pink chromophore. Rats subjected to $CCl_4$ treatment exhibited a substantial ($p < 0.05$) increase in hepatic TBARS levels (expressed as MDA) by 44% compared to the normal group, as shown in Fig. 5. However, pretreatment with EECA at 100, 500, and 1,000

mg/kg b.wt. significantly ($p < 0.05$) reduced TBARS levels in a dose-dependent manner by 15%, 33%, and 57%, respectively, with the highest dose showing the greatest inhibition.

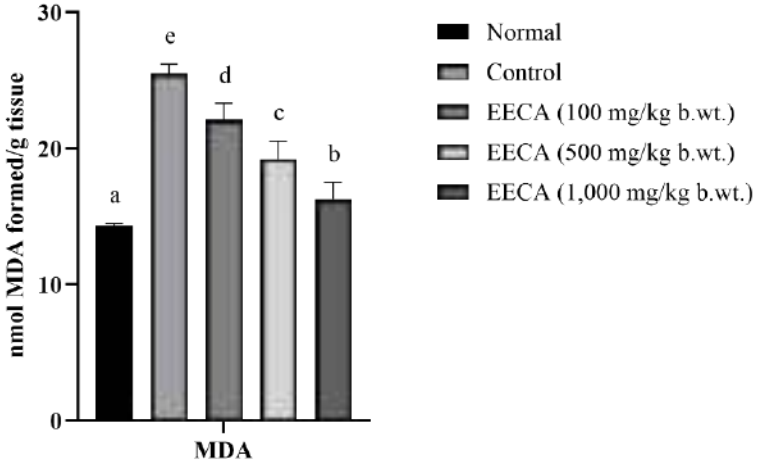


**Figure 5:** Effects of EECA on MDA levels across various groups. Data are presented as mean ± standard deviation (n = 6). Different letters indicate significant differences between groups (one-way ANOVA, Tukey's HSD, $p < 0.05$).

## Effects of EECA on GSH activity

Hepatic GSH levels showed a significant ($p < 0.05$) reduction of 40% in the control group compared to the normal group, indicating oxidative stress (Fig. 6). However, pretreatment with EECA at 100, 500, and 1,000 mg/kg b.wt. significantly ($p < 0.05$) mitigated GSH depletion, increasing GSH levels by 3%, 17%, and 23%, respectively, in a dose-dependent manner. Notably, the 1,000 mg/kg b.wt. dose provided the highest level of protection.

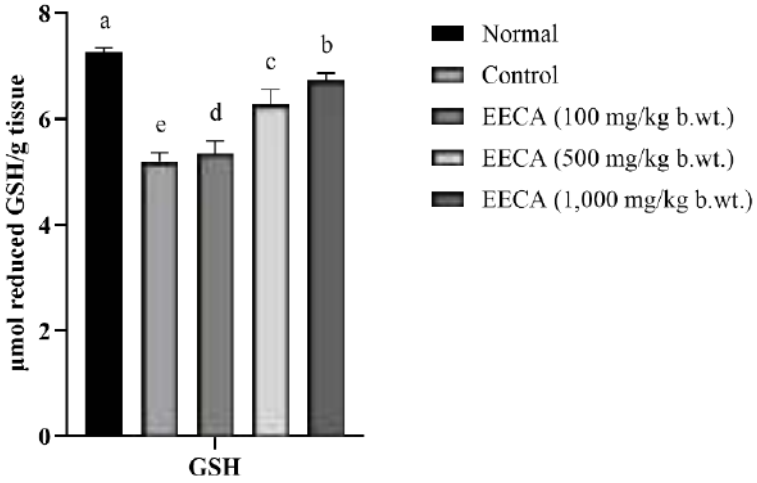


**Figure 6:** Effects of EECA on GSH levels across various groups. Data are presented as mean ± standard deviation (n = 6). Different letters indicate significant differences between groups (one-way ANOVA, Tukey's HSD, $p < 0.05$).

## DISCUSSION

Phenolic compounds are widely recognised for their antioxidant activity, chain-breaking properties, physiological functions, and hepatoprotective effects. These effects arise from redox potential, enabling neutralisation of free radicals, decomposition of peroxides, and suppression of singlet and triplet oxygen (Shahidi & Ambigaipalan, 2015). The Folin–Ciocalteu method, employed to determine TPC, produces a blue solution with maximum absorption at 765 nm through the reduction of metal oxides by phenolic acids (Pérez et al., 2023). According to Moon and Shibamoto (2009), numerous studies have utilised these assays to evaluate antioxidant capacity in various food products. The DPPH assay is widely used due to its simplicity, high sensitivity, minimal technical expertise required, and reliance on a basic spectrophotometer. The reaction involves a colour change from purple to pale yellow as DPPH radicals are reduced and hydrogen atoms are abstracted from antioxidants, indicating effective scavenging activity (Moon & Shibamoto, 2009). Similarly, the ferric reducing antioxidant power assay assesses the ability of antioxidants to convert ferric iron ($Fe^{3+}$-TPTZ) into the more stable divalent $Fe^{2+}$ ion at low pH, producing a violet-blue colour that ensures consistent and timely results (Nwachukwu & Aluko, 2019). Overall, the study demonstrated the antioxidant properties of EECA, as shown by its high TPC and effective elimination of the stable free radical DPPH, although its reducing power, despite being concentration-dependent, was comparatively weak. Previous research has established a correlation between TPC, DPPH radical scavenging activity, and reducing power (Benjamin et al., 2022), suggesting that the observed antioxidant effects of EECA are attributed to its high concentration of phenolic compounds.

$CCl_4$ has long been recognised as a hepatotoxin capable of causing liver damage (Thanebal et al., 2021), primarily entering water sources through industrial waste due to its use in chlorofluorocarbon synthesis (Borzelleca et al., 1990). Its toxicity is associated with accumulation in liver adipose tissue and centrilobular necrosis, with hepatotoxicity initiated in the endoplasmic reticulum of hepatocytes via cytochrome P-450 enzymes. Frequently employed as a model for studying hepatotoxicity (Vun-Sang et al., 2024), $CCl_4$ induces tissue damage through oxidative stress mediated by LPO. Cytochrome P-450 converts $CCl_4$ into highly reactive trichloromethyl ($CCl_3$•) and trichloromethylperoxyl ($CCl_3O_2$•) radicals. These unpaired electron species trigger chain reactions targeting lipid-rich membranes, including mitochondria and the endoplasmic reticulum, disrupting cellular structures and leading to oxidative, mitochondrial, and endoplasmic reticulum stress. These events contribute to apoptosis, necrosis, ferroptosis, and autophagy (Unsal et al., 2021). Free radicals, produced under specific environmental conditions and normal cellular metabolism, lack an electron and achieve stability by acquiring or donating one to nearby molecules. Antioxidants neutralise free radicals before chain reactions responsible for oxidative damage can begin (Chaudhary et al., 2023). To evaluate the potential of EECA in mitigating oxidative stress and free radical-induced damage, its antioxidant properties were assessed in a rat model of $CCl_4$-induced hepatotoxicity.

Liver health can be assessed by measuring serum ALT and AST levels, as these cytoplasmic enzymes are released into circulation following hepatocyte membrane damage (Wang et al., 2017). ALT catalyses the conversion of alanine to pyruvate, while AST catalyses the conversion of aspartate to oxaloacetate. EECA may have protected hepatocyte membranes from damage caused by reactive metabolites during $CCl_4$ exposure, thereby reducing enzyme leakage (McGill, 2016). The lower expression of these transaminases in EECA-treated rats supports this hypothesis. One proposed mechanism suggests that free radical derivatives of

$CCl_4$ contribute to LPO, leading to hepatopathy, and that protection against $CCl_4$ toxicity requires significant antioxidant activity or inhibition of free radical generation (Yin et al., 2011). A substantial increase in MDA levels was observed in the $CCl_4$-only group, indicating that elevated LPO levels contributed to tissue damage and overwhelmed antioxidant defence systems. Treatment with EECA significantly reduced MDA levels, demonstrating its antioxidant potential. GSH levels are critical in determining tissue susceptibility to oxidative injury, as this tripeptide acts as a non-enzymatic antioxidant, providing protection on both intracellular and extracellular surfaces (Kurutas, 2016). GSH, a non-protein sulphydryl compound, helps maintain the reduced state of membrane protein-SH groups, while persistent oxidative stress can lead to cellular deformities (Bansal, 2015). The lower GSH levels observed in rats exposed to $CCl_4$, compared to those treated with both EECA and $CCl_4$, suggest that EECA mitigated susceptibility to $CCl_4$-induced hepatic injury. These findings are consistent with those reported by Sivakumar et al. (2018) and Park et al. (2021), who observed reduced serum hepatic markers and LPO, along with restoration of endogenous antioxidant enzymes, following treatment with *C. asiatica* extract in acetaminophen-induced hepatotoxicity. Hence, the hepatoprotective effects observed in this study further support the therapeutic potential of *C. asiatica*.

Prior research has established that antioxidative enzymes act as a primary defence against ROS and other free radicals. This study evaluated the antioxidant potential of EECA using a rat hepatotoxicity model induced by $CCl_4$ intoxication, with a focus on serum ALT and AST levels, as well as MDA and GSH in hepatic tissues. The findings contribute to understanding the role of *C. asiatica* administration in mitigating oxidative liver damage, suggesting a potential nutritional alternative to pharmacological interventions for reducing hepatotoxicity. However, a key limitation of the study is the absence of histopathological analysis, which is crucial for providing comprehensive insights into hepatic tissue integrity and for complementing biochemical assessments. Such analyses are essential for evaluating structural damage and tissue inflammation caused by toxic substances such as $CCl_4$. Future research should incorporate more extensive histopathological examinations to gain deeper insights into liver tissue architecture, the mechanisms underlying $CCl_4$-induced damage, and the hepatoprotective effects of EECA.

## CONCLUSIONS

The results indicate that EECA exhibits significant antioxidant activity, providing effective protection against $CCl_4$-induced hepatic toxicity in a dose-dependent manner at 100, 500, and 1,000 mg/kg b.wt. This protective effect is demonstrated by improvements in ALT and AST levels, reductions in MDA levels, and prevention of GSH depletion in hepatic cells. The findings suggest that EECA mitigates $CCl_4$-induced hepatic injury by enhancing antioxidant defence mechanisms and reducing oxidative stress. These outcomes highlight the potential of *C. asiatica* in counteracting ROS-related liver damage.

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## DECLARATIONS

**Research permit(s).** Not applicable.
**Ethical approval/statement**. All experimental procedures were approved by the Animal Ethics Committee of Universiti Malaysia Sabah under approval code UMS/IP7.5/M3/4-2012.
**Generative AI use.** AI was not used in this study or in the writing of this article.

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**Research Article**

# Characterisation of Fruticose Lichen Genus *Stereocaulon* from Sabah Based on Morphology, Chemotyping, and Molecular Typing

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## ABSTRACT

Lichens in the genus *Stereocaulon* are commonly found in high-elevation mountains in tropical regions. Taxonomy of *Stereocaulon* is always an ongoing topic discussed among lichenologists, especially for species-level identification due to polymorphism and chemical diversity of *Stereocaulon*. In this study, we examined *Stereocaulon* spp. from Mount Kinabalu, the highest mountain in Borneo (4095 m). A total of 42 *Stereocaulon* specimens were included in this study; 40 were newly collected between 1900 m and 3700 m a.s.l. along the summit trail of Mount Kinabalu, and two specimens from Mount Alab (1900 m) were previously published. We used integrative approaches to delimit the specimens into Operational Taxonomic Units (OTUs) based on macro-morphology, chemical profiles, and molecular phylogeny. Macro-morphological characters, including apothecia (reproductive organ), phyllocladia, pseudopodetia and cephalodia were examined, while chemical profiles were obtained from spot tests and High-performance thin-layer chromatography (HPTLC). A phylogenetic tree was constructed based on the ITS gene by using Bayesian and Maximum Likelihood analyses. A total of six Operational Taxonomic Units (OTUs) were identified and were provisionally named according to their diagnostic morphological and chemical characters, namely, RF (ramular-fluorescence), RN (ramular-nonfluorescence), GF (granular-fluorescence), GN (granular-nonfluorescence), RU1 (ramular-curved 1) and RU2 (ramular-curved 2). The monophyly of each of the OTUs is supported by phylogenetic analysis. Due to lack of reliable identification keys and reference genetic data for this genus, we could not determine the species identities for the OTUs revealed in this study. Nevertheless, the findings of this study provide a baseline for future studies on the taxonomy of *Stereocaulon* species in Mount Kinabalu, integrating morphology, chemistry, and genetics. The implementation of high-performance thin-layer chromatography (HPTLC) profiles show potential for distinguishing samples. However, further research with more *Stereocaulon* samples from different taxa and regions is necessary to verify the reliability of this method.

**Keywords**: Snow lichen; integrative taxonomy; alpine ecosystem; montane forest; thin-layer chromatography; high-performance thin-layer chromatography (HPTLC); mountain ecosystem; Borneo.

## INTRODUCTION

Genus *Stereocaulon* is a widely distributed fruticose lichen genus. These lichens grow mostly in upland regions on siliceous rock, particularly on recent volcanic rock, on metal-rich spoil heaps, and on acidic soil among mosses (Oset, 2014; Ismed et al., 2018). There are around 140 species of *Stereocaulon*, including varieties and forms of the same species, that have been recorded worldwide (Kirk, 2001; Oset, 2014). For *Stereocaulon*, the key morphological characters that have been used for species-level identification are pseudopodetia, phyllocladia, cephalodia, apothecia, and the shape and number of microspores (Fries, 1858; Lamb, 1978; Oset, 2014; Park et al., 2018). Some of the key morphological characters show polymorphism due to the rock types, elevations, and environmental factors, which include light intensity, humidity, air quality and chemical composition of the surroundings (Lamb, 1977; Huang, 2010; Oset, 2014; Athukorala et al. 2016; Park et al., 2018; Lõhmus et al. 2023; McCune et al., 2023). This high polymorphism has made species identification difficult (Myllys et al., 2001, Orange et al., 2001; Oset, 2014). Hence, chemical profiling and phylogenetic approaches, in addition to the morphological characters, have been used to improve the taxonomy of *Stereocaulon* (Lamb, 1951, 1977, 1978; Tønsberg, 1977; Orange et al., 2001; Huang, 2008, 2010; Huyen et al., 2017; McCune et al., 2023; Torres et al., 2023).

The first comprehensive worldwide review of *Stereocaulon* was conducted by Lamb (1951, 1977, 1978), which provided identification keys based on morphological and chemical profile data, including spot tests and thin-layer chromatography. There are very few studies that investigate the detailed chemical composition of *Stereocaulon* (Tønsberg, 1977). Currently, chemical profiling is an essential part of *Stereocaulon* taxonomy and overall lichen taxonomy, especially for species-level identification (Lamb, 1978; Tønsberg, 1977; Orange et al., 2001; Oset, 2014; Huyen et al., 2017; Park et al., 2018; Torres et al., 2023). The commonly used method for the taxonomy of lichen is thin layer chromatography (TLC) and frequently used solvent systems for development include Toluene:Dioxane:Acetic acid (180:45:5 v/v/v), Hexane:Methyl ter-butyl ether:Formic acid (140:72:18v/v/v), and Toluene:Acetic acid (170:30 v/v), which have improved the taxonomic classification (Orange et al., 2001). Atranorin, lobaric acid, stictic acid and norstictic acid are common compounds found in the genus *Stereocaulon* (Lamb, 1978; Tønsberg, 1977; Orange et al., 2001; Oset, 2014; Park et al., 2018).

Due to the high morphological and chemical polymorphism of *Stereocaulon* species, genetic sequences of selected genes have been used to resolve taxonomy problems, both in terms of classification and identification. Many genera and species of lichen have been re-identified and reclassified over the years due to the introduction of genetic analysis (Huang, 2008; Leavitt et al., 2013; Oset, 2014 Park et al., 2018). Many species have a wide distribution across different continents and large geographical areas, but comprehensive studies comparing their morphological and chemical profiles across their full range are rare. This makes it difficult to establish reliable references for species identification (Huang, 2010; Huyen et al., 2017).

In Malaysia, the records of *Stereocaulon* are from the high-elevation habitats on mountains. To date, six taxa that were recorded from Mount Kinabalu, Sabah, which include *S. graminosum* Schaer., *S. granulans* Sipman, *S. halei* I.M. Lamb, *S. massartianum* Hue, *S. massartianum* var. *chlorocarpoides* (Zahlbr.) I.M. Lamb, and *S. staufferi* var. *borneense* I.M. Lamb (Sipman, 1993). Previous studies have shown that these species occupy different elevational zones, and that dominance of the foliose lichens at higher elevations is due to higher humidity and higher light intensity (Sipman, 1993; Huang, 2008, 2010; Hyde et al., 2023). In this study, we used integrative approaches to delimit *Stereocaulon* spp. from Mount Kinabalu

into Operational Taxonomic Units (OTUs) based on macro-morphology, chemical profiles, and molecular phylogeny. We also discussed the possible species identity of the OTUs based on available references for macro-morphology and chemical profiles from the literature. Our study will provide information for this region about the morphological, chemical and genetic variations.

## METHODOLOGY

### The study site and collection of *Stereocaulon* lichens

This research was primarily conducted at Mount Kinabalu (4096 meters above sea level, hereinafter a.s.l.), Sabah, Malaysia. Transect sampling was conducted on the mountain climbing route (Summit Trail) from the Sabah Parks Kinabalu headquarters (1500 m a.s.l.) to the peak (4096 m a.s.l.) (Fig. 1). In addition, two *Stereocaulon* specimens from Mount Alab, collected from the sampling conducted between 1200 m a.s.l. and 1900 m a.s.l. (as previously described in Lim, 2019), were also incorporated in this study. *Stereocaulon* can be easily seen in the field due to their white colour and prominent short-stalked structure (secondary thallus with pseudopodetia), which can only be found growing in large clumps on substrates such as soil and rock in areas with high light intensity (usually open areas with direct sunlight) (Oset, 2014).

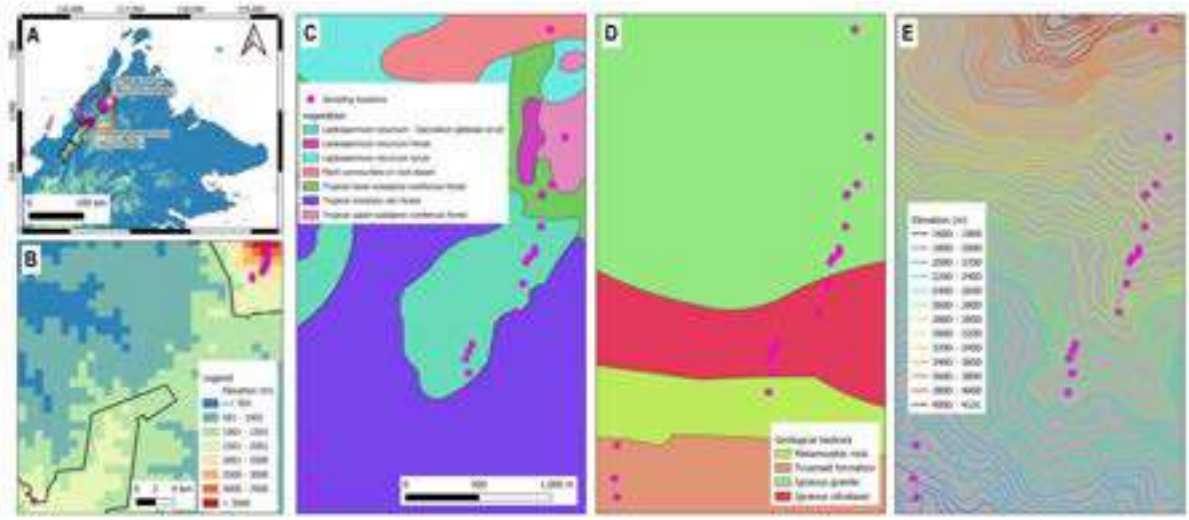


**Figure 1:** Study area and sampling locations on Mount Kinabalu and Mount Alab, Sabah. **A.** Overview map of Sabah showing Kinabalu Park and Crocker Range Park as the study area (red box). **B.** Enlarged map of the study area within Kinabalu Park and Crocker Range Park showing sampling locations (pink dots). **C.** Vegetation map of the summit trail area with sampling locations on Mount Kinabalu; source for vegetation type: Kitayama (1991). **D.** Geological map of the summit trail area with sampling locations on Mount Kinabalu; source for geological type: Badang (1999). **E.** Topographic map of the summit trail area showing detailed elevation contours and sampling locations.

For each population (the clump) of *Stereocaulon* that was observed in the field, habitat information like soil type, elevation, and vegetation type around the lichen population was recorded. Then, a sample of a 10 cm × 10 cm small patch of lichen, each consisting of around 100 g of fresh specimen, was collected by using a small shovel at each of the 42 locations along the Summit Trail (Fig. 1). The samples were immediately processed in the field by removing debris and contamination, and sorting them into three subsamples: one for morphological

examinations, one for genetic sequencing, and one for chemical analysis. The subsample for DNA analysis was kept in a separate tea bag and stored in air-tight containers filled with silica gel in the field (Din et al., 2010; Oset, 2014). Upon returning from the field, the containers with specimens for genetic isolation were stored in a –80°C freezer for further analyses. The specimens for morphological and chemical analysis were air-dried and then were kept in paper bags which were then stored in containers with silica gel. All samples were deposited at BORNEENSIS Herbarium (BORH) of Institute for Tropical Biology and Conservation, Universiti Malaysia Sabah with accession codes: BORH 5854 – BORH 5898 (Table 1).

**Morphological examination of the collected *Stereocaulon* lichens**

Upon return to the laboratory, the morphological characteristics of all the collected specimens were examined using a stereo microscope (Olympus SZ61, .0.67× – 4.5×) and a compound microscope (Olympus CX23). The key characters of *Stereocaulon*, namely, pseudopodetia (C-shape – curved vs. Y-shape – branched, Fig. 2A and Fig. 2B), location of apothecia (Lateral – alongside the lichen body vs. Terminal – at the tip of the lichen body, Fig. 2C and Fig. 2D), phyllocladia (Ramular or Coral-like vs. Granular or Clumpy, Fig. 2E and Fig. 2F), and cephalodia (location of cyanobacteria) were examined, measured, and photographed. Based on these morphological characters, we assigned the samples into 4 morphological groups as shown in Fig. 2. Both the identification of these key morphological characters and for determination of the possible species identity of the OTUs were done based on Sipman (1993), as it provides the most recent record with morphological descriptions of *Stereocaulon* species from the region.

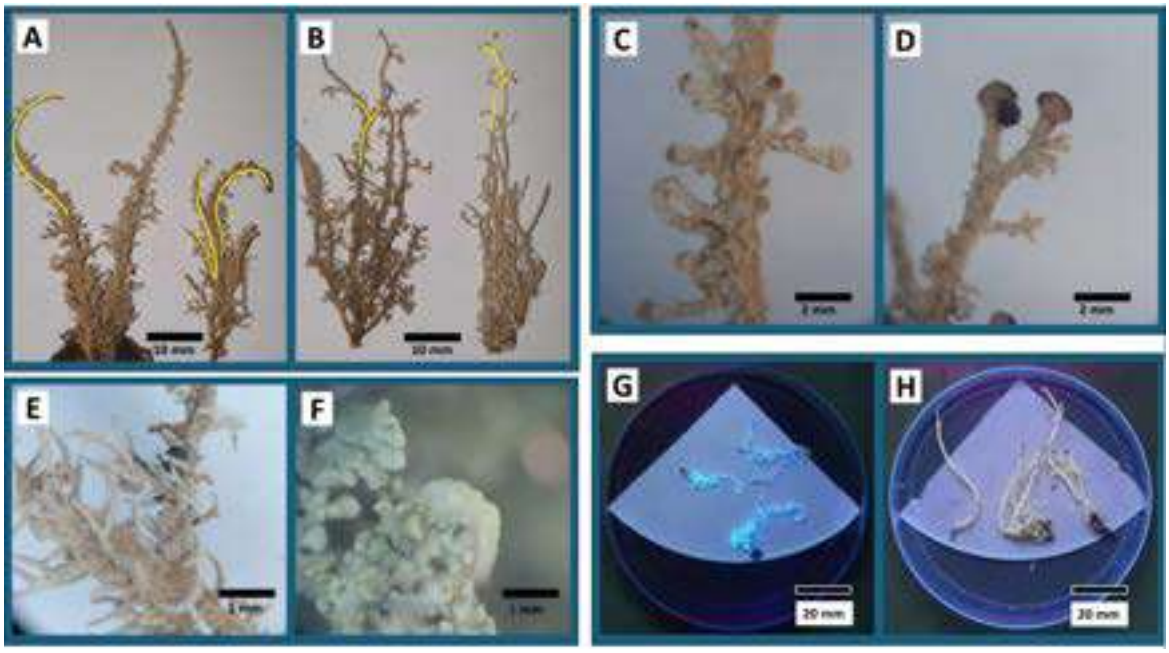


**Figure 2:** Morphological characteristics of the lichen *Stereocaulon* from Mount Kinabalu, Sabah. Pseudopodetia: **A.** (1) C-shape / Curved shown **B.** (2) Y-shape / Branched shown **C.** Location of apothecia: (1) Lateral / Alongside the lichen body **D.** (2) Terminal / At the tip of the lichen body **E.** Type of phyllocladia: (1) Ramular / Coral-like **F.** (2) Granular / Clumpy **G.** UV (Ultraviolet) light observation (254 nm and 365 nm): (1) Fluorescence (Light-blue) **H.** (2) Non-fluorescence . **A** and **B** were photographed by a digital camera. **C**, **D**, **E** and **F** were viewed under a stereo microscope with 2.0 × magnification. **G** and **H** were photographed under ultraviolet light.

**Chemical analysis of the collected *Stereocaulon* specimens**

For each sample, a total of 10 g of the lichen was soaked in acetone at room temperature for 7 days, then each solvent was filtered and concentrated under vacuum using a rotary evaporator with low pressure and temperature at 28 °C. Concentrated crude extracts were diluted and transferred to pre-weighed vials. Then the crude extracts were reconcentrated and weighed again to calculate the crude extract weight. After that, the crude extracts were desiccated and stored in a freezer at –20 °C for later processing.

Spot tests were conducted directly on the thallus of each sample by observing the colour changes under a stereo microscope (Ismed et al., 2018) after applying the different treatments/reagents in the following order: (1) Ultraviolet (UV-test), (2) Potassium hydroxide solution (K-test), (3) Sodium hypochlorite solution (C-test), (4) KC-test, (5) *para*-phenylenediamine solution (PD-test), and (6) Iodine solution (I-test).

Next, thin-layer chromatography (TLC) was conducted following the methods by Orange et al. (2001) on silica gel plates 10 cm × 20 cm (Kieselgel 60 F258, Merck, Germany). Then, a few drops of each concentrated crude extract, dissolved in acetone, were spotted at the line of origin (1 cm from the bottom) on TLC gel plates. A 10 mL volume of the solvent system Toluene:Dioxane:Acetic acid (180:45:5 v/v/v, corresponding to 7.8 mL:2.0 mL:0.2 mL respectively) was prepared and poured into a labelled developing chamber. The solvent front was allowed to migrate to 0.5 cm from the top of the plate (the ending line). TLC plates were placed into the chamber for chromatographic development.

After the TLC plate was fully developed in the chamber, the plate was taken out and air dried. The TLC plate was then observed and recorded under ultraviolet (UV) light (254 nm and 365 nm). After observation under ultraviolet light, the plate was sprayed with a molybdophosphoric acid visualisation reagent and heated on a hot plate.

For high-performance thin-layer chromatography (HPTLC), solvent system A (Toluene: Dioxane: Acetic acid, 180:45:5 v/v/v) was prepared. Approximately 2 µL of each crude extract was applied to the HPTLC plate for development. Each extract was sprayed automatically by the HPTLC system onto an HPTLC glass plate. The plate was then placed into the developing chamber. The glass plate was then observed in the visualisation chamber of the HPTLC system under 254 nm, 365 nm and white light. Retention factor ($R_f$) values were measured by using the CAMAG software. All results from TLC and HPTLC were compared with reference chemical data provided by Orange et al. (2001).

**Genetic sequence isolation of the *Stereocaulon* lichens**

A total of selected 24 *Stereocaulon* samples that represent all morphological groups from different localities were included in a genetic analysis. A specimen of *Cladonia* sp. was included as an outgroup because *Cladonia* is closely related to *Stereocaulon* (Genbank Accession no. PP158545). DNA was extracted from the thallus (around 1 cm long) by using QIAGEN Plant Mini Kit and its extraction protocols. The nuclear internal transcribed spacer (ITS rDNA; ITS1–5.8S–ITS2) was amplified with primers (1) ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and (2) ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (Gardes & Bruns, 1993; White et al., 1990). PCR was performed under the following conditions: an initial denaturation at 94 °C for 1 min, followed by 35 cycles of denaturing at 94 °C for 1 min, annealing at 55 °C for 1 min, and elongation at 72 °C for 1 min. The cycles were followed by a final extension at 72 °C for 8 min. All samples were amplified in a total reaction volume of 25 µL (20 µL of Qiagen® Taq PCR Master Mix Kit and 5 µL DNA extract).

The positive PCR samples were sequenced at Apical Scientific Sdn. Bhd. All the sequences were deposited in NCBI GenBank.

The phylogenetic relationships of the selected samples were estimated by using Bayesian and Maximum Likelihood (ML) approaches (Fig. 4). Bayesian analyses were run in MrBayes v3.2.7 for 10 million iterations, with tree sampling every 100 iterations; the first 25,000 trees (20 %) were discarded as burn-in, while the rest were used to obtain the final consensus tree. The ML analyses were run in IQ-Tree v 2.2.2.7 with the TNe+I model and 1000 rapid bootstrap iterations. The genetic interspecific (i.e., inter-OTUs) and intraspecific (i.e., intra-OTU) distances were also calculated in MEGA ver. 10 (Kumar et al., 2018).

**Delimitation of specimens as Operational Taxonomic Units (OTUs)**

The 42 specimens were initially assigned to five operational taxonomic units (OTUs) based on morphological similarities as follows: (1) RF (ramular-fluorescence), (2) RN (ramular-nonfluorescence), (3) GF (granular-fluorescence), (4) GN (granular-nonfluorescence), and (5) RU (ramular-curved). To cross-validate these morphologically-defined OTUs, their chemical compositions were examined to assess homogeneity within each OTU and differentiation between them, and the obtained phylogeny was used to further evaluate their delimitation based on monophyly and genetic distances among these OTUs.

## RESULTS

A total of 40 *Stereocaulon* specimens were collected between 1900 m and 3700 m a.s.l. along the summit trail of Mount Kinabalu. Based on our observation, the genus appeared to be most common and abundant at elevations between 2600 m and 3300 m a.s.l. The habitats in this elevation zone are dominated by *Leptospermum recurvum* forests and scrubs on ultrabasic and granite bedrock (Table 1; Fig. 1). In addition, two specimens were obtained from Mount Alab, collected at 1900 m a.s.l.. All 42 samples were found in open areas with direct sunlight exposure, directly attached to soils or rock surfaces and growing in patches.

**Table 1:** Details of *Stereocaulon* samples collected from Mount Kinabalu, Sabah, with collector number, BORNEENSIS Herbarium (BORH) accession number, operational taxonomic unit (OTU) number, location information, and GenBank accession number for the ITS sequences.

| Collector No. | Herbarium Accession No. | *Stereocaulon* OTUs | Location | Coordinates | Elevation (m) | Genbank accession no. |
|---|---|---|---|---|---|---|
| **JK01** | BORH 5854 | RU2 | Mt. Kinabalu, Summit trail | N 6.03651, E 116.55039 | 2112 | PP158542 |
| **JK02** | BORH 5855 | RU2 | Mt. Kinabalu, Summit trail | N 6.03767, E 116.55023 | 2129 | PP158543 |
| **JK03** | BORH 5856 | RU2 | Mt. Kinabalu, Summit trail | N 6.03973, E 116.55036 | 2223 | PP158544 |
| **JK05** | BORH 5858 | RN | Mt. Kinabalu, Summit trail | N 6.04302, E 116.55978 | 2648 | PP158546 |
| **JK06** | BORH 5859 | RU2 | Mt. Kinabalu, Summit trail | N 6.04421, E 116.56005 | 2679 | - |
| **JK09** | BORH 5862 | RU2 | Mt. Kinabalu, Summit trail | N 6.04510, E 116.55987 | 2691 | - |
| **JK10** | BORH 5863 | RF | Mt. Kinabalu, Summit trail | N 6.04510, E 116.55987 | 2691 | PP158547 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **JK11** | BORH 5864 | RU2 | Mt. Kinabalu, Summit trail | N 6.04556, E 116.56008 | 2708 | - |
| **JK12** | BORH 5865 | RU2 | Mt. Kinabalu, Summit trail | N 6.04600, E 116.56026 | 2724 | PP158548 |
| **JK13** | BORH 5866 | RN | Mt. Kinabalu, Summit trail | N 6.04600, E 116.56026 | 2724 | PP158549 |
| **JK14** | BORH 5867 | RU2 | Mt. Kinabalu, Summit trail | N 6.04799, E 116.56295 | 2880 | - |
| **JK15** | BORH 5868 | RU1 | Mt. Kinabalu, Summit trail | N 6.04975, E 116.56342 | 2921 | - |
| **JK16** | BORH 5869 | RF | Mt. Kinabalu, Summit trail | N 6.04975, E 116.56342 | 2921 | PP158550 |
| **JK17** | BORH 5870 | RU1 | Mt. Kinabalu, Summit trail | N 6.05137, E 116.56378 | 2932 | - |
| **JK18** | BORH 5871 | RU1 | Mt. Kinabalu, Summit trail | N 6.05108, E 116.56360 | 2943 | - |
| **JK19** | BORH 5872 | RF | Mt. Kinabalu, Summit trail | N 6.05136, E 116.56381 | 2964 | - |
| **JK20** | BORH 5873 | RU2 | Mt. Kinabalu, Summit trail | N 6.05163, E 116.56415 | 2982 | - |
| **JK21** | BORH 5874 | RF | Mt. Kinabalu, Summit trail | N 6.05190, E 116.56414 | 2994 | - |
| **JK22** | BORH 5875 | RF | Mt. Kinabalu, Summit trail | N 6.05328, E 116.56454 | 3038 | - |
| **JK23** | BORH 5876 | RF | Mt. Kinabalu, Summit trail | N 6.05527, E 116.56460 | 3118 | PP158551 |
| **JK24** | BORH 5877 | RU1 | Mt. Kinabalu, Summit trail | N 6.05589, E 116.56523 | 3130 | - |
| **JK25** | BORH 5878 | GF | Mt. Kinabalu, Summit trail | N 6.05883, E 116.56601 | 3239 | - |
| **JK26** | BORH 5879 | RU1 | Mt. Kinabalu, Summit trail | N 6.05883, E 116.56601 | 3239 | PP158552 |
| **JK27** | BORH 5880 | RF | Mt. Kinabalu, Summit trail | N 6.05883, E 116.56601 | 3239 | - |
| **JK28** | BORH 5881 | RF | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3668 | PP158553 |
| **JK29** | BORH 5882 | RF | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3668 | PP158554 |
| **JK30** | BORH 5883 | RN | Mt. Alab | N 5.82371, E 116.34117 | 1943 | PP158555 |
| **JK31** | BORH 5884 | RU2 | Mt. Alab | N 5.82371, E 116.34117 | 1920 | PP158556 |
| **JK32** | BORH 5885 | RF | Mt. Kinabalu, Summit trail | N 6.05521, E 116.56457 | 3089 | - |
| **JK33** | BORH 5886 | RF | Mt. Kinabalu, Summit trail | N 6.05521, E 116.56457 | 3089 | PP158557 |
| **JK34** | BORH 5887 | RN | Mt. Kinabalu, Summit trail | N 6.05521, E116.56457 | 3089 | PP158558 |
| **JK35** | BORH 5888 | RU1 | Mt. Kinabalu, Summit trail | N 6.05521, E 116.56457 | 3089 | PP158559 |
| **JK36** | BORH 5889 | RF | Mt. Kinabalu, Summit trail | N 6.05527, E 116.56460 | 3259 | - |
| **JK37** | BORH 5890 | GF | Mt. Kinabalu, Summit trail | N 6.05527, E 116.56460 | 3259 | PP158560 |
| **JK38** | BORH 5891 | GF | Mt. Kinabalu, Summit trail | N 6.05527, E 116.56460 | 3259 | PP158561 |
| **JK39** | BORH 5892 | RF | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | - |
| **JK40** | BORH 5893 | RF | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | PP158562 |

| | | | | | | |
|---|---|---|---|---|---|---|
| **JK41** | BORH 5894 | RU1 | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | PP158563 |
| **JK42** | BORH 5895 | GN | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | PP158564 |
| **JK43** | BORH 5896 | RF | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | - |
| **JK44** | BORH 5897 | RN | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | PP158565 |
| **JK45** | BORH 5898 | GN | Mt. Kinabalu, Summit trail | N 6.06548, E 116.56509 | 3677 | PP158566 |

## Characterisation of the genus *Stereocaulon* morphology

All 42 *Stereocaulon* samples were of the fruticose type and belonged to five morphological groups, namely, RF (ramular-fluorescence), RN (ramular-nonfluorescence), GF (granular-fluorescence), GN (granular-nonfluorescence), and RU (ramular-curved), based on the shape of the secondary thallus (pseudopodetia), type of phyllocladia, and colour changes of the thallus under UV light. Other morphological features examined, such as cephalodia characteristics and various character measurements, did not show significant differences in the classification of *Stereocaulon* collected from Mount Kinabalu.

## High-performance Thin-layer Chromatography (HPTLC)

The samples in each of the morphological groups, except for RU (ramular-curved), exhibited unique and internally homogeneous chemical profiles (e.g., shape of development, colour of bands, and $R_f$ values of specific compounds) (Fig. 3). All samples in the RU group shared identical morphological characteristics, but these samples had two distinctive chemical profiles (hereafter RU1 and RU2). RU2 exhibited a large, smeared spot around $R_f$ 0.40 to 0.45, and RU1 exhibited a large, smeared spot around $R_f$ 0.50 to 0.60 under 254 nm UV observation (Fig. 3). RU1 exhibited a large, light blue coloured smeared spot around $R_f$ 0.60, while RU2 did not exhibit any such smeared spot under 365 nm UV observation. Based on their $R_f$ values and comparison with reference data (Orange et al., 2001; Supplementary Data 1), putative chemical compounds identified included atranorin ($R_f$ ~ 0.90), lobaric acid ($R_f$ ~ 0.45), and stictic acid ($R_f$ ~ 0.47) (Fig. 3).

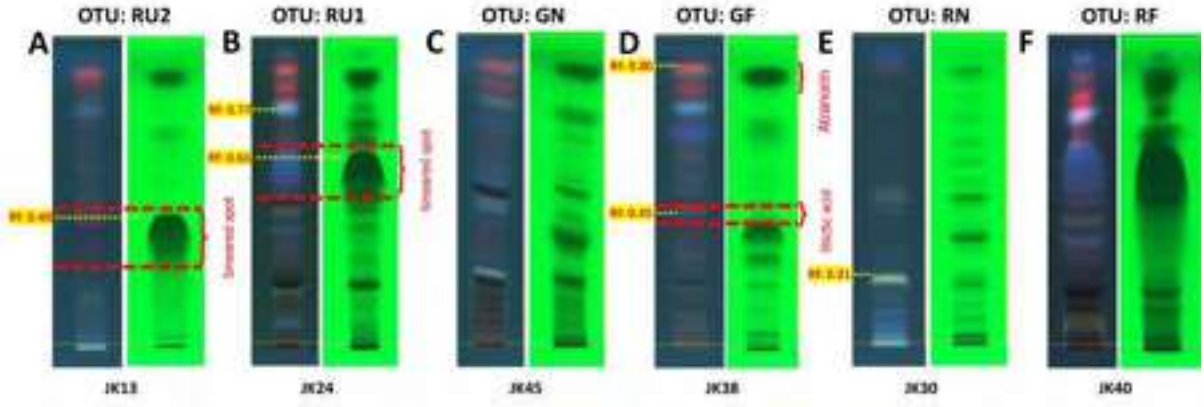


**Figure 3:** Comparison of chemical profiles obtained from High-performance Thin-layer Chromatography (HPTLC) of the *Stereocaulon* operational taxonomic units (OTUs) from Mount Kinabalu, Sabah: RF (ramular-fluorescence), RN (ramular-nonfluorescence), GF (granular-fluorescence), GN (granular-nonfluorescence), RU1 (ramular-curved 1) and RU2 (ramular-curved 2). The green images (right) show HPTLC profiles under UV 254 nm, and dark blue images (left) show HPTLC profiles under UV 365 nm for each *Stereocaulon* OTU**.** Selected distinctive chemical spots that contribute to the unique profile of each OTU, with the examples of *Stereocaulon* secondary metabolites, such as atranorin and stictic acid, are noted where identified with $R_f$ values.

**Genetic analysis and phylogenetic trees based on ITS rDNA of Mount Kinabalu *Stereocaulon* specimens**

The monophyly of the six OTUs, which were determined by the morphological and chemical data, was confirmed by phylogenetic analysis with high support values (PP > 0.95; ML > 95) (Fig. 4). The sister clades of GN–GF and RU1–RU2 were phylogenetically closely related. The genetic distances among most of the OTU pairs were higher than 0.160 (16%), with a range between 0.160 and 0.222 (Table 2). The smallest genetic distances were between GN and GF (0.043) and between RU1 and RU2 (0.058). These small inter-OTU genetic distances were still much larger than the genetic distances among samples within each OTU.

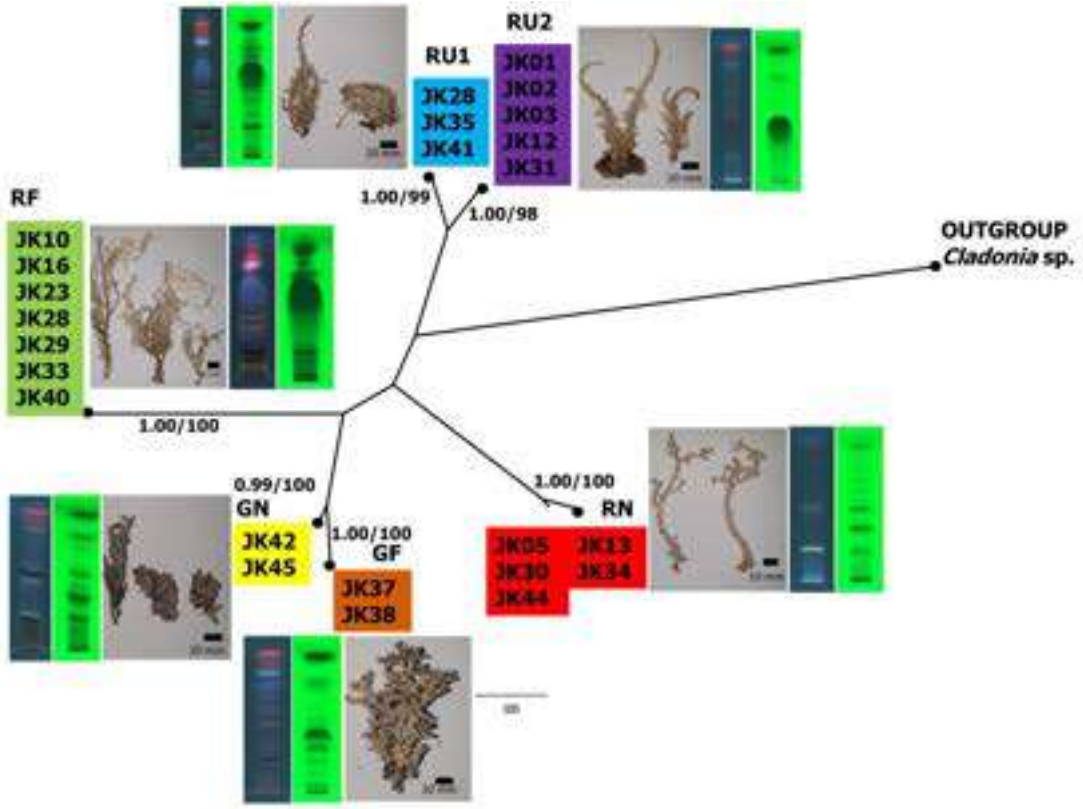


**Figure 4:** Phylogenetic tree (ITS rDNA) of *Stereocaulon* from Mount Kinabalu, Sabah constructed using Bayesian inference (BI) and was rooted using *Cladonia* sp. as an outgroup. Values at the nodes represent Bayesian posterior probability (left) and Maximum Likelihood bootstrap support values (right). The scale bar shows the estimated number of substitutions per site. Clades are labelled and colour-coded as follows: RN (Red), GF (Orange), GN (Yellow), RF (Green), RU1 (Blue), and RU2 (Purple).

**Table 2:** The average Kimura 2-Parameter (K2P) genetic distances of ITS region sequences between OTUs (inter-OTU) and within each OTU (intra-OTU; bolded values) for *Stereocaulon* from Mount Kinabalu, Sabah.

| | **RU2** (n = 10) | **RU1** (n = 7) | **RN** (n = 5) | **GF** (n = 3) | **GN** (n = 2) | **RF** (n = 15) |
|---|---|---|---|---|---|---|
| **RU2** | **0.001** | | | | | |
| **RU1** | 0.058 | **0.000** | | | | |
| **RN** | 0.196 | 0.202 | **0.015** | | | |
| **GF** | 0.187 | 0.180 | 0.183 | **0.000** | | |
| **GN** | 0.169 | 0.160 | 0.175 | 0.043 | **0.000** | |
| **RF** | 0.222 | 0.209 | 0.203 | 0.177 | 0.169 | **0.001** |

**Summary of Diagnostic Characters**

The full morphological description and chemical profile information, and genetic data for each of the six OTUs, namely, RF (Ramular-Fluorescent), RN (Ramular-Nonfluorescent), GF (Granular-Fluorescent), GN (Granular-Nonfluorescent), RU1 (Ramular-Unbranching 1) and RU2 (Ramular-Unbranching 2) are given in this section.

**OTU 1: RN (Ramular-Nonfluorescent) (Fig. 5)**

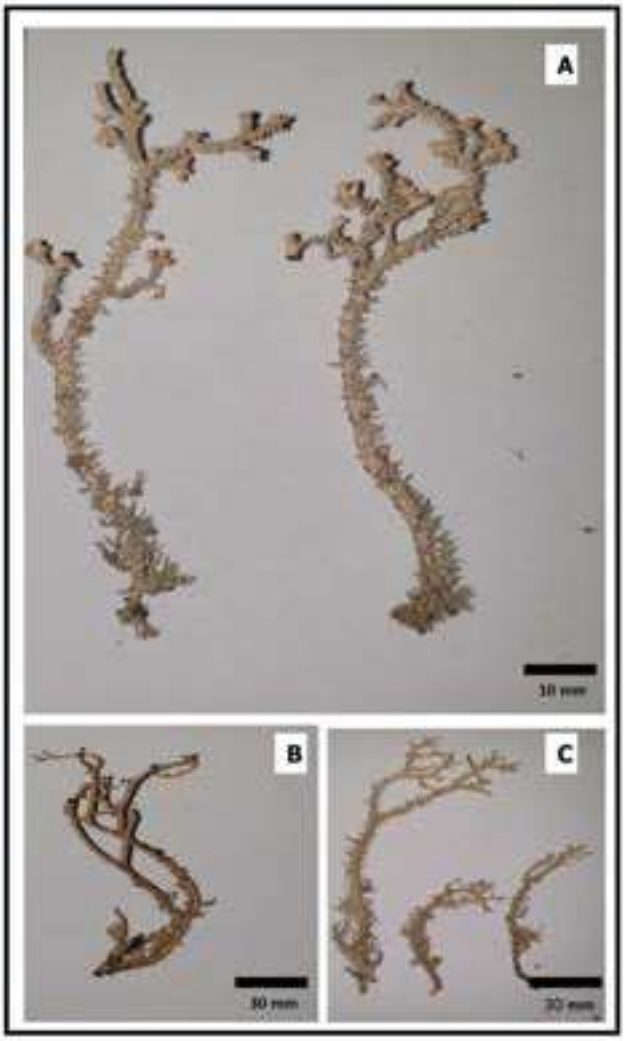


**Figure 5:** *Stereocaulon* specimens collected from Mount Kinabalu, Sabah that belong to the RN (Ramular-Nonfluorescent) OTU with branching secondary thallus.

**Description.** Pseudopodetia, 9.0–11.0 cm tall, highly branched, especially at the base and terminal parts; phyllocladia as highly branched, phyllocladioid branchlets, 0.1–5.0 mm long; cephalodia whitish-grey, wrinkled, wart-like, located randomly on pseudopodetia, 0.5–4.0 mm in diameter; apothecia 0.8–1.0 mm in diameter, dark brown, abundant terminally on pseudopodetia.

**Chemistry.** Atranorin and stictic acid. Spot tests: K+ yellow, PD+ yellow.

**Notes.** Characterised by its tall and branching pseudopodetia, phyllocladia in the form of phyllocladioid branchlets, large apothecia, and no fluorescence under UV lights (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open sites such as on soil or rocky habitats (e.g., cliffs, rock surfaces) with no or low vegetation coverage on Mount Kinabalu and Mount Alab.

**Distribution.** Ranging from 2648 m to 3089 m a.s.l. on Mount Kinabalu and at 1943 m a.s.l. on Mount Alab.

**Possible species as compared to previously recorded species.** *Stereocaulon massartianum*: Cephalodia sacculate. Spot tests: K+ yellow or occasionally K+ red; the red reaction regularly produces the characteristic red spicular crystals of the potassium salt of norstictic acid.

**OTU 2: RF (Ramular-Fluorescent) (Fig. 6)**

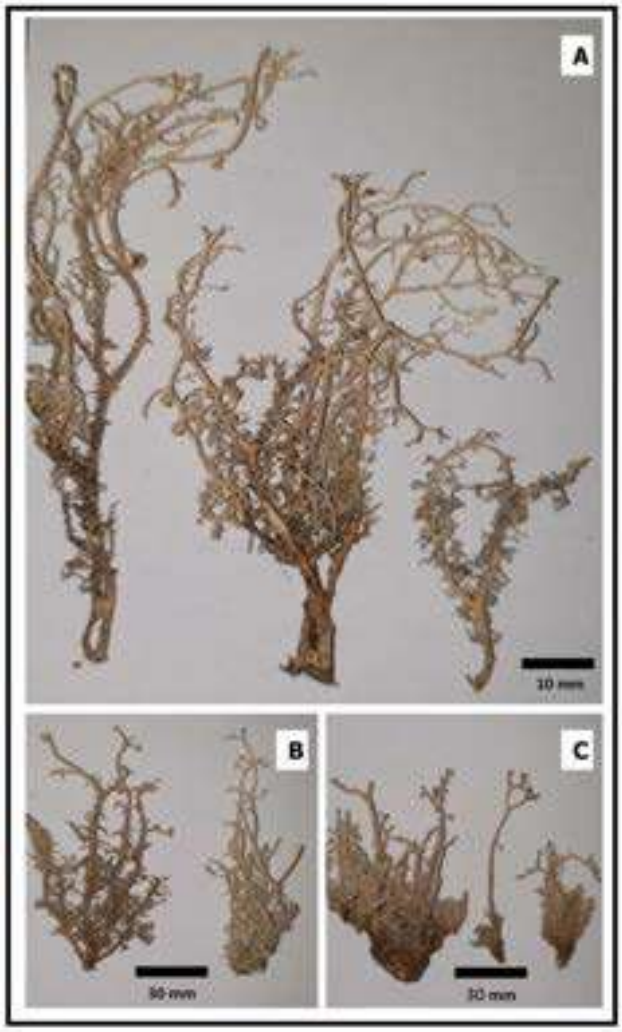


**Figure 6:** *Stereocaulon* specimens from Mount Kinabalu, Sabah that belong to the RF (Ramular-Fluorescent) OTU with branching secondary thallus.

**Description.** Pseudopodetia, 5.5–13.0 cm tall, highly branched, especially at the base and terminal parts; phyllocladia ramular, highly branched (especially at the basal areas of pseudopodetia), 1.0–8.0 mm long; cephalodia whitish-grey, wrinkled, wart-like, located randomly on pseudopodetia, 1.0–4.0 mm in diameter; apothecia 0.6–1.2 mm in diameter, dark brown, abundant terminally on pseudopodetia.

**Chemistry.** Atranorin, stictic acid, and lobaric acid. Spot tests: K+ yellow, PD+ yellow, UV+ white-blue fluorescence.

**Notes.** Characterised by its tall and branching pseudopodetia; phyllocladioid (as phyllocladia branchlets) that are highly dense at the base of the pseudopodetia; large apothecia, and white-blue fluorescence under UV light (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open sites such as on soil, rocky habitats (e.g., cliffs, rock surfaces) with no or low vegetation coverage on Mount Kinabalu (on hiking trails and open areas).

**Distribution on Mount Kinabalu.** Ranging from 2691 m to 3677 m a.s.l.

**Possible species as compared to previously recorded species.** *Stereocaulon granulans*: Similar descriptions provided by Sipman (1993), yet with a shorter height (2–4 cm).

**OTU 3: GN (Granular-Nonfluorescent) (Fig. 7)**
**Description.** Pseudopodetia 0.5–2.5 cm tall, slightly branched terminally; phyllocladia granular and papilliform, 0.2–0.5 mm in diameter, densely aggregated terminally on pseudopodetia; cephalodia absent from the specimens that were examined in this study; apothecia 1.0–2.0 mm in diameter, dark brown to black, terminal on pseudopodetia.

**Chemistry.** Atranorin and stictic acid. Spot tests: K+ yellow, PD+ yellow.

**Notes.** Characterised by its granular and papilliform phyllocladia (abundant terminally on pseudopodetia), terminal apothecia, and no fluorescence under UV lights (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open rocky habitats such as on rock surfaces with no vegetation coverage on Mount Kinabalu (on hornblende granite rock surface in summit zone).

**Distribution on Mount Kinabalu.** At 3677 m a.s.l., located in the summit zone, near the peak.

**Possible species as compared to previously recorded species.** *Stereocaulon graminosum*: Similar, differing only in the small size of its phyllocladia.

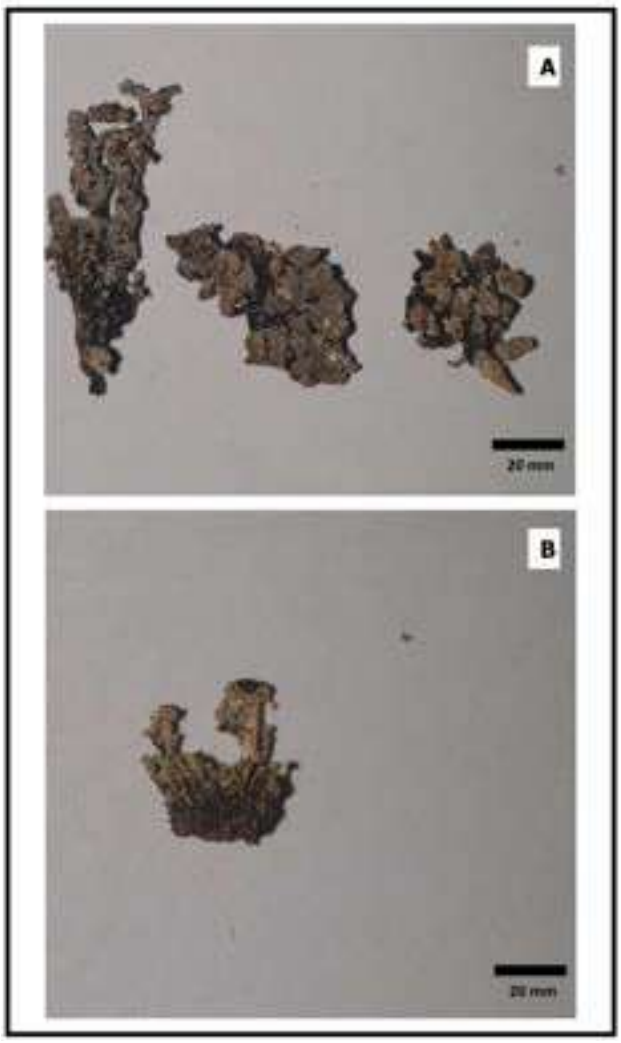


**Figure 7:** *Stereocaulon* specimens from Mount Kinabalu, Sabah that belong to the GN (Granular-Nonfluorescent) OTU with branching secondary thallus.

**OTU 4: GF (Granular-Fluorescent) (Fig. 8)**

**Description.** Pseudopodetia 3.0–10.0 cm tall, highly branched terminally; phyllocladia granular and papilliform, 1.0–1.5 mm long; cephalodia whitish-grey, wrinkled, wart-like, located at the basal areas of pseudopodetia, 0.8–1.5 mm in diameter; apothecia 0.5–1.0 mm in diameter, dark brown to black, highly abundant terminally on pseudopodetia.

**Chemistry.** Atranorin, stictic acid, and lobaric acid. Spot tests: K+ yellow, PD+ yellow, UV+ white-blue fluorescence.

**Notes.** Characterised by its granular and papilliform phyllocladia, large and abundant apothecia, and white-blue fluorescence under UV light (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open sites on soil or rocky habitats (e.g., rock surfaces) with no or low vegetation coverage on Mount Kinabalu (on porphyritic granite rock surfaces in open areas).

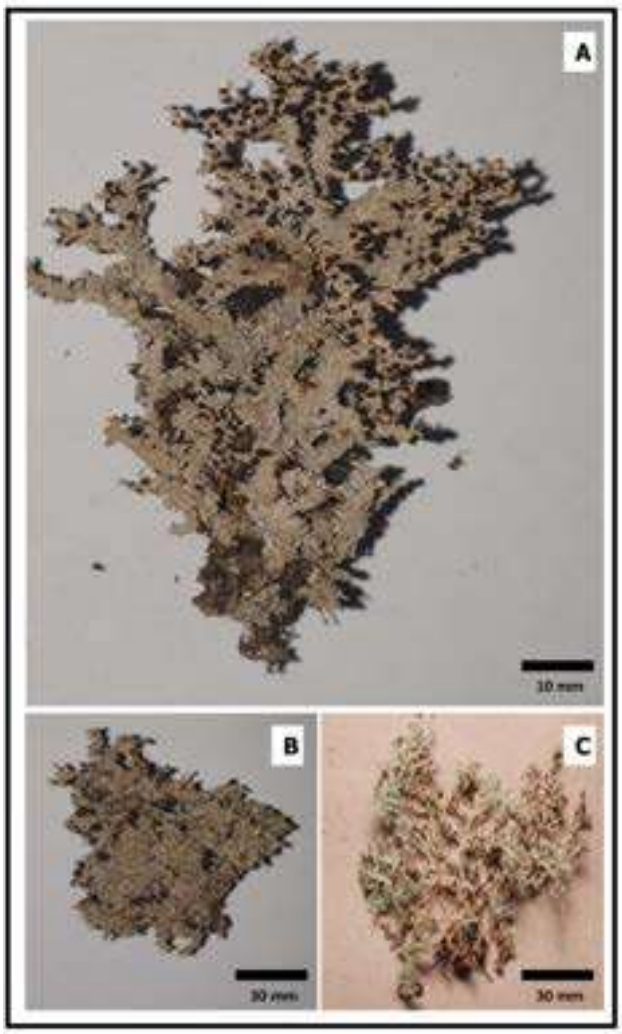


**Figure 8:** *Stereocaulon* specimens from Mount Kinabalu, Sabah that belong to the GF (Granular-Fluorescent) OTU with unbranching secondary thallus.

**Distribution on Mount Kinabalu.** Ranging between 3239 m and 3259 m a.s.l., in areas near Laban Rata.

**Possible species as compared to previously recorded species.** This OTU does not closely match any of the six taxa previously recorded from Mount Kinabalu by Sipman (1993). Morphologically similar species, typically found in other regions, include:

1. *Stereocaulon tomentosum*: Shorter height (2–4 cm) and larger apothecia (0.4–1.5 mm wide). Found near water sources such as lakes and river shores (Henssen, 1974; Jørgensen et al., 1998).
2. *Stereocaulon alpinum*: Shorter height (1–4 cm), and smaller cephalodia (0.3–0.8 mm diameter). Usually located in snow-covered areas; mainly circumpolar, arctic-alpine, ranging south to New Hampshire, Colorado, and Washington in North America (Lamb, 1977).

*Stereocaulon grande*: Slightly shorter (4–8 cm), with slightly shorter phyllocladia (0.4–0.8 mm), and slightly larger apothecia (2 mm wide). Spot tests: P– (Lamb, 1977).

**OTU 5: RU 1 (Ramular-Unbranching) (Fig. 9)**

**Description.** Pseudopodetia curved or slightly bent, 4.0–9.0 cm tall, unbranched; phyllocladia as slightly branched, phyllocladioid branchlets, 1.0–7.0 mm long, densely aggregated at the base of pseudopodetia; cephalodia whitish-grey, wrinkled, wart-like, located at the middle to basal areas of pseudopodetia, 0.5–4.0 mm in diameter; apothecia 0.1–0.8 mm in diameter, black or white, located laterally on the ends of phyllocladia, covering the middle to terminal parts of pseudopodetia.

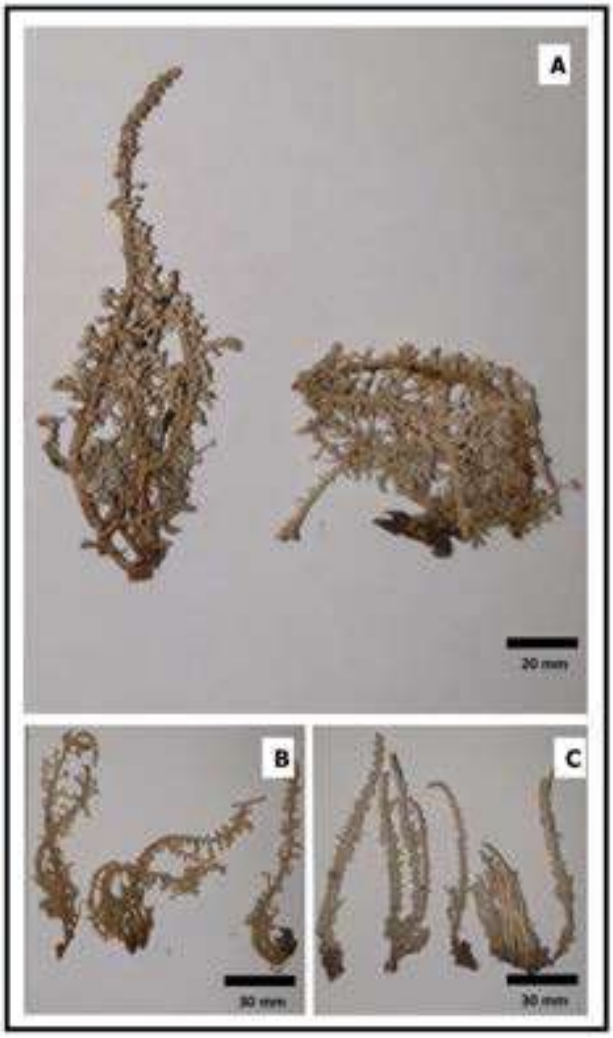


**Figure 9:** *Stereocaulon* specimens from Mount Kinabalu, Sabah that belong to the RU1 (Ramular-Unbranching 1) out with unbranching secondary thallus.

**Chemistry.** Atranorin, stictic acid, α-alectoronic acid, and lobaric acid. Spot tests: K+ yellow, KC+ red, PD+ yellow, UV+ white-blue fluorescence.

**Notes.** Characterised by its unbranched and curved pseudopodetia, phyllocladia as phyllocladioid branchlets; small apothecia located laterally on the end of phyllocladia, distributed from the middle to terminal areas of pseudopodetia; and white-blue fluorescence under UV light (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open sites, on soil or rocky habitats (e.g., cliffs, rock surfaces) with no or low vegetation coverage on Mount Kinabalu (on hiking trails and open areas near Laban Rata and summit zone).

**Distribution on Mount Kinabalu.** Ranging from 2921 m to 3677 m a.s.l.

**Possible species as compared to previously recorded species.** *Stereocaulon halei*: Description similar to that provided by Lamb (1977).

**OTU 6: RU 2 (Ramular-Unbranching) (Fig. 10)**
**Description.** Pseudopodetia curved or slightly bent, 4.5–7.0 cm tall, unbranched; phyllocladia as slightly branched, phyllocladioid branchlets, 1.0–5.0 mm long; cephalodia whitish-grey, wrinkled, wart-like, located mainly at the basal areas of pseudopodetia, 1.0–3.0 mm in diameter; apothecia 0.1–1.0 mm in diameter, reddish-brown, black or white, located laterally on the end of phyllocladia, covering from the base to the terminal parts of pseudopodetia.

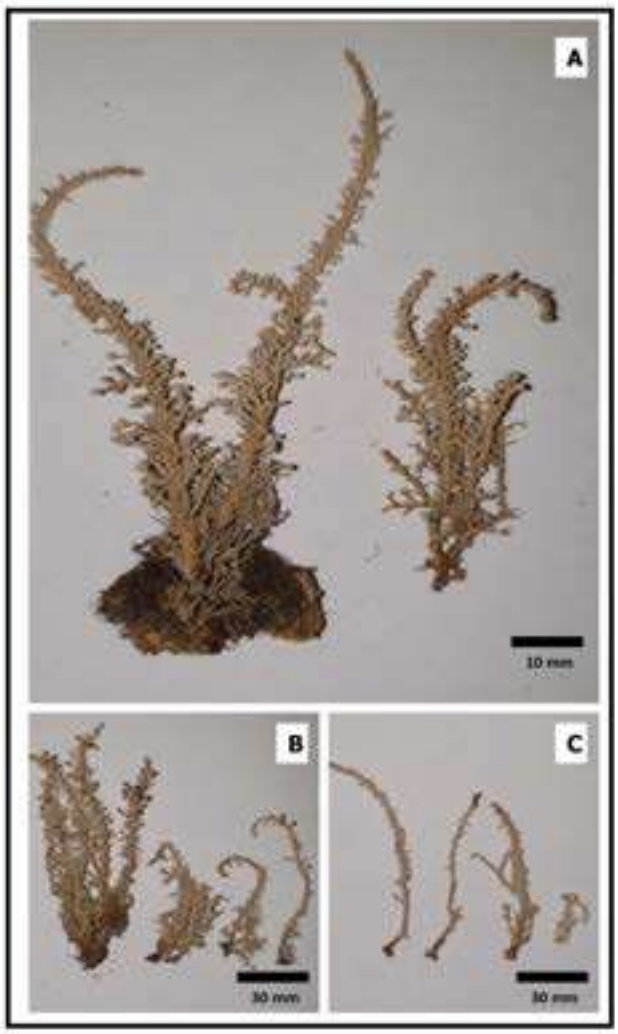


**Figure 10:** *Stereocaulon* specimens from Mount Kinabalu, Sabah that belong to the RU2 (Ramular-Unbranching 2) OTU with unbranching secondary thallus.

**Chemistry.** Atranorin, stictic acid, α-alectoronic acid, and lobaric acid. Spot tests: K+ yellow, KC+ red, PD+ yellow, UV+ white-blue fluorescence.

**Notes.** Characterised by its unbranched and curved pseudopodetia; phyllocladia as phyllocladioid branchlets; small apothecia located laterally on the end of phyllocladia, covering the pseudopodetia from base to terminal parts, and white-blue fluorescence under UV light (254 nm and 365 nm).

**Habitat.** Occurs exclusively in exposed, open sites, on soil or rocky habitats (e.g., cliffs, rock surfaces) with no or low vegetation coverage.

**Distribution.** Ranging from 2112 m to 2982 m a.s.l. on Mount Kinabalu and at 1920 m a.s.l. on Mount Alab.

**Possible species as compared to previously recorded species.** *Stereocaulon halei*: Description similar to that provided by (Lamb, 1977).

## DISCUSSION

The most recent survey by Sipman (1993) recorded six *Stereocaulon* taxa from Mount Kinabalu, namely, *Stereocaulon graminosum*, *Stereocaulon granulans*, *Stereocaulon halei*, *Stereocaulon massartianum*, *Stereocaulon massartianum* var. *chlorocarpoides*, and *Stereocaulon staufferi* var. *borneense*. Although the previous species number is the same as the number of OTUs revealed in this study, we could only narrow down the possible species identities of a few OTUs. For example, RN OTU matched Sipman's morphological description of *Stereocaulon massartianum* or *Stereocaulon massartianum var. chlorocarpoides*. RF samples corresponded to *Stereocaulon granulans*, and GN OTU matched *Stereocaulon graminosum*. GF OTU similar to *Stereocaulon tomentosum*, *Stereocaulon alpinum* and *Stereocaulon grande*, despite these species are not in Sipman (1993). Both RU1 and RU2 OTUs aligned morphologically with *Stereocaulon halei*. Although only a small set of macro-morphological characters were examined in this study and successfully separated the OTUs, it is still important for future, more detailed taxonomic studies to examine other micro characters (e.g., ascus and ascospores).

Although the chemical profile data for three of the six *Stereocaulon* species from Mount Kinabalu were available, these data could not be used to make reliable species identifications because of the chemical variabilities in the same species (Sipman, 1993). Lamb (1977) reported 123 chemical compounds from *Stereocaulon* species for identification purposes, but high morphological polymorphism and chemical variability challenge the usefulness of these reference data (Ismed et al., 2018). Compared to spot tests, the use of secondary metabolite profiles data for lichen taxonomy is still underutilised (Orange et al., 2001; Huang, 2008, 2010; Oset, 2014). Our study shows that HPTLC secondary metabolite profiles are useful for the delimitation of the *Stereocaulon* specimens into OTUs and potentially into species. In our example, RU1 and RU2, which were genetically distinct but morphologically indistinguishable, could be separated by the secondary metabolite profiles. However, further research with more comprehensive datasets is necessary to verify the reliability of this method, as Huang (2008) has also noted the challenges of using chemical characters in *Stereocaulon* taxonomy.

Phylogenetic analysis supports the monophyly of the six morpho-chemically defined OTUs, suggesting distinct evolutionary lineages (Fig. 4). A critical aspect for the taxonomic interpretation in our study of these closely related sister OTU clades is that even their ITS gene genetic distances (0.043–0.058) were found to be substantially greater than the maximum intra-OTU genetic distances (0.015). This suggests a consistent genetic gap that distinguishes even these more closely related OTUs from one another (e.g. RU1–RU2; GF–GN). This interpretation aligns with findings where similar or even lower genetic distance thresholds have been applied to differentiate species in other lichen studies (Del-Prado et al. 2010, 2011; Leavitt et al., 2013; Divakar et al., 2016).

These findings underscore the power of integrating genetic sequence data, especially when attempting to resolve problematic groups that exhibit overlapping morphological and chemical profiles, including the crucial identification of potential cryptic species, as shown in *Stereocaulon alpinum* (Ekman & Tonsberg, 2002; Del-Prado et al., 2010, 2011; Leavitt et al., 2013; Fontaine et al., 2013; Divakar et al., 2016; Torres et al., 2023).

Therefore, both genetic and chemical data are valuable tools for lichenologists in making taxonomic decisions (Lamb, 1977; Lumbsch, 2002; Del-Prado et al., 2010; Ismed et al., 2018). Furthermore, it is important to include morphological, chemical and genetic data of the specimens of *Stereocaulon* species from type localities, in order to establish reliable reference data for species identification and to expand sampling efforts for each *Stereocaulon* species across multiple regions and environments to improve our understanding of the extent of morphological, chemical, and genetic variability.

## CONCLUSIONS

In this study, six operational taxonomic units (OTUs) of *Stereocaulon* were identified from Mount Kinabalu, Sabah. The RN samples were identified as potentially *Stereocaulon massartianum*, the GF samples as *Stereocaulon tomentosum*, the GN samples as *Stereocaulon graminosum*, the RF samples as *Stereocaulon granulans*, and both RU1 and RU2 as *Stereocaulon halei*. Additionally, our study shows that HPTLC secondary metabolite profiles hold promise for the taxonomy of lichens. Our data provide a baseline for future studies on the taxonomy of *Stereocaulon* species in Mount Kinabalu, integrating morphology, chemistry, and genetics. Given that *Stereocaulon* species can have broad distribution ranges, further comparison of populations from different regions could shed light on the consistency of their genetic and chemical profiles that could potentially serve as diagnostic characters.

## ACKNOWLEDGEMENTS

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## DECLARATIONS

**Research permit(s).** Sabah Biodiversity Center: JKM/MBS.1000-2/2 JLD. 13(39) and Sabah Parks: TTS/IP/100-6/2 JLD.13.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** The authors declare that generative AI has been used in compliance with the JTBC policies for final proofreading to correct the language error. We have reviewed and edited the content after using this tool and we take full responsibility for the content of the publication.

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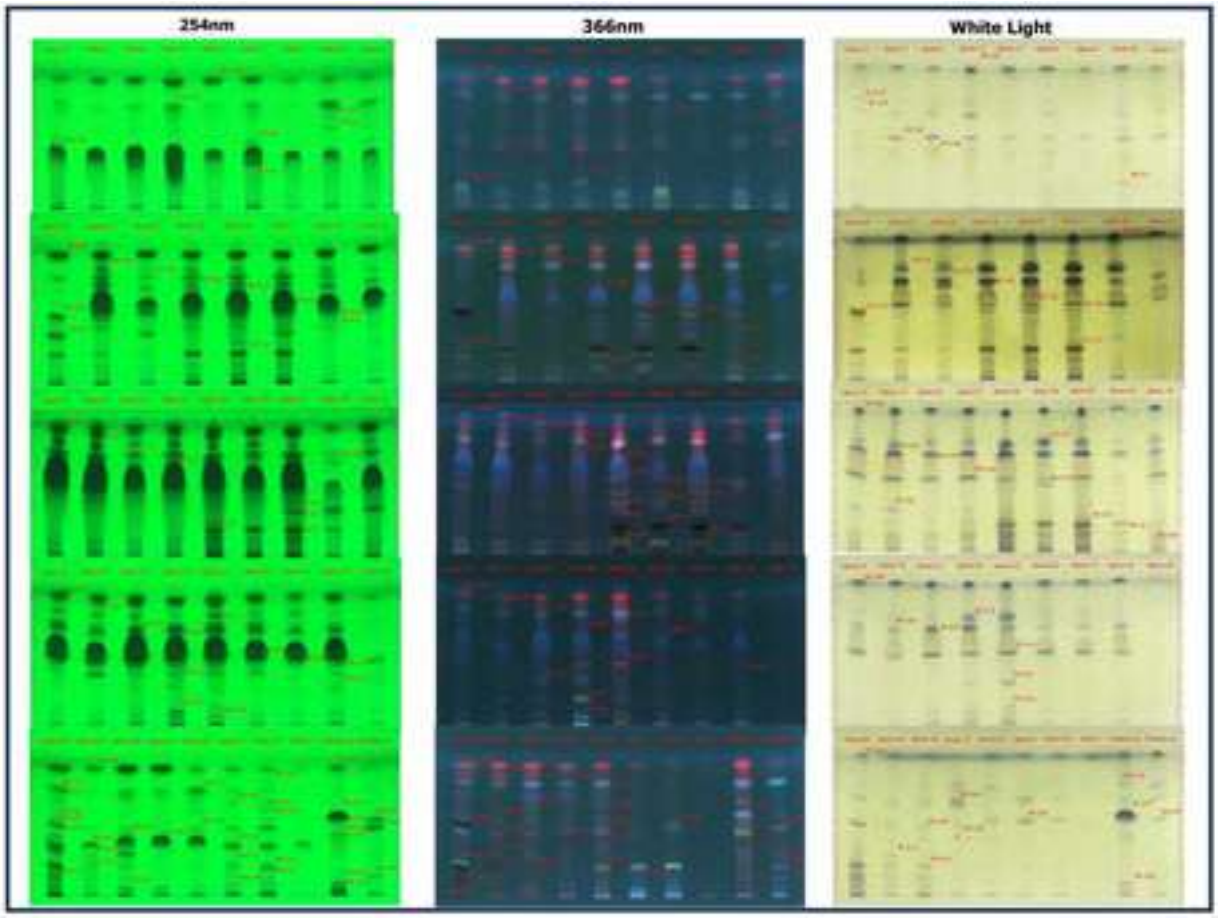
## APPENDIX

**Appendix 1:** HTPLC (High-performance thin-layer chromatography) image of all 42 samples of crude extracts of *Stereocaulon* developed using Solvent System A, under UV 254nm, 365 nm, and White light after spraying with phosphormolybdic acid.



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**Research Article**

# Bat (Mammalia: Chiroptera) Diversity of the Taliwas River Conservation Area, Lahad Datu, Sabah

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## ABSTRACT

Understanding species diversity and distribution is essential for informing conservation strategies, particularly in lesser-explored forest habitats. This study provides the first species checklist of bats in the Taliwas River Conservation Area (TRCA), a forest reserve adjacent to the Danum Valley Conservation Area (DVCA) in Sabah, Malaysia. A four-night bat survey was conducted from June 8–11, 2022, using mist nets and harp traps placed along three forest interior trails. A total of 16 bat species comprising 61 individuals were recorded across four families: Pteropodidae (4 spp.), Rhinolophidae (6 spp.), Hipposideridae (3 spp.), and Vespertilionidae (3 spp.). The insectivorous *Hipposideros* cf. *saevus* (formerly *Hipposideros ater*) was the most frequently captured species (21 individuals), while *Pteropus vampyrus* (n = 4) was visually observed feeding on *Octomeles sumatrana* (Binuang) nectar near the main camp. Two species of high conservation concern were recorded: the Endangered *P. vampyrus* and the Vulnerable *Hipposideros ridleyi*. The detection of multiple forest-interior species, including individuals showing reproductive signs, suggests that the TRCA provides suitable habitat for both foraging and roosting. These results highlight the conservation relevance of the TRCA as a complementary area to the broader DVCA landscape. Future studies incorporating long-term monitoring, acoustic detection, and habitat quality comparisons between protected and adjacent modified landscapes are recommended to inform site-based conservation management.

**Keywords**: Bat assemblage; forest understorey; harp trapping; species richness; Southeast Asian biodiversity.

## INTRODUCTION

As the second largest order, Chiroptera includes diverse species with significant importance especially in the forest ecosystem (Simmons & Cirranello, 2018; Soliman & Emam, 2022). Bats are one of the more extensively dispersed taxa, and they occupy a wide range of feeding niches in forests, including their interiors, edges, and open areas above or outside of the forest (Soliman & Emam, 2022; Law et al., 2015). The bat assemblages are influenced by different factors: roost site availability, the presence of suitable foraging habitats, and food supply (Froidevaux et al., 2021; Kaňuch & Krištín, 2005). In Borneo, bat assemblages are facing structural changes as the result of habitat degradation (Furey et al., 2010). Most forest specialist bats are in threat of extinction as the effect of forest fragmentation (Struebig et al., 2008, 2011) and land conversion (Phommexay et al., 2011). Therefore, bat assemblages are well-suited to serve as bioindicators for forest landscapes in the Paleotropics and Borneo, where they are structured over a forested landscape. Many species of bats combine a variety of traits that enable them to provide essential ecological services, particularly in tropical habitats, such as pollination, seed dispersal, and arthropod population control (Ramírez-Fráncel et al., 2021, Aziz et al., 2021).

Nearly a third of Sabah's overall forest cover and a comparable percentage of the commercially permitted (Class II) forest reserves are contained inside the Yayasan Sabah Forest Management Area (YSFMA) (Reynolds et al., 2011). The YSFMA also involves substantial and quickly growing plantation interests as well as numerous massive forest restoration initiatives (Reynolds et al., 2011). Three of Southeast Asia's largest and most significant protected primary forests, the Danum Valley Conservation Area (DVCA), Maliau Basin Conservation Area (MBCA), and Imbak Canyon Conservation Area (ICCA), are included in the YSFMA (Reynolds et al., 2011; Conservation & Environmental Management - Yayasan Sabah Group, 2022). The DVCA and MBCA remnants were not the only remaining areas of primary forest; very little of the YSFMA had been logged more than once by the end of the 1990s (Reynolds et al., 2011). These important conservation areas are part of the Heart of Borneo and provide significant landscape for conservation, supporting megadiversity, and important ecological processes (Sloan et al., 2019).

The bat assemblages within the YSFMA have not yet been fully uncovered. Given that the YSFMA still contains substantial portions of primary forest and old-growth secondary forest bordered by other landscapes like oil palm plantations (Conservation & Environmental Management - Yayasan Sabah Group, 2022), these places could potentially host a variety of bat species as well as new species discoveries. Bat checklists were recorded in the ICCA (Bunya et al., 2012, Bansa et al., 2020, Senawi et al., 2020), the MBCA (Shukor et al., 2010; Mahyudin et al., 2010; Turner, 2011; Hemprich-Bennett et al., 2021) and the DVCA (Struebig et al., 2008; Roslan, 2018; Hemprich-Bennett et al., 2021), where the latter includes the Taliwas River Conservation Area (TRCA), Silam Coast Conservation Area (SCCA) and INFAPRO (Conservation & Environmental Management - Yayasan Sabah Group, 2022). The checklist of bats is still far from complete for DVCA, especially in the TRCA. Therefore, this study aims (1) to document a bat species checklist within the TRCA, Sabah; (2) to assess the composition and diversity of bat assemblages in the TRCA as baseline data for monitoring impacts of habitat degradation and fragmentation; and (3) to evaluate the conservation value of the TRCA by identifying species associated with primary forest habitats and highlighting priorities for future conservation and management efforts.

## METHODOLOGY

### Study area

The Taliwas River Conservation Area (TRCA) 4° 59′ 32.72″ N 118° 4′ 16.06″ E is located about 36 km from Lahad Datu town, 24 km to the west of Silam and about 45 km east of the Danum Valley Conservation Area (DVCA) (Taliwas River Conservation Area, 2022). This area is approximately 29.5 km away from the DVCA Field Centre. About 9,546 hectares of lowland forest are covered by TRCA, with some areas having been treated with agroforestry techniques and silvicultural treatments through girdling and enrichment planting by the Sabah Forestry Department from 1970 to 1980 (Taliwas River Conservation Area, 2022). The TRCA was then elevated to a Class 1 (Protection) Forest Reserve in 2012 (Taliwas River Conservation Area, 2022). These areas are rich in dipterocarps and the oldest silvicultural forest that was designed for research, teaching, training, and ecotourism (Taliwas River Conservation Area, 2022).

### Field methods

A bat survey was conducted in the TRCA from June 8th to 11th, 2022, covering three sites, namely the Vanilla Trail (VT), the Gading Trail (GT), and the Arboretum Trail (AT) (Fig. 1).

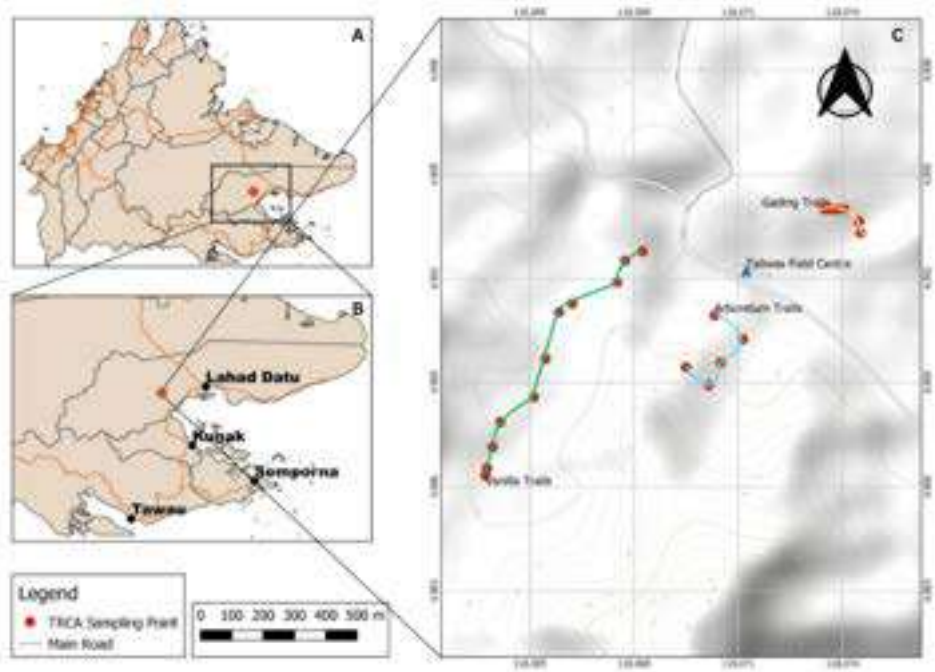


**Figure 1: A.** Map of Sabah, Malaysia in the northern region of Borneo. **B.** The location of the Taliwas River Conservation Area, in Lahad Datu, Sabah. **C.** Sampling locations indicate the trapping points in the Taliwas River Conservation Area, which includes 3 trails: the Vanilla Trail (green), Gading Trail (yellow), and Arboretum Trail (blue).

Four trapping nights were used to conduct the survey utilising 10 conventional mist-nets (13–15 mm) and three sets of four-bank harp traps (5×6 ft, lined with 0.18 mm/6 lb string and 10 mm gap between the line) (Kunz & Kurta, 1988; Francis, 1989). The nets and traps were set up along the routes, in the narrow pathways of the forest understorey, and across small streams. This deployment was designed to catch the forest understorey bats. Both nets and traps were checked frequently (every 15–30 minutes) from 1900 hrs and 2200 hrs and finally at 0600 hrs

(Kingston et al., 2003). The total survey effort was 208 trap hours (mist nets: 10 × 4 hours/night × 4 nights = 160 net-hours; harp trap – 3 × 4 hours/night × 4 nights = 48 trap hours).

**Bat identification, samples processing and preservation**

Captured bats were held in individual cloth bags and identified following Payne & Francis (1985) and Phillips & Phillips (2016) and taxonomic nomenclature following Simmons & Cirranello (2018). Their maturity was determined by the amount of diaphyseal fusion on the third, fourth and fifth metacarpals (Kunz & Anthony, 1982). For all individuals, sex is identified by the presence of a prominent penis for males, and the nipples on both sides for females. Three individuals per species were taken as voucher specimens. The standard morphological measurements were taken, namely, the forearm (FA), ears (E), tibia length (TB), hind foot (HF), head body (HB) and tail to ventral (TVL) by using a digital calliper (Mitutoyo) and weighed by using a spring balance (Pesola) (Hall et al*.,* 2004). The bats were photographed for identification records and future reference and released within 12 hours.

Selected bats individuals were euthanised by using isopropene in accordance to approval by the Animal Ethics Committee, UMS (AEC-0005/2020). Liver and muscle tissue were minced and preserved into lysis buffer for further molecular work (Longmire et al., 1997). The whole specimen was preserved in 70% ethanol as voucher specimens and deposited in the BORNEENSIS Wet Collection of the Institute for Tropical Biology and Conservations, UMS.

**Species composition and diversity of bat assemblages**

Species abundance matrix (species × site) was compiled, and the following diversity indices were calculated using the vegan package in R (Oksanen et al., 2022): species richness (S), Shannon diversity index (H'), Simpson's diversity index (D) and Evenness (J'). Higher values for H' indicate greater species diversity and evenness (Shannon, 1948); value closer to 1 in D indicate higher diversity while close to 0 indicates dominance by a few species (Simpson, 1949); while value close to 1 in J' indicates even species distribution while otherwise indicates dominance (Pielou, 1966).

Sampling completeness was evaluated using species accumulation curves (`specaccum( )` from the vegan package) and individual-based rarefaction curves (iNEXT package; Hsieh et al., 2016) to determine whether sampling effort was adequate for characterizing species richness. Principal Coordinates Analysis (PCoA) was performed based on the Bray-Curtis dissimilarity matric to visualise community composition. Ordination was conducted using `cmdscale( )` and species scores were overlaid using `wascores( )` to display species associations with particular sites. This method was chosen due to its effectiveness in handling abundant data and visualising similarities in community structure (Cisneros et al., 2015; Struebig et al., 2008). The closer two sites or species in the plot, the more similar their species assemblages are.

**Species conservation statuses and ecology**

The IUCN Red List status of each recorded species was obtained from the online database (International Union for Conservation of Nature, 2025). Species were categorised as Least Concern (LC), Near Threatened (NT), Vulnerable (VU), or Endangered (EN). The number of species within each category was summarised and visualised using a bar chart. Species accounts are provided for species with NT, VU and EN statuses, and the three most abundant species with the following information: Family name, species, relevant remarks, general body measurements (forearm length in mm) and a short note on each species occurrence and ecology. Foraging strategy of each species was also included to reflect species ecological role based on

their echolocation calls as published in previous studies (Kingston et al., 1999; Delaval & Charles-Dominique, 2006; Sedlock, 2001).

## RESULTS

A total of 65 bat individuals representing 16 species were recorded through traps or observation from the TRCA. These were represented by four families namely, Pteropodidae (three genera, four species), Hipposideridae (one genus, three species), Rhinolophidae (one genus, six species), and Vespertilionidae (one genus, three species). From the survey, *Hipposideros* cf. *saevus* (formerly *Hipposideros ater*) recorded the highest relative abundance with 21 individuals (32.3%), followed by *Rhinolophus trifoliatus* with nine individuals (13.8%), and *Cynopterus minutus* with eight individuals (12.3%) (Table 1, Fig. 2 and Fig. 3).

Less than five individuals were recorded for the rest, namely *Pteropus vampyrus* (the only species recorded through observation) and *Rhinolophus sedulus* with four individuals (6.2%) each; and four species with two individuals (3.1%) each, namely *Macroglossus minimus*, *Rhinolophus acuminatus*, *Rhinolophus borneensis*, and *Rhinolophus creaghi*. Five species were recorded as singletons (1.5%), namely *Cynopterus brachyotis*, *Hipposideros ridleyi, Kerivoula intermedia, Kerivoula lenis,* and *Kerivoula papillosa*.

**Table 1:** List of bat species, conservation status, relative abundance and foraging strategy of bats recorded based on four nights bat surveys at the Taliwas River Conservation Area. The three most abundant bat species are highlighted in bold. Bat species with important and critical conservation status based on the IUCN Red List are marked with an asterisk (*).

| Family | Species | Common name | Relative abundance (%) | Conservation status (IUCN Red list) | References (IUCN) | Foraging strategy |
|---|---|---|---|---|---|---|
| Pteropodidae | *Cynopterus brachyotis* | Lesser short-nosed fruit bat | 1.5 | LC | Csorba et al., 2019 | Uf |
| | ***Cynopterus minutus*** | Minute fruit bat | 12.3 | LC | Ruedas & Suyanto, 2019 | Uf |
| | *Macroglossus minimus* | Long-tongued nectar bat | 3.1 | LC | Waldien et al., 2021 | Uf |
| | *Pteropus vampyrus* | Large flying fox | 6.2 | EN* | Mildenstein et al., 2022 | Cf |
| Hipposideridae | ***Hipposideros* cf. *saevus*** **(**formerly *Hipposideros ater*) | Dusky leaf-nosed bat | 32.3 | LC | Armstrong, 2021 | Ni |
| | *Hipposideros diadema* | Diadem Leaf-nosed Bat | 4.6 | LC | Aguilar & Waldien, 2021 | Ni |
| | *Hipposideros ridleyi* | Ridley's Leaf-nosed Bat | 1.5 | VU* | Khan et al., 2020 | Ni |
| Rhinolophidae | *Rhinolophus acuminatus* | Acuminate Horseshoe Bat | 3.1 | LC | Thong et al., 2019 | Ni |

| | | | | | | |
|---|---|---|---|---|---|---|
| | *Rhinolophus borneensis* | Bornean Horseshoe Bat | 3.1 | LC | Jayaraj, 2020 | Ni |
| | *Rhinolophus creaghi* | Creagh's Horseshoe Bat | 3.1 | LC | Jayaraj, 2020 | Ni |
| | *Rhinolophus luctus* | Greater Wooly Horseshoe Bat | 4.6 | LC | Thong et al., 2019 | Ni |
| | *Rhinolophus sedulus* | Lesser Wooly Horseshoe Bat | 6.2 | NT* | Jayaraj, 2020 | Ni |
| | ***Rhinolophus trifoliatus*** | *Trefoil Horseshoe Bat* | 13.8 | NT* | Huang, 2020 | Ni |
| Vespertilionidae | *Kerivoula intermedia* | Small Woolly Bat | 1.5 | NT* | Nor Zalipah, 2020 | Ni |
| | *Kerivoula lenis* | Lenis Woolly Bat | 1.5 | LC | Srinivasulu & Srinivasulu, 2019 | Ni |
| | *Kerivoula papillosa* | Papillose Woolly Bat | 1.5 | LC | Hutson & Kingston, 2021 | Ni |
| | Total number of individuals | | 11 | | | |
| | Total number of families | | 4 | | | |
| | Total number of species | | 16 | | | |

*na-Data not available;
[a]LC=Least Concern, NT=Near Threatened, VU=Vulnerable, EN = Endangered;
[b]Cf- Canopy frugivore; Uf – understorey frugivore; Ni – narrow-space insectivore

Diversity indices indicated a moderately high species richness and evenness (H'=2.45) with high diversity and no single species being overly dominant (1-D = 0.89; E = 0.88) (Table 2). The species accumulation graph for four nights of survey at three sites showed increasing number of species accumulated throughout the trapping nights and did not reach asymptote (Fig. 4). Rarefaction curves showed that the Vanilla Trail (sampling night = 1) has the highest species richness or better sampling efficiency, while the Arboretum Trail (sampling night = 2) and the Gading Trail (sampling night = 1) each showed moderate and low species richness, respectively. It also indicates incomplete sampling for all trails.

**Table 2:** Diversity indices of bat species in the Taliwas River Conservation Area indicates high taxonomic diversity and well-distributed community without single species dominance.

| Metric | Value |
|---|---|
| Species Richness | 16.0000000 |
| Shannon Index (H') | 2.4534145 |
| Simpson Index (1-D) | 0.8906250 |
| Evenness (E) | 0.8848822 |

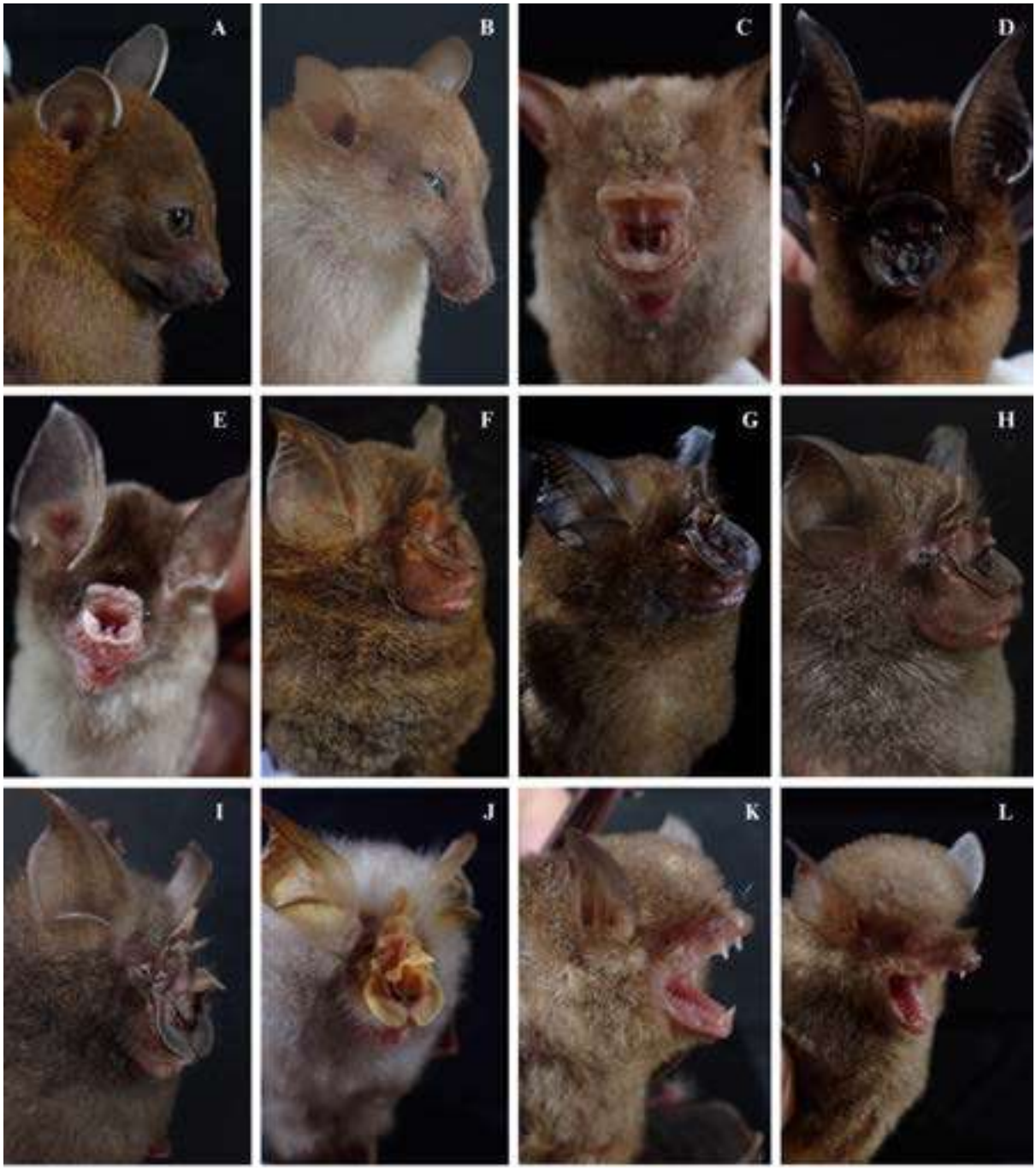


**Figure 2:** Chiroptera species recorded from the Taliwas River Conservation Area. Family Pteropodidae **A.** *Cynopterus brachyotis.* **B.** *Macroglossus minimus;* Family Hipposideridae. **C.** *Hipposideros diadema.* **D.** *Hipposideros ridleyi.* **E.** *Hipposideros ater (Hipposideros* cf. *saevus);* Family Rhinolophidae **F.** *Rhinolophus borneensis,* **G.** *Rhinolophus creaghi.* **H.** *Rhinolophus acuminatus.* **I.** *Rhinolophus luctus.* **J.** *Rhinolophus trifoliatus;* and Family Vespertilionidae **K.** *Kerivoula papillosa.* **L.** *Kerivoula lenis.*

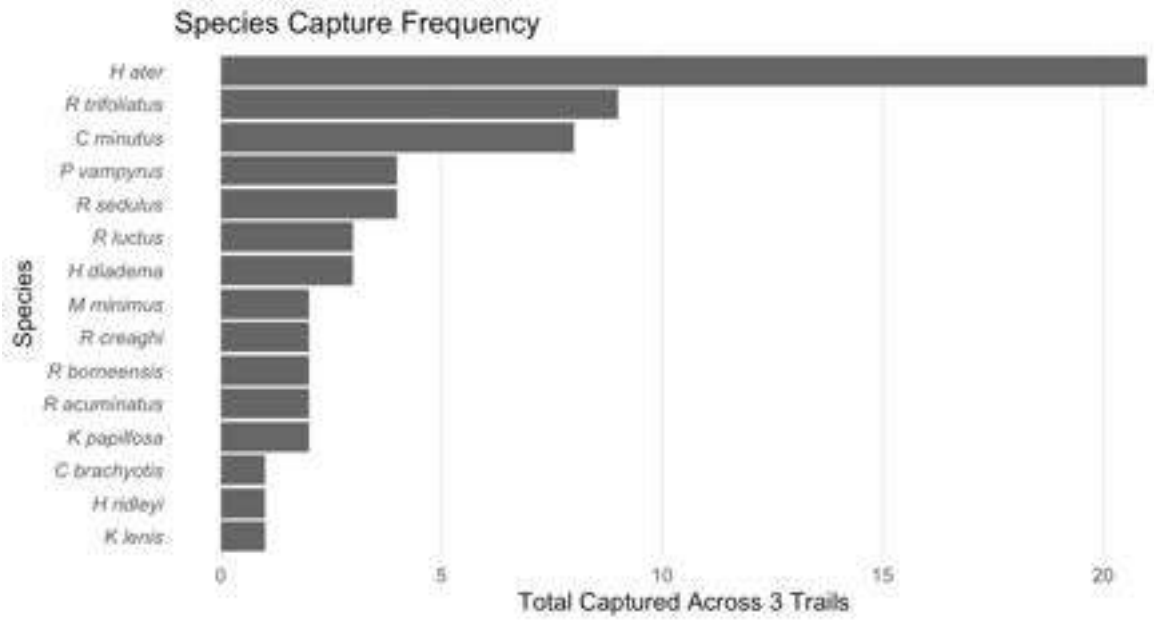


**Figure 3:** Species capture frequency recorded during the surveys across the 3 trails in the Taliwas River Conservation Area, with more common species recorded on the top, and rarer towards the bottom.

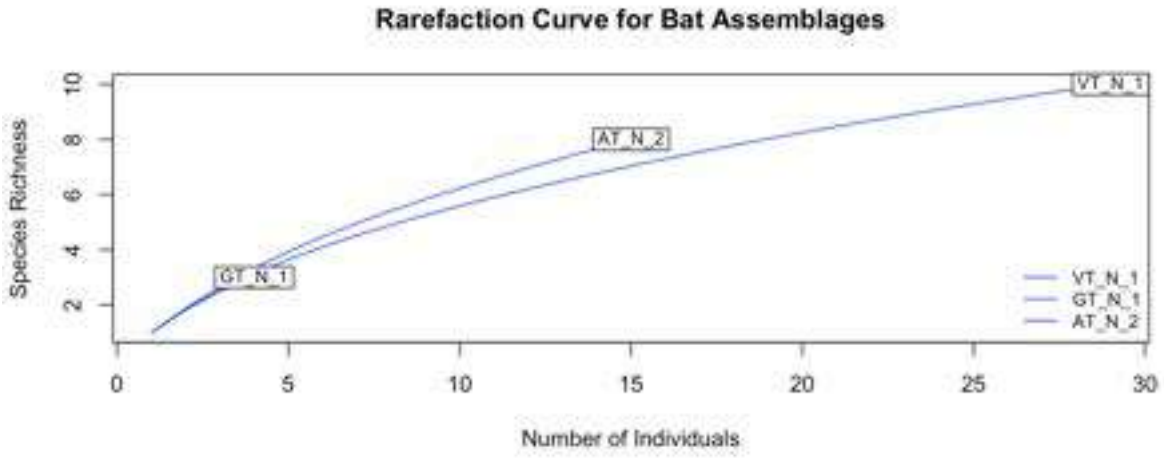


**Figure 4:** Rarefaction curve for bat assemblages in the Taliwas River Conservation Area showed the still rising curve indicating incomplete sampling from the area. Vanilla Trail (VT_N_1) showed the highest species richness (sampling night = 1), followed by Arboretum Trail (AT_N_2) (sampling night = 2) and Gading Trail (GT_N_1) (sampling night = 1) with moderate and lowest species richness, respectively.

The PCoA plot indicated a homogenous bat assemblage across all sites, with the Arboretum Trail (AT) and the Vanilla Trail (VT) showing close species clustering in ordination spaces, suggesting species such as *R. luctus, R. trifoliatus, R. borneensis* was more frequently captured or consistently present in both trails (Fig. 5). The Gading Trail was slightly separated with minor variation in species, such as *M. minimus* and *R. sedulus,* that is indicative of less widespread or site-specific occurrence across the sites.

According to the IUCN Red List, this survey identified five bat species of conservation concern, namely the large flying fox*, P. vampyrus* (Pteropodidae), listed as Endangered (EN),

and the Ridley's leaf-nosed bat, *H. ridleyi* (Hipposideridae), listed as Vulnerable (VU) (Fig. 6). *Rhinolophus sedulus*, *R. trifoliatus*, (Rhinolophidae) and *K. intermedia* (Vespertilionidae) are three of the known species that are designated as Near Threatened (NT), and 11 other species are listed as Least Concern (LC) (Fig. 6).

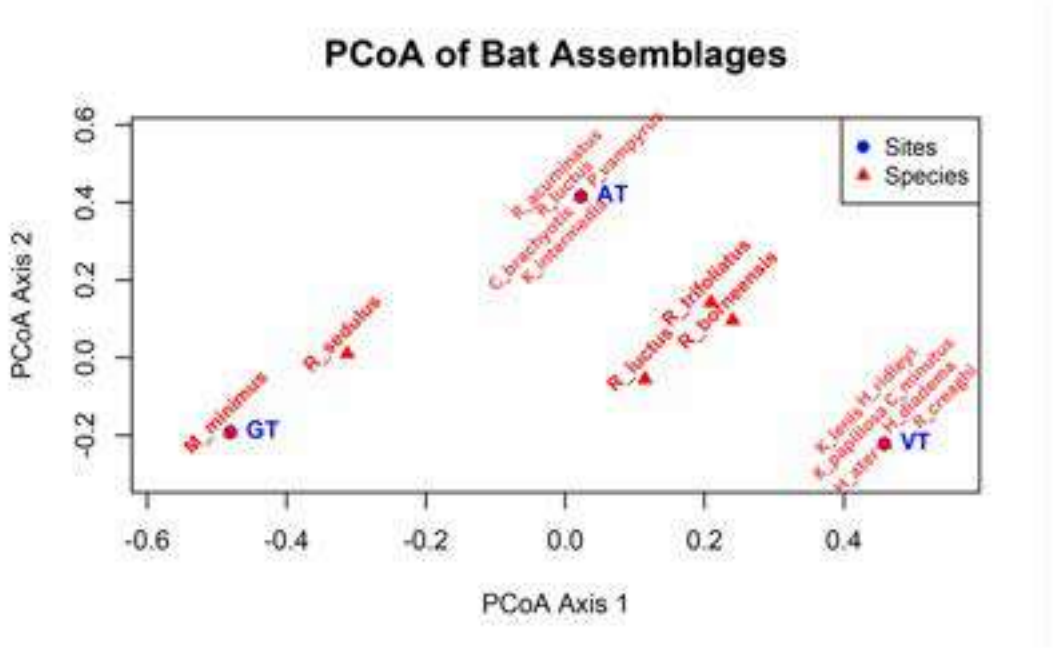


**Figure 5:** Principle Coordinates Analysis plot showing the community composition of bats in the Taliwas River Conservation Area across three trails, Vanilla Trail (VT), Arboretum Trail (AT), Gading Trail (GT).

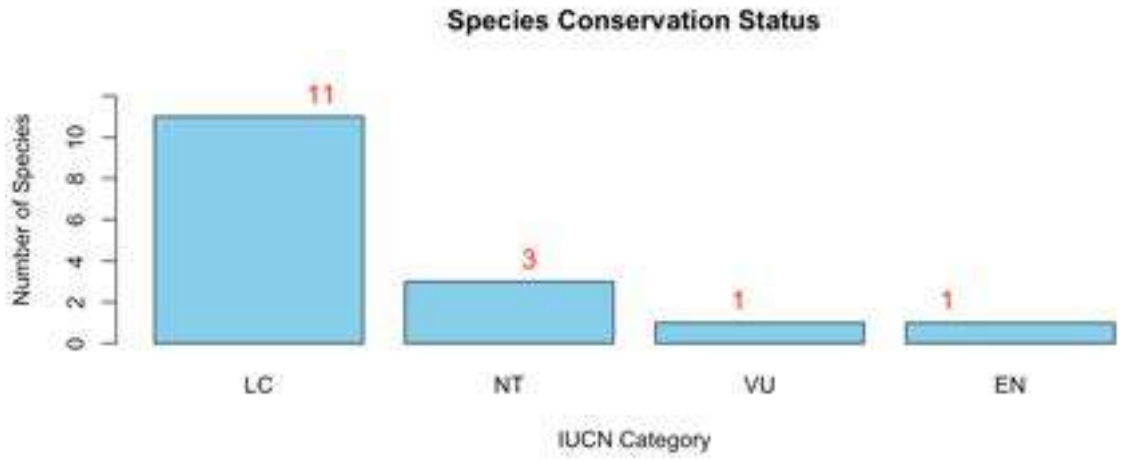


**Figure 6:** Summary of the IUCN Red List conservation statuses of bat species from the Taliwas River Conservation Area. LC: Least Concern, NT: Near Threatened, VU: Vulnerable, E: Endangered.

**Species account**

**Family Pteropodidae.**

*Cynopterus minutus* (Miller, 1906)

Collected voucher specimen – 1 (BOR MAL10677); Forearm (mm): 53.67–58.52

Two individuals of *Cynopterus minutus* were recorded during this survey along the Vanilla Trail, captured using mist nets deployed along the trail. Typically found in primary forests, *C. minutus* has also been recorded in villages and disturbed habitats (Ruedas & Suyanto, 2019), reflecting its adaptability to a range of habitat conditions. Its presence in the TRCA, particularly within intact forest, may reflect a preference for semi-closed canopy areas. This frugivorous species likely contributes to short-distance seed dispersal, particularly for understorey plants and shrubs (Sheherazade et al., 2017). In Borneo, it has been observed roosting in banana leaves, palms, and low foliage, indicating its reliance on lower strata vegetation for roosting (Payne & Francis, 1985). The species is currently listed as Least Concern on the IUCN Red List (Ruedas & Suyanto, 2019). Individuals encountered in this study frequently exhibited a pale-yellow fur collar around the neck. In contrast to *C. brachyotis*, where bright yellow to orange collars is commonly documented in adult males, the markings in *C. minutus* were subtler but observed in both males and females. This characteristic, along with a noticeably calmer disposition when handled (except for pregnant female), may aid in field identification and species differentiation.

*Pteropus vampyrus* (Linnaeus, 1758)

Remarks – 4 individuals observed

Four individuals of the large flying fox (*Pteropus vampyrus*) were observed feeding on Binuang (*Octomeles sumatrana*) nectar near the main camp of TRCA. This migratory species is listed as Endangered on the IUCN Red List due to significant population declines across its range (Mildenstein et al., 2022). While *P. vampyrus* forages in both forested and agricultural landscapes, it prefers undisturbed native forests, particularly mangroves, peat swamps, and freshwater swamps for roosting (Lim, 1966; Payne & Francis, 1985; Mildenstein et al., 2005, 2022). Major threats include habitat loss, overhunting, roost site disturbance, and conflict with fruit producers (Mildenstein et al., 2022). As one of Southeast Asia's largest fruit bats, *P. vampyrus* serves as a vital pollinator and seed disperser for large-canopy forest trees (Nakamoto et al., 2009; Aziz et al., 2017). Its presence in the TRCA reinforces the importance of protecting mature fruiting trees that support these ecological services. Observations of this species feeding on Binuang nectar have also been reported from other forested sites such as the Maliau Basin Conservation Area. The repeated observation of *P. vampyrus* in the TRCA suggests that the area may function as part of a broader foraging corridor (Epstein et al., 2009). Conservation of such sites is particularly important as suitable roosting habitats continue to decline.

**Family Hipposideridae.**

*Hipposideros* cf. *saevus* (Templeton, 1848)

Collected voucher specimens – 3 (BOR MAL10670, MAL10669, MAL10674); Forearm (mm): 39.30–43.73

A total of 21 individuals of *Hipposideros* cf. *saevus* (formerly *Hipposideros ater*) were captured at the Vanilla and Gading trails. This species is known to roost in caves and man-made structures such as tunnels, often forming colonies of up to several hundred individuals (Payne & Francis, 1985; Sedlock, 2001). It is currently listed as Least Concern on the IUCN Red List (Armstrong, 2021). The high number of captures suggests it may be among the more commonly encountered insectivores in the area. Its frequent detection using harp traps aligns with its low, manoeuvrable flight and clutter-adapted foraging behaviour (Pavey, 2021). As a

small constant-frequency echolocator, *H.* cf. *saevus* likely plays an important role in insect population regulation within forest understorey environments (Schnitzler & Kalko, 2001). Wongwaiyut et al. (2023) proposed reclassification of this taxon as *Hipposideros* cf. *saevus*, highlighting the need for detailed morphological comparisons—particularly of the internarial septum and noseleaf base—with other similar species. Genetic and morphometric assessments are recommended to confirm the identity of Bornean populations.

*Hipposideros ridleyi* (Robinson and Kloss, 1911)
Remarks – 1 individual (Released); Forearm (mm): 48.88
A single female *Hipposideros ridleyi* was captured in a harp trap at the Vanilla Trail, showing clear signs of post-lactation. This species is listed as Vulnerable on the IUCN Red List due to population declines across its range (Khan et al., 2020). It has been reported from lowland dipterocarp and Kerangas forests (Payne & Francis, 1985) and has been recorded roosting in culverts and drainpipes rather than caves (Mohd-Ridwan et al., 2011). The capture occurred along a shaded, interior trail consistent with its narrow-space foraging ecology. Although only a single record, the observation of a post-lactating individual may indicate the presence of suitable breeding conditions in the TRCA.

**Family Rhinolophidae.**
*Rhinolophus sedulus* (K. Andersen, 1905)
Collected voucher specimens – 3 (BOR MAL10672, MAL10665, MAL10676); Forearm (mm): 49.06–51.11
Four individuals of *Rhinolophus sedulus* were captured using harp traps across all three trails, including one post-lactating female. This species is generally associated with primary lowland forests, where it forages in the dense understorey (Payne & Francis, 1985; Corbet & Hill, 1992; Jayaraj, 2020). It is currently listed as Near Threatened due to suspected population declines driven by forest loss (Jayaraj, 2020). Its detection in multiple locations, including a breeding female, suggests that parts of the TRCA may still offer adequate foraging and roosting conditions.

*Rhinolophus trifoliatus* (Temminck, 1834)
Collected voucher specimens – 2 (BOR MAL10668, MAL10680); Forearm (mm): 50.2–51.47
Nine individuals of *Rhinolophus trifoliatus* were recorded, making it one of the more frequently captured species during this survey. A solitary-roosting species, *R. trifoliatus* is typically found beneath palm, rattan, or other large leaves in the understorey (Kingston et al., 2006; Francis, 2008). It is known to occupy a variety of habitats, including primary and secondary forests as well as mangroves (Phillipps & Phillipps, 2018). Although currently listed as Near Threatened due to habitat fragmentation (Huang, 2020), its consistent detection in protected areas across Sabah suggests a wider ecological tolerance. The relatively high number of individuals captured suggests that suitable microhabitats for this species are available within the TRCA.

**Family Vespertilionidae.**
*Kerivoula intermedia* (Hill and Francis, 1984)
Remarks – 1 individual (Released)
A single individual of *Kerivoula intermedia* was captured in a harp trap along the Arboretum Trail. This species is typically associated with forest understorey habitats, where it roosts in foliage or tree cavities (Payne & Francis, 1985). Although infrequently encountered in surveys, it has been documented in multiple forest reserves across Sabah. It is currently listed as Near Threatened, with population declines linked to logging, forest fires, and plantation expansion (Nor Zalipah, 2020). Its detection in this study contributes to the understanding of its local

distribution and indicates that suitable forest structure persists in some parts of the TRCA. Continued protection of vegetation complexity and understorey conditions may benefit this and other similar forest-dependent species.

## DISCUSSION

### TRCA as a conservation site for threatened and forest-specialist bats

This study represents the first documentation of bat diversity within the Taliwas River Conservation Area (TRCA), providing baseline data on species composition and community structure. A total of 16 species were recorded over 70 trap hours—a moderately diverse assemblage when compared to the Danum Valley Conservation Area (DVCA), which has previously reported 35 species over 1341 trap hours (Kingston et al., 1995; Hazebroek et al., 2004). Although direct comparisons are limited due to data availability, the diversity observed in TRCA supports its value as a forest site of conservation significance. More species are likely to be recorded with extended sampling across multiple seasons.

The habitats within TRCA—including primary lowland rainforest, streamside vegetation, and dipterocarp-enriched silvicultural zones—provide structurally diverse environments that support a variety of bat guilds. In this survey, insectivorous species were more numerous than frugivores and nectarivores, likely due to both their greater reliance on intact forest structure for roosting and the effectiveness of harp traps in targeting forest-interior gleaners. For example, *Hipposideros* cf. *saevus (*formerly *H. ater*) and *Rhinolophus trifoliatus*—both gleaning insectivores active in the forest understorey—were the most frequently captured species. The detection of these species reinforces the importance of intact understorey conditions for supporting forest-specialist bats (Kingston, 2013; Struebig et al., 2008, 2012).

Notably, several Near Threatened and Vulnerable species were recorded, including *Kerivoula intermedia*, *Rhinolophus sedulus*, *R. trifoliatus*, and the Endangered *Pteropus vampyrus*. These species are known forest specialists and are highly sensitive to habitat degradation and fragmentation (Struebig et al., 2008, 2011; Kingston, 2013; Jayaraj, 2020; Huang, 2020). The observation of a post-lactating *Hipposideros ridleyi* (Vulnerable) further suggests that TRCA may support breeding populations of rare species, strengthening the case for its conservation priority.

### Ecological and cultural importance of *Pteropus vampyrus*

The repeated presence of *P. vampyrus* feeding on *Octomeles sumatrana* (Binuang) trees near the TRCA camp highlights the site's importance as a foraging ground for this wide-ranging keystone species. In addition to its ecological roles in pollination and seed dispersal, *P. vampyrus* is facing population declines due to overharvesting and habitat loss (Lane et al., 2006; Bates et al., 2008). Although it is protected under wildlife laws in parts of Malaysia, hunting is still permitted under license in Sabah, where the species is consumed for its perceived medicinal properties (Mildenstein et al., 2022; Mohd-Azlan et al., 2022). Observations from this study support the need to protect nectar-producing trees and their surrounding habitats to ensure continued ecosystem services are provided by this species.

### TRCA and the role of bats as bioindicators

Bats are increasingly recognized as useful bioindicators due to their ecological diversity, sensitivity to environmental changes, and wide geographic distribution (Jones et al., 2009; Pulscher et al., 2020). However, effective use of bats as indicators requires accurate and

localized species inventories. This study contributes to filling that gap for TRCA and complements existing data from DVCA and other forest reserves in Sabah. The presence of forest-interior specialists such as *K. papillosa* and *K. intermedia*, as well as wide-ranging species like *P. vampyrus*, suggests that TRCA retains a relatively intact forest structure capable of supporting diverse bat guilds.

A recent study on tropical birds noted that protected areas are particularly effective in conserving forest-dependent, locally endemic, and threatened species (Cazalis et al., 2020). The bat community in the TRCA reflects this pattern, with the detection of multiple forest specialists and Near Threatened species that rely on the availability of continuous canopy cover, intact roosting sites, and a functional understorey. *K. papillosa* and *K. intermedia*, for instance, are known to be vulnerable to changes in their habitat, while *R. sedulus* and *R. trifoliatus* have been reported to roost solitarily or in shared sites within the forest understorey (Corbet & Hill, 1992; Payne & Francis, 1985).

**Caveats and limitations**

While this study provides valuable baseline data, several caveats should be noted. First, the sampling effort was limited in duration and scope, representing only a short-term, single-season survey, as this factor is known to have a negative impact on capture rates (Yoh et al., 2020; Meyer, 2015). Some species may have been missed due to seasonal variation of bat species composition, especially among frugivores and nectarivores. Additionally, the survey covered only three trails within the conservation area, which may not fully reflect the spatial heterogeneity of the TRCA.

Second, the reliance on harp traps without concurrent acoustic monitoring likely biased the results toward low-flying, forest-interior insectivores, underrepresenting high-flying or edge-adapted species such as *Taphozous* or *Miniopterus*. Third, while species identifications were based on morphological characteristics, some taxa—notably within *Hipposideros* and *Kerivoula*—may represent cryptic species complexes that require genetic confirmation (Khan et al., 2010).

Lastly, while comparisons were made to other conservation areas, such as the DVCA, inconsistencies in sampling design, effort, and available species lists limit the strength of such comparisons. Despite these limitations, the findings from the TRCA still offer meaningful insights into the area's conservation value and can serve as a foundation for future, more comprehensive surveys.

**Implications for conservation and future monitoring**

The presence of sensitive and threatened bat species within the TRCA highlights its value as a key conservation area within the larger Yayasan Sabah Forest Management Area. The diversity recorded suggests that the TRCA still maintains good forest quality. However, to better understand seasonal and long-term trends in bat diversity and abundance, future surveys should incorporate acoustic monitoring, stratified sampling across forest strata, and longer-term trapping efforts. These efforts will strengthen the baseline and enable more informed management decisions.

In summary, the findings from this study not only provide the first detailed bat inventory for the TRCA but also demonstrate the area's ecological importance in supporting a diverse and conservation-relevant bat community. This supports the case for the continued protection and integration of TRCA into Sabah's wider conservation planning framework.

## CONCLUSIONS

This study presents the first detailed assessment of bat diversity in the TRCA, highlighting its ecological value as a primary lowland forest that supports a diverse bat community. With 16 species recorded in a relatively short sampling effort—including several forest-interior specialists and threatened taxa—the TRCA emerges as an important habitat for both common and conservation-priority species. The detection of *Pteropus vampyrus* and *Hipposideros ridleyi*, alongside multiple Near Threatened species, underscores the role of the TRCA in maintaining populations of bats that are sensitive to environmental change.

The diversity and evenness observed in this assemblage suggest a relatively undisturbed forest structure, supporting a mix of foraging strategies and roosting requirements. These findings not only add to the growing body of data on Sabah's bat fauna but also serve as a crucial reference point for long-term monitoring and biodiversity planning. As pressures on natural forests continue to rise, sites like the TRCA provide a valuable stronghold for species that depend on intact, functioning forest ecosystems.

To strengthen the current baseline and better understand seasonal variation in bat activity, further monitoring is recommended. This includes periodic surveys across different months and using complementary methods such as acoustic detectors, which can capture high-flying and echolocating species that are not easily sampled with mist nets or harp traps. A consistent monitoring program would allow forest managers and conservationists to detect changes in species composition over time and respond proactively to ecological shifts.

Finally, the TRCA's role as part of the larger Yayasan Sabah Forest Management Area should not be overlooked. Its connectivity to surrounding forest blocks provides crucial movement corridors for bats, especially migratory and wide-ranging species. The conservation value of the TRCA would be further strengthened by integrating it into broader habitat linkage or buffer zone planning, ensuring that bat diversity remains protected within a connected forest landscape.

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## DECLARATIONS

**Research permit(s).** This study was conducted under the reference number JKM/SaBC.1000-2/14 JLD. 1(55) provided to the Yayasan Sabah as the organizer for the Taliwas River Conservation Area Scientific Expedition.
**Ethical approval/statement**. Animal handling procedures were approved by the Animal Ethics Committee of Universiti Malaysia Sabah (Approval Code: AEC-0005/2020).

**Generative AI use.** The author(s) declare that generative AI has been used in compliance with the JTBC policies, and that I/we have reviewed and edited the content after using this tool and we take(s) full responsibility for the content of the publication.

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**Research Article**

# Inventory and Assessment of Lycophytes in the Selected Forest Patches of Kalabugao, Impasug-ong, Bukidnon

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## ABSTRACT

Lycophytes, a group of ancient vascular plants, represent a fascinating evolutionary lineage with significant ecological and evolutionary importance. This study was carried out to determine species composition, describe the diagnostic characteristics, and assess the conservation status and endemism of lycophyte species in Kalabugao, Impasug-ong, Bukidnon. A total of nine lycophyte species were recorded in Kalabugao, namely *Phlegmariurus banayanicus* Herter, *Huperzia* sp., *Phlegmariurus salvinioides* (Herter) Ching, *Palhinhaea cernua* (L.) Vasc. & Franco, *Selaginella alligans* Hieron., *S. involvens* (Sw.) Spring, *S. llanosii* Hieron, *S. negrosensis* Hieron., and *Selaginella* sp. Assessment of conservation status and endemism revealed that two species are endangered (EN) and two species are of least concern (LC). This study contributes to the broader understanding of lycophyte biology and ecology, emphasizing their role in terrestrial ecosystems and their evolutionary significance.

**Keywords:** Club moss; spike moss; quillworts; vascular plants; Mindanao.

## INTRODUCTION

The Philippines comprises *ca.* 1,079 species of ferns and lycophytes that are distributed to 180 genera and 40 families (Pelser et al., 2011 onwards). In Mindanao, a total of 163 species have been recorded, encompassing 73 genera and 26 families. Of these, 144 species are ferns and 19 species are lycophytes (Silverio et al., 2021). These species account for about 15% of fern diversity and 29% of lycophyte diversity in the Philippines and Mindanao Island, respectively (Coritico & Amoroso, 2020).

Lycophytes are seedless vascular plants that have existed for thousands of years and belong to the most fundamental group of vascular plants (Pryer et al., 2001). They are widely distributed across various habitats in subtropical, tropical, temperate, and boreal climates, demonstrating their remarkable adaptability (Moran & Smith, 2001). The living members of lycophytes are divided into three families: Lycopodiaceae, Isoetaceae, and Selaginellaceae (Kenrick & Crane, 1997). Lycophytes are characterized by several distinctive features, including small, scale-like leaves called microphylls (Schneider & Smith, 2001). Additionally, lycophytes do not produce seeds but reproduce through the production of spores (Cranfill, 2001). While lycophytes have limited direct economic importance, they are valued for their ornamental uses in landscaping, potential pharmacological applications, and their role in scientific research and education, indirectly enhancing our understanding of plant diversity and ecosystem health (Brummitt et al., 2015).

Selaginellaceae species are often small and delicate, and thrive predominantly in tropical zones worldwide and have practical uses as sources of natural medicines, vegetables, and ornamental plants (Setyawan, 2011). Lycopodiaceae or clubmosses are ecologically significant, serving as habitats and food sources to animals, contributing significantly to biodiversity. In the Philippines, their roles are influenced by local context and species diversity (Amoroso et al., 2016). The Isoetaceae family, on the other hand, includes the single genus *Isoetes*, with around 250 living species globally (Brunton & Troia, 2018). In the Philippines, *Isoetes philippinensis* Merr. and L.M. Perry is the sole representative and was recently recollected by Amoroso et al. (2022) after a lapse of 52 years.

Kalabugao Mountain is home to the Talaandig tribe, an indigenous community that has lived in this area for generations. The Talaandig people have a deep connection with the land, practicing sustainable living and fostering a harmonious relationship with the diverse wildlife that share this pristine environment. However, there are no published reports on the lycophyte flora in the area. In particular, *Selaginella*, commonly known as spikemosses, is essential to the lycophyte diversity of the Philippines, especially in the mountain ecosystems of Mindanao. Despite their abundance, the morpho-taxonomy of these plants in the region is poorly documented (Bautista et al., 2018). Therefore, this study was conducted to inventory and assess the conservation status and endemism of the lycophyte species found in the selected forest patches of Kalabugao, Impasug-ong, Bukidnon.

## METHODOLOGY

### Site description

The study was conducted in selected forest patches of Kalabugao in the municipality of Impasug-ong, Bukidnon province, Mindanao, Southern Philippines, from December 2023 to April 2024, utilizing the Wildlife Gratuitous Permit (WGP) obtained by the first author, with

WGP number R10-2024-26 (Fig. 1). Four sampling sites were employed in the selected forest patches in the area. Site 1 was located at the mid-elevation, which is characterized as a mossy forest at Sitio Nasandigan (8.4594967 N, 125.1197441) and has an elevation of 1,230 m a.s.l. This area is comprised of diverse species of understory flowering plants, such as *Alocasia heterophylla* (C.Presl) Merr. (Araceae), *Psychotia* sp. (Rubiaceae), *Etlingera fimbriobracteata* (K.Schum.) R.M.Sm. (Zingiberaceae), and *Zingiber* sp. (Zingiberaceae), and some *Elatostema* spp. that can be found near the stream. Site 2 was located in the lower montane area and is a primary forest that is situated at a higher elevation in Mt. Palusonga, Sitio Nasandigan (8.4721673 N, 125.1072541 E) with an elevation of 1,246 m a.s.l. This area is composed of fern species, such as *Diplazium esculentum* (Retz.) Sw. and *Asplenium* spp., gingers such as *Etlingera philippinensis* (Ridl). R.M.Sm., and *Habenaria* sp. (Orchidaceae). Sites 3 and 4 are in a dipterocarp forest in Sitio Sigayan (8.4328504 N, 125.2255214 E) with an elevation of 1182 m a.s.l. and 1045 m a.s.l., respectively. In Site 3, some parts of its forest lands have been converted to roads, which pose a great threat to the species of lycophytes in the area, while Site 4 is partially open with some forest fragments (Fig. 2).

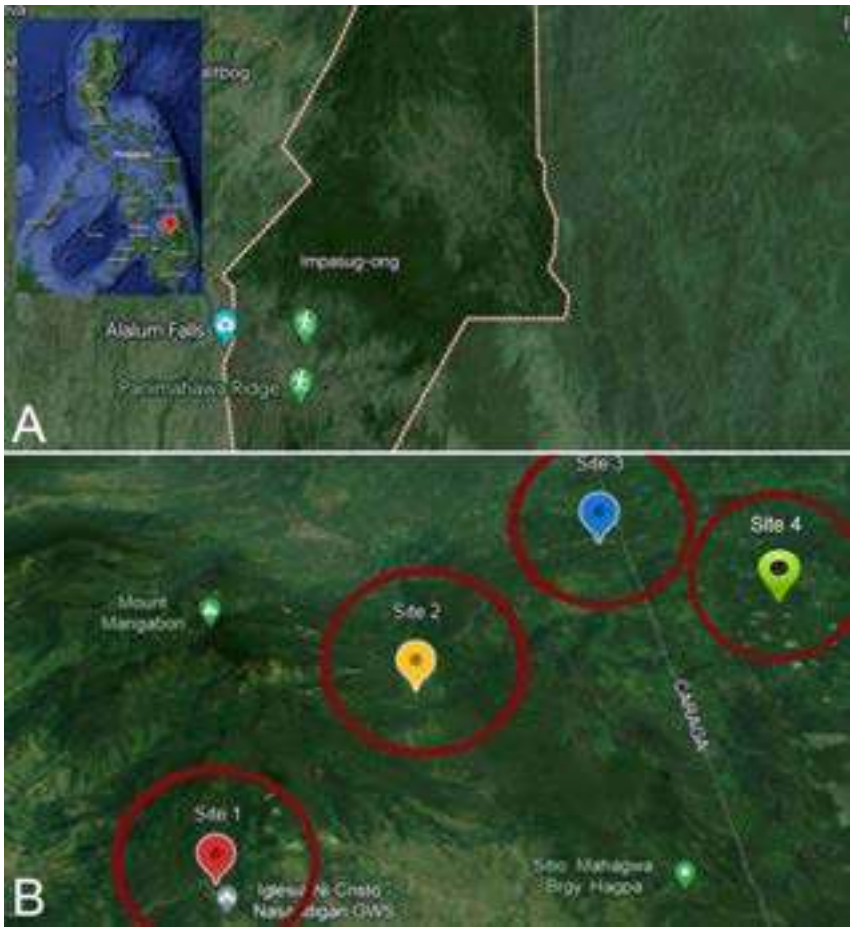


**Figure 1:** Location of the study site. **A.** Map of Mindanao showing the location of Impasug-ong, Bukidnon (inset: Philippine map). **B.** Map of Kalabugao showing the four sampling sites (Google Earth, 2024).

## Sampling procedure

This study was carried out in the selected forest patches of Kalabugao, Impasug-ong, Bukidnon. A total of four transect lines (one transect line per site) were established, and each

of these were  2 km long. Each site contains a single trail, which was inventoried repeatedly to record lycophyte species. Repeated transect walks were carried out to collect lycophyte species present in the transect line, with 5 m on each side of the trail. Opportunistic sampling was also conducted to list and collect other lycophyte species that were found outside the transect line and beyond a 5 m distance on each side.

**Collection of specimens and herbarium preparation**

Three duplicates of each species were collected and stored at the Central Mindanao University Herbarium (CMUH). The entire plant was completely pulled out to expose its roots. With the use of pruning shears and a pair of scissors, the collected specimens were placed in a plastic sack to lessen dehydration. To avoid damage, specimens were placed in plastic bags and placed in a sack along with their field information. Field data such as the date of collection, name of the collector, and habitat were recorded in the field notebook. Other data, such as description and other measurable characteristics, were also recorded. Small lycophytes were collected by uprooting the whole plant, removing the soil, and pressing the plant intact (Amoroso et al., 2016; Coritico et al., 2020).


**Figure 2:** Portion of Sampling sites. **A.** Site 1 – mid-elevation at Sitio Nasan-digan. **B.** Site 2 – higher elevation at Mt. Palusonga. **C–D.** Sites 3 and 4 are in lower elevation at Sitio Sigayan in Kalabugao, Impasug-ong, Bukidnon.

The specimens were placed in cellophane bags for transport of specimens. Afterwards, the collected specimens were placed between newspaper sheets with the corresponding collection number and their initial identification. These were cleaned by removing the dew, debris, and other unwanted materials. After pressing, all specimens were soaked with denatured alcohol following the wet method by Hodge (1947). The specimens were then dried and mounted. These specimens were then deposited at the CMUH.

### Morphological description, classification, and identification

Morphological description of lycophyte species was done using pictorial keys, published papers, and the online database of the Co's Digital Flora of the Philippines (Pelser et al., 2011 onwards) as bases for initial identification. Image comparisons and protologues from Internet sources were also accessed. Additionally, specimen identification was also referred to in the following monographs, floras, and other publications, such as Smith et al. (2006), Amoroso et al. (2007), Copeland's Fern Flora of the Philippines (1958–1961), Bada et al. (2023), and Pteridophyte Phylogeny Group I (PPG I, 2016). The final verification of the specimens was done at the CMUH by Dr. Fulgent P. Coritico, a taxonomic specialist working on Pteridophytes.

### Assessment of conservation status and endemism

The conservation status of lycophytes, whether critically endangered, endangered, or vulnerable, was based on the International Union for Conservation of Nature (IUCN, 2025) and the book of Fernando et al. (2022). The endemism of each species was based on the database of Pelser et al. (2011 onwards).

## RESULTS AND DISCUSSION

### Species composition

Nine species of Lycophytes belonging to four genera, three subfamilies, and three tribes were recorded in the four sampling sites in the selected forest patches of Kalabugao, Impasug-ong, Bukidnon. The most diverse in terms of species number was Selaginelleae (5 species), followed by Huperzioideae and Lycopodioideae (2 species each). In terms of genus level, *Selaginella* obtained the highest number of species distributed across the four sites.

The species collected in the area were *Huperzia* sp., *Palhinhaea cernua* (L.) Vasc. & Franco, *Phlegmariurus banayanicus* (Herter) A.R.Field & Bostock, *Phlegmariurus salvinioides* (Herter) Ching, *Selaginella alligans* Hieron., *Selaginella llanosii* Hieron., *Selaginella involvens* (Sw.) Spring, *Selaginella negrosensis* Hieron., and *Selaginella* sp. These species belong to Lycopodiaceae and Selaginellaceae (Table 1; Fig. 3).

**Table 1:** Species Composition and Distribution of Lycophytes in the Selected Forest Patches of Kalabugao, Impasug-ong, Bukidnon.

| | Species | Site | | | |
|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 |
| 1 | *Huperzia* sp. | / | | | / |
| 2 | *Palhinhaea cernua* (L.) Vasc. & Franco | / | / | / | / |
| 3 | *Phlegmariurus banayanicus* (Herter) A.R.Field & Bostock | | | | / |
| 4 | *Phlegmariurus salvinioides* (Herter) Ching | / | | | / |
| 5 | *Selaginella alligans* Hieron. | / | / | / | / |
| 6 | *Selaginella involvens* (Sw.) Spring | / | / | | / |
| 7 | *Selaginella llanosii* Hieron. | / | / | | / |
| 8 | *Selaginella negrosensis* Hieron. | / | / | | / |
| 9 | *Selaginella* sp. | | / | | / |


**Figure 3:** Lycophytes of Kalabugao, Impasug-ong, Bukidnon. **A.** *Huperzia* sp. **B.** *Palhinhaea cernua* (L.) Vasc. & Franco. **C.** *Phlegmariurus banayanicus* **(**Herter) A.R.Field & Bostock. **D.** *Phlegmariurus salvinioides* (Herter) Ching. **E.** *Selaginella alligans* Hieron. **F.** *Selaginella involvens* (Sw.) Spring. **G.** *Selaginella llanosii* Hieron. **H.** *Selaginella negrosensis* Hieron. **I.** *Selaginella* sp.

The Philippines harbours around 15.47% of the world's lycophyte species, although this estimate is subject to further research. The current study covers only about 1% of the known 1,300 lycophyte species worldwide, underscoring the need for continued exploration and study. Lycophyte species richness observed in the selected forest patches of Kalabugao, Impasug-ong, Bukidnon, is notably lower than that of the Mt. Pantaron Range, Natampod, San Fernando, Bukidnon, which recorded 14 species (Palange, 2023). Similarly, it falls short of the six lycophyte species documented across the four protected areas in Mindanao, which include Mt. Apo Natural Park (Cotabato), Mt. Kitanglad Range Natural Park (Bukidnon), Mt. Malindang Range Natural Park (Misamis Occidental), and Mt. Hamiguitan Range Wildlife Sanctuary (Davao Oriental) (Coritico & Amoroso, 2020). Additionally, the diversity of lycophytes in Kalabugao is relatively low compared to that of Mt. Sinaka, Arakan, North Cotabato, Southern Philippines, with 19 species (Silverio et al., 2021). The low number of lycophyte species in the area could also be attributed to the area covered in this study, as similarly observed by Kessler (2010).

The species of lycophytes observed in the four sites were *Phlegmariurus banayanicus*, *Huperzia* sp., *Palhinhaea cernua*, *Phlegmariurus salvinioides*, *S. alligans*, *S. involvens*, *S. llanosii*, *S. negrosensis*, and *Selaginella* sp. The common species observed in sites 1 and 2 were *Palhinhaea cernua* and *S. alligans*, while the only common species found in sites 1 and 4 was *P. cernua*. Most of the common species were found in sites 1 and 2, while in site 3, only *P. cernua* and *S. alligans* were recorded.

These findings highlight the variations in the adaptations of lycophytes across the four transects in the selected forest patches of Kalabugao. The presence and distribution of these species may be influenced by several factors, including the availability of diverse microhabitats, consistent environmental conditions, humidity levels, and rapid decomposition rates (Coritico & Amoroso, 2020). Kalabugao is a primary forest, and its climate falls under the tropical forest category, meaning that the forest is characterized by a distinct dry season (PAG-ASA, 2023), while most lycophyte species prefer moist environments. Additionally, several factors can affect the species richness of local montane forests in the Philippines, such as anthropogenic disturbances, including conversion of forests to agricultural or industrial lands, and pollution (Amoroso et al., 2016). It has been observed that Site 3 has experienced road construction and widening in the area, which could explain the low numbers of lycophytes recorded at this sampling site. Climate conditions, soil type, and geographic location might also influence the number of lycophyte species in the area (Kessler, 2010).

**Description of lycophytes in the selected forest patches of Kalabugao, Impasug-ong, Bukidnon**

Among the nine species of lycophytes found in the area, two species were not identified up to species level. The morphological descriptions of these species, as well as their ecology and distribution in the Philippines, are presented below.

**1. *Huperzia* sp. (Fig. 3A)**

Epiphytic, pendulous, 20 cm in length, branching, green to reddish-brown. Microphylls small, narrow, and scale-like, arranged densely, lanceolate or ovate, slightly toothed margin, cylindrical or club-shaped. Strobili pendent, elongated, along the axis of sporophylls.

**Collection number.** AYMA007

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.) and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.)

**Distribution in the Philippines.** The only known distribution in this locality.

**2. *Palhinhaea cernua* (L.) Vasc. & Franco (Fig. 3B)**
Terrestrial, creeping, ascending, or caulescent, stems dichotomously branching, yellowish. Microphylls light green, linear, small, entire with acute apex, needle-like, dichotomously branched. Strobili pendent, oblong, along the axis of sporophylls.

**Collection number.** AYMA002

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.), lower montane area in Mt. Palusonga, Sitio Nasandigan (1,246 m a.s.l.), dipterocarp forest in Sitio Sigayan (1182 m a.s.l.), and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.).

**Distribution in the Philippines.** Native and widely distributed in the different islands of the Philippines (Basilan, Batan, Biliran, Bohol, Bucas Grande, Camiguin, Catanduanes, Cebu, Dinagat, Leyte, Luzon, Mindoro, Negros, Palawan, Panay, Polillo, Romblon, Sabtang, Siargao, Siasi, and Sibuyan (Pelser et al., 2011 onwards).

**3. *Phlegmariurus banayanicus* (Herter) A.R.Field & Bostock (Fig. 3C)**
Epiphytic, pendulous, 19–27 cm. Creeping habit, segmented, green. Stems erect, slender, and branching. Microphylls alternate in whorls, narrow, lanceolate, and toothed margins. Strobili short, oblong.

**Collection number.** AYMA009

**Vegetation, locality, and elevation.** Dipterocarp forest in Sitio Sigayan (1045 m a.s.l.)

**Distribution in the Philippines.** Native and endemic to the Philippines (Camiguin, Luzon, Mindanao, Mindoro, Negros, Palawan, and Panay) (Pelser et al., 2011 onwards).

**4. *Phlegmariurus salvinioides* (Herter) Ching (Fig. 3D)**
Epiphytic, pendulous, 18 cm. Stem creeping, ascending, greenish brown, longitudinal. Microphylls alternate, linear, leaf apex lanceolate, margin entire. Scale-like leaves are light green, with entire margins. Strobili elongated, terminal.

**Collection number.** AYMA004

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.) and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.).

**Distribution in the Philippines.** Native to the Philippines (Catanduanes, Leyte, Luzon, Mindanao, Mindoro, Negros, Palawan, Panay, Polillo, and Samar) (Pelser et al., 2011 onwards).

**5. *Selaginella alligans* Hieron. (Fig. 3E)**
Epiphytic, 16 cm, climbers, usually growing on the trunk. Median microphylls with short arista, serrate with cuspidate leaf apex, lateral microphylls non-ciliated, serrate with acute apex,

axillary microphylls auricled, strobilus tetragonous, decussate leaf arrangement. Scale-like leaf dark green, dichotomously branched, monomorphic sporophyll, unbranched vein. Strobili arranged in a distinct pattern on the sporophylls.

**Collection number.** AYMA008

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.), lower montane area in Mt. Palusonga, Sitio Nasandigan (1,246 m a.s.l.), dipterocarp forest in Sitio Sigayan (1182 m a.s.l.), and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.).

**Distribution in the Philippines.** Native to the Philippines (Biliran, Catanduanes, Dinagat, Leyte, Luzon, Mindanao, Mindoro, and Negros) (Pelser et al., 2011 onwards).

**6. *Selaginella involvens* (Sw.) Spring (Fig. 3F)**
Hemi-epiphytic climbers, median microphylls with long arista, serrated with cuspidate apex, lateral. Microphylls non-ciliated, serrated with acute apices, and axillary microphylls are auricled. Strobili tetragonous, scale-like, decussate leaf arrangement, dichotomously branched, light green, monomorphic sporophylls, unbranched vein arrangement.

**Collection number.** AYMA005

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.), lower montane area in Mt. Palusonga, Sitio Nasandigan (1,246 m a.s.l.), and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.)

**Distribution in the Philippines.** Native and widely distributed to the Philippines (Bohol, Camiguin, Jolo, Leyte, Luzon, Mindanao, Mindoro, Negros, Panay, Samar, and Sulu Archipelago) (Pelser et al., 2011 onwards).

**7. *Selaginella llanosii* Hieron. (Fig. 3G)**
Epiphytic, erect, rooting at the base, median microphylls with long arista, serrate with cuspidate apex, lateral microphylls are ciliated, serrate with obtuse apex, axillary microphylls are non-auricled, decussate leaf arrangement. Scale-like microphylls dark green, dichotomously branched, monomorphic sporophylls with unbranched veins. Strobilus tetragonous.

**Collection number.** AYMA001

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.), lower montane area in Mt. Palusonga, Sitio Nasandigan (1,246 m a.s.l.), and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.)

**Distribution in the Philippines.** Native and widely distributed in the Philippines (Bohol, Dinagat, Luzon, Mindoro, Negros, Palawan, Panay, Samar, and Sibuyan) (Pelser et al., 2011 onwards).

**8. *Selaginella negrosensis* Hieron. (Fig. 3H)**
Terrestrial, creeping, ascending, or caulescent, median microphylls with short arista, serrate, cuspidate apex. Lateral microphylls non-ciliated, margin serrate, apex acute, axillary microphylls non-auricled. Scale-like leaf dark green, dichotomously branched, monomorphic sporophyll, unbranched vein. Strobilus tetragonous, decussate leaf arrangement.

**Collection number.** AYMA003

**Vegetation, locality, and elevation.** Mossy forest at Sitio Nasandigan (1,230 m a.s.l.), lower montane area in Mt. Palusonga, Sitio Nasandigan (1,246 m a.s.l.), and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.).

**Distribution in the Philippines.** Native and endemic to the Philippines (Leyte, Luzon, Mindanao, Negros, Panay, Samar, and Sibuyan (Pelser et al., 2011 onwards).

**9. *Selaginella* sp. (Fig. 3I)**
Erect, rooting at the base. Stem monostele. Median microphylls with short arista. Strobilus tetragonous, concave sides, broadly rounded corners, greenish yellow.

**Collection number.** AYMA006

**Vegetation, locality, and elevation.** Lower montane area in Mt. Palusonga and dipterocarp forest in Sitio Sigayan (1045 m a.s.l.).

**Distribution in the Philippines.** The only known distribution in this locality.

**Conservation status and endemism**
Two endangered (EN) species, two least concern (LC) species, and five data deficient (DD) species were recorded in Kalabugao (Table 2). The endangered species are *Phlegmariurus banayanicus* and *Phlegmariurus salvinioides*, which are both pendulous in their habit. *Palhinhaea cernua* and *Selaginella involvens* are the least concern species, meaning that these species are widespread, abundant, have a low risk of extinction, and there are no significant threats observed in their habitats. Three species are Data Deficient, which means that there is not enough scientific information available to assess the species' conservation status, and there are unclear population trends.

Among the recorded species, only *Selaginella negrosensis* is endemic to the Philippines, and six species are native to the Philippines. Two species – *Huperzia* sp. and *Selaginella* sp. – were not identified up to the species level due to lack of adequate distinguishing reproductive features necessary for accurate taxonomic identification. It is also noteworthy that *Selaginellaceae* is one of the least investigated lycophyte families, with several taxa facing extinction (Ebihara et al., 2012).

**Table 2:** Conservation status and Endemism of Lycophytes in the forest patches of Kalabugao, Impasug-ong, Bukidnon (Fernando et al., 2022; IUCN, 2025).

| No | Species | Conservation Status | Endemism |
|---|---|---|---|
| 1 | *Huperzia* sp. | | |
| 2 | *Palhinhaea cernua* | LC | Native |
| 3 | *Phlegmariurus banayanicus* | EN | Native |
| 4 | *Phlegmariurus salvinioides* | EN | Native |
| 5 | *Selaginella alligans* | | Native |
| 6 | *S. involvens* | LC | Native |
| 7 | *S. llanosii* | | Native |
| 8 | *S. negrosensis* | | Endemic |
| 9 | *Selaginella* sp. | | |

## CONCLUSIONS AND RECOMMENDATIONS

A total of nine species of lycophytes distributed to four genera, three subfamilies, and three tribes were recorded in Kalabugao, Impasug-ong, Bukidnon. The genus *Selaginella* had the highest number of species distributed across the four sites. This study revealed two endangered (EN) species (*Phlegmariurus banayanicus* and *Phlegmariurus salvinioides*) and two least concern (LC) species (*Palhinhaea cernua* and *Selaginella involvens*). In this study, only *Selaginella negrosensis* is recorded as endemic to the Philippines.

This study recommends the need for further exploration and monitoring in the selected forest patches in Kalabugao, Impasug-ong, Bukidnon, to record other lycophyte species. Policymakers should also develop and implement policies to protect and conserve these lycophyte species for future studies.

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## DECLARATIONS

**Research permit(s).** A Wildlife Gratuitous Permit (WGP) was obtained from the Department of Environment and Natural Resources (DENR) Region X with a holder number R10 2024-26 in compliance to R.A 9147 known as Wildlife Resources Conservation and Protection of Act of 2001.
**Ethical approval/statement.** Not applicable.
**Generative AI use.** AI was not used in this study or in the writing of this article.

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**Short Note**

# The Correct Scientific Name for Kacip Fatimah is *Labisia pumila* (Primulaceae), not *Marantodes pumilum*

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**Keywords**: Kacip Fatimah; *Labisia*; Myrsinoideae; Malesia; nomenclature.

Kacip Fatimah is a small, woody-herbaceous plant in the Primulaceae family. It is commonly found in the understorey of tropical rainforests across western Malesia, with distribution extending from Thailand to New Guinea (Stone, 1988; Sunarno, 2005). The species is highly valued in traditional medicine, particularly among Malay communities, for its use in childbirth and postpartum recovery (Rahmi et al., 2020; Hairi et al., 2024).

Although the species has attracted substantial attention in pharmacological and phytochemical research (e.g., Rahmi et al., 2020; Ibrahim et al., 2022), it has been inconsistently cited in the literature under two scientific names: *Labisia pumila* (Blume) Fern.-Vill. and *Marantodes pumilum* Kuntze. This dual usage has led to taxonomic confusion, particularly in non-taxonomic disciplines where researchers may rely on outdated databases (Schellenberger Costa et al., 2023). This note clarifies the correct scientific name by reviewing its nomenclatural history, examining current usage trends, and applying the International Code of Nomenclature for algae, fungi, and plants (ICN – Turland et al., 2018).

To assess the prevalence and trends in usage of the competing names, we conducted a literature search on Google Scholar using the keywords "*Labisia pumila*" and "*Marantodes pumilum*". The search was performed on May 15, 2025. The results indicated 4370 citations for "*Labisia pumila*" and 357 for "*Marantodes pumilum*," with continued usage of the latter in recent publications, including 72 since 2024. The search term "Kacip Fatimah" alone returned approximately 2390 results. This trend highlights the predominant use of *Labisia pumila* among researchers, although *Marantodes pumilum* continues to appear in recent studies, primarily in pharmacological contexts within Malaysia. Selected articles were reviewed to understand the naming rationale and citation sources, revealing frequent references to The Plant List—a static resource last updated in 2013—despite the existence of other actively curated taxonomic plant lists (Schellenberger Costa et al., 2023).

Taxonomists have long used the name *Labisia pumila* for the species known as Kacip Fatimah (Ridley, 1923; Stone, 1988; Sunarno, 2005). In contrast, as outlined above, the usage of *Marantodes pumilum* persists, contributing to confusion, especially in contexts where taxonomic verification is not a primary concern. The generic name *Labisia* was first validly published by Lindley in 1845. In comparison *Marantodes* was published by Post & Kuntze in 1903, based on a section of *Ardisia* originally described by De Candolle in 1844. Importantly, the spelling "*Marantodes*" is an incorrect orthographic variant, and the correct citation should be *Marantoides* (A.DC.) T.Post & Kuntze. Additionally, under Article 62.4 of the ICN (Turland et al., 2018), generic name ending -oides are treated as feminine, so the correct species citation would be *Marantoides pumila* (Blume) T.Post & Kuntze.

According to Article 11 of the ICN (Turland et al., 2018), priority applies only at the same taxonomic rank. Since *Labisia* was validly published at the genus level before *Marantoides*, it holds nomenclatural priority. Furthermore, because the name *Labisia* was included in synonymy under *Marantoides* (erraneously as '*Marantodes*') when it was published (Post & Kuntze, 1903), and because *Labisia* has priority, *Marantoides* is considered superfluous and cannot be used under current nomenclatural rules.

Stability in scientific naming is essential for maintaining consistency across disciplines. Inconsistent use of names may lead to fragmentation in research data, hinder literature retrieval, and complicate applications in policy, conservation, and ethnobotany. A unified naming approach facilitates effective communication, especially between taxonomists and professionals in pharmacology, ethnomedicine, and biodiversity conservation. The continued reference citation of The Plant List in recent publications is concerning, as it does not reflect updated taxonomic consensus. This case highlights the need for ongoing taxonomic awareness and training, particularly in interdisciplinary research teams.

In conclusion, we reaffirm that the correct and accepted name for Kacip Fatimah is *Labisia pumila*. Using this name is consistent with historical usage, current nomenclatural rules, and global taxonomic consensus. We recommend that future research, especially in pharmacological and phytochemical studies, consistently apply this name and actively consult maintained databases such as IPNI and POWO.

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## DECLARATIONS

**Research permit(s).** Not applicable.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Dereplication of Oligostilbenes in The Crude Extracts of Dipterocarpaceae Plants from Kadamaian, Sabah

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## ABSTRACT

Oligostilbenes are a class of polyphenolic compounds with notable bioactivities, predominantly produced by Dipterocarpaceae, a major tree family in Southeast Asian tropical rainforests. Given their ecological and pharmacological significance, efficient identification of oligostilbenes from natural sources is essential, particularly to avoid re-isolation of known compounds. This study aimed to apply an LC-ESI-$MS^n$-based dereplication approach for the rapid identification of known oligostilbenes directly from crude extracts of Dipterocarpaceae species collected from Kadamaian, Sabah. The selected species; *Parashorea tomentella*, *Dryobalanops lanceolata*, *Dipterocarpus caudiferus*, *Shorea xanthophylla*, and *Shorea seminist*, represent ecologically important flora from one of the most biodiverse forest regions in Malaysia. An in-house $MS^1$–$MS^5$ spectral database of authenticated oligostilbenes was used to match fragmentation profiles and retention times from ten crude extracts (bark and heartwood). A total of 11 known oligostilbenes were confidently identified, with species- and tissue-specific variations observed in their distribution. *P. tomentella* showed the richest profile in bark, while certain trimeric and tetrameric stilbenes were more prevalent in heartwood, suggesting tissue-specific biosynthetic patterns. Additionally, several unidentified peaks with consistent stilbene-like fragmentation were detected, indicating the presence of potentially novel oligostilbenes. This dereplication method significantly enhanced the speed and reliability of compound identification in complex matrices, demonstrating its utility in streamlining phytochemical workflows. The findings also provide valuable chemotaxonomic insights into the Dipterocarpaceae of Sabah and support their potential as reservoirs of bioactive natural products.

**Keywords**: Dereplication; LC-$MS^n$; oligostilbenes; Dipterocarpaceae; Kadamaian Sabah; natural products.

## INTRODUCTION

Approximately 60% of Sabah's land area remains forested, representing one of the most significant expanses of tropical rainforest in Malaysia. However, much of this forest cover has been subjected to extensive logging activities, resulting in areas at various stages of ecological succession. A substantial portion of these forests comprise lowland dipterocarp rainforest, which is recognized as one of the most species-rich and biologically diverse terrestrial ecosystem. These forests are predominantly composed of species from the Dipterocarpaceae family, which plays a vital role in Sabah's forestry sector, an important contributor to the state's economy and revenue generation (Eschenbach et al., 1998).

Members of the Dipterocarpaceae family are well known for producing a wide range of oligostilbenes. Notably, nearly one-third of all stilbene derivatives reported to date have been isolated from this family (Shen et al., 2009). Previous work by this laboratory led to the isolation of four novel resveratrol oligomers, along with thirteen known oligostilbenes, from the heartwood of *Neobalanocarpus heimii* (Jalal et al., 2018). As analytical methodologies have advanced, particularly in the areas of chromatographic and spectroscopic techniques, the discovery of new oligostilbenes from various plant sources have continued to increase (Lim et al., 2023). However, this also raises the likelihood of re-isolating previously identified compounds.

The structural complexity of natural products necessitates the use of sophisticated spectrometric methods and considerable analytical expertise. Given the time and resources involved, there is a critical need for rapid and efficient methods to identify known compounds directly from crude extracts. Such dereplication strategies are essential to prevent redundant isolation of previously characterized metabolites, thereby allowing researchers to focus on the discovery of novel or pharmacologically relevant compounds.

A previous study demonstrated the application of liquid chromatography-electrospray ionization-mass spectrometry (LC-ESI-MS) for the dereplication of oligostilbenes, including its ability to distinguish closely related diastereoisomers (Manshoor & Weber, 2015a, b). The method, originally optimized for a triple quadrupole mass spectrometer ($MS^2$), has since been adapted for use with ion trap instrumentation. This study builds upon that work by employing a targeted dereplication strategy using a curated library of MS fragmentation data for known oligostilbenes.

## METHODOLOGY

### Plant materials and extraction method

A field expedition was conducted from 21st to 25th October 2019 in Kadamaian, Kota Belud, as part of the Borneo Geographic Expedition. This initiative was jointly organized by the Institute for Tropical Biology and Conservation (ITBC), Universiti Malaysia Sabah (UMS), and Sabah Parks. The primary aim was to collect bark and heartwood samples from selected Dipterocarpaceae species. Sampling sites included areas near a waterfall adjacent to the expedition base camp, along the Pinolobu and Meliawa rivers, and near Melangkap Noriou. Ten samples, including bark and heartwood from five dipterocarp species were collected.

Bark samples were obtained by carefully scraping the tree trunks at breast height (approximately 1.3 meters above ground level) using sterile knives to prevent contamination.

The underlying heartwood was then accessed and sliced from the area beneath the removed bark.

The species collected, all members of the Dipterocarpaceae family, were identified based on morphological characteristics and referenced to taxonomic descriptions by Meijer and Wood (1964) and Cockburn (1980):
1. *Parashorea tomentella* (Urat mata beludu); a very large tree, the height can exceed 60 m, with a dense crown.
2. *Dryobalanops lanceolata* (Kapur paji); A large tree with a diameter of up to 160 cm at breast height and a dense, oval crown, endemic to Borneo and widely distributed except in the south.
3. *Dipterocarpus caudiferus* (Keruing puteh, white Seraya); A large, lowland species common in Sabah, endemic to Borneo.
4. *Shorea xanthophylla* (Seraya kuning); Found only in northern Borneo (Sabah, Sarawak, Brunei), this species grows up to 28 m and commonly occurs in lowland forests.
5. *Shorea seminis* (Selangan batu terendak); Reaches heights of up to 50 m and is typically found along slowly flowing rivers; widely distributed in the lowland forests of Sabah.

Freshly collected bark and heartwood samples were chopped and oven-dried at 40°C until a constant weight was achieved. Bark samples (300 g each) were first defatted with *n*-hexane by maceration (1 L, overnight at room temperature) to remove non-polar constituents. The defatted material was then extracted with acetone by maceration and lixiviation (1 L, 3 cycles × 24 hours) at room temperature to obtain phenolic-rich extracts.

Heartwood samples (300 g each) were also delipidated with *n*-hexane and subsequently extracted using a water:acetone mixture (30:70, v/v). The extraction involved maceration for 20 hours followed by lixiviation with fresh solvent for 4 hours. The combined extracts were concentrated under reduced pressure and subjected to liquid–liquid partitioning using water and a methanol:ethyl acetate (1:1, v/v) mixture. The organic phase was collected and evaporated to dryness to yield the crude extracts. All extractions were done at RT, without mechanical shaking or centrifuging. All extracts were filtered through 0.45 µm PTFE membranes before chromatographic analysis. The extraction yields are shown in Table 1.

**Table 1:** Extraction yields of crude bark and heartwood extracts from five Dipterocarpaceae species collected in Kadamaian, Sabah. Yields were calculated based on the dry weight of starting material (300 g per sample).

| No | Plant species | Bark<br>g (% w/w) | Heartwood<br>g (% w/w) |
|---|---|---|---|
| 1 | *Parashorea tomentella* | 8.77 (2.92) | 4.71 (1.57) |
| 2 | *Dryobalanops lanceolata* | 7.22 (2.41) | 3.06 (1.03) |
| 3 | *Dipterocarpus caudiferus* | 5.89 (1.96) | 5.33 (1.77) |
| 4 | *Shorea xanthophylla* | 5.22 (1.74) | 3.12 (1.04) |
| 5 | *Shorea seminis* | 6.25 (2.08) | 5.15 (1.72) |

## Reference standards

Pure compounds were isolated from the wood extract of *Neobalanocarpus heimii* through successive chromatographic separation techniques. Fractionated samples were subjected to repeated purification steps until chemical homogeneity was achieved. For each fraction, appropriate chromatographic parameters and solvent systems were carefully optimized based on its unique composition. The structures of the purified compounds were subsequently

elucidated using advanced spectroscopic techniques, including nuclear magnetic resonance (NMR) and mass spectrometry (MS), ensuring accurate identification and structural confirmation. The compounds were identified as heimiol A, heimiol B, balanocarpol, copaliferol A, vaticanol A, vaticaphenol A, heimiol D, heimiol E, hemsleyanol D, hopeaphenol, and isohopeaphenol (Bayach et al., 2015).

**Chromatography**

High-performance liquid chromatography (HPLC) and ultra-high-performance liquid chromatography (UHPLC) analyses were carried out using a Thermo Scientific™ UltiMate™ 3000 system (Thermo Fisher Scientific, Waltham, MA, USA). Both systems were equipped with a quaternary pump, autosampler, column oven, and a diode array detector (DAD-3000). The detector was set to monitor UV absorbance at 270 nm, which corresponds to the maximum absorbance of oligostilbenes. The DAD parameters were as follows: sampling rate of 5 Hz, slit width of 4 nm, and bandwidth of 20 nm. Column temperature was maintained at 35°C for both systems. The solvent systems and gradient profiles were optimized as described earlier to ensure effective separation of oligostilbenes.

*HPLC Analysis.* High-performance liquid chromatography (HPLC) was conducted using an ODS Hypersil® column (150 × 4.6 mm, 5 µm particle size; Thermo Fisher Scientific, Waltham, MA, USA) maintained at 35 °C. The mobile phase consisted of acetonitrile (ACN) and ultrapure water ($H_2O$), delivered through a combination of isocratic and gradient elution as follows: 5% ACN (0–3 min), 5–16% ACN (3–6 min), isocratic at 16% ACN (6–36 min), followed by a linear gradient to 34% ACN (36–39 min). At the end of each run, the column was flushed with 85% ACN for 6 minutes and re-equilibrated with the initial solvent composition for 10 minutes. The total run-time was 50 minutes, including pre-/post-equilibration, and the injection volume was 10 µL, with a flow rate of 1.0 mL/min.

*UHPLC Analysis.* Ultra-high-performance liquid chromatography (UHPLC) was carried out using an ODS Hypersil® column of the same stationary phase but with reduced internal diameter and particle size (150 × 2.1 mm, 3 µm particle size; Thermo Fisher Scientific). The solvent gradient was adapted from the HPLC protocol. Injections were made at 2 µL with a flow rate of 0.2 mL/min.

**Mass spectrometry**

Mass spectrometric analyses were performed using an Agilent 6300 Series Ion Trap LC/MS system (Agilent Technologies, Santa Clara, CA, USA) equipped with an electrospray ionization (ESI) source operating in positive ion mode. Nitrogen served as both the nebulizing and drying gas, with the nebulizer temperature set at 350 °C. Spectra were acquired in positive ion mode across a mass range of *m/z* 100–1000. $MS^n$ experiments were carried out up to $MS^5$, using helium as the collision gas. The isolation width was set to 2.0 *m/z*, and the fragmentation amplitude was maintained at 0.90 V.

**Compound identification**

An in-house compound library was constructed using mass spectral data of authenticated reference standards. Fragmentation patterns ($MS^1$–$MS^5$) of each pure compound were recorded and stored using Agilent MassHunter software. Subsequently, LC-MS data from crude plant extracts were acquired and analysed. The identification of known compounds was accomplished by matching the $MS^n$ spectra of sample peaks with those in the reference in-house library, enabling confident dereplication (Ramli et al., 2015).

## RESULTS

As part of the Borneo Geographic Expedition in Kadamaian, Sabah, five Dipterocarpaceae species were selected for analysis: *Parashorea tomentella*, *Dryobalanops lanceolata*, *Dipterocarpus caudiferus*, *Shorea xanthophylla*, and *Shorea seminis*. For each species, both bark and heartwood were sampled to assess tissue-specific variations in oligostilbene content. Figure 1 presents photographs of the trees to provide visual context for the samples used in this study.


**Figure 1:** Representative photographs of Dipterocarpaceae species sampled during the Borneo Geographic Expedition in Kadamaian, Sabah. The trees selected for this study include: **A.** *Parashorea tomentella*, **B.** *Dryobalanops lanceolata*, **C.** *Dipterocarpus caudiferus*, **D.** *Shorea xanthophylla*, and **E.** *Shorea seminis*. Bark and heartwood samples were collected from each tree species for chemical profiling and dereplication analysis.

### Chromatographic profile of the crude extracts

A polar heartwood extract was obtained via liquid–liquid partitioning of an aqueous–acetone extract using a methanol: ethyl acetate (1:1) and water biphasic system. Chromatographic profiles of all crude extracts were first generated using conventional high-performance liquid chromatography (HPLC) prior to liquid chromatography-mass spectrometry (LC-MS) analysis.

The resulting chromatograms revealed the chemical complexity of the extracts, characterized by numerous overlapping peaks, particularly among metabolites present in low abundance. Optimal dereplication requires baseline separation of individual compounds; however, the method must also remain time-efficient and robust.

The complexity of the crude extracts, particularly the presence of structurally similar oligostilbenes with varying degrees of polymerization, posed a significant challenge for chromatographic separation. These compounds often differ only slightly in polarity and molecular weight, leading to co-elution if the chromatographic conditions are not carefully optimized.

To improve resolution, we adjusted the solvent gradient to achieve a balance between polar and non-polar interactions with the stationary phase. A reversed-phase ODS column was selected to exploit hydrophobic interactions, allowing better separation of the phenolic stilbenes based on their increasing hydrophobicity with higher oligomerization. The early

gradient phase (5–16% acetonitrile) allowed more polar compounds to elute slowly, enhancing separation, while the later gradient (up to 34%) gradually increased elution strength to resolve more hydrophobic trimers and tetramers.

The 35-minute run time was chosen as an optimal point to allow sufficient separation without unnecessarily extending the analysis duration, maintaining throughput for multiple sample runs. An additional 10-minute high-acetonitrile flush ensured removal of strongly retained compounds and re-equilibration of the column, preventing carryover and preserving reproducibility. These adjustments aimed to ensure baseline separation of closely related metabolites, thereby increasing confidence in compound identification and enhancing dereplication efficiency.

**Mass spectrometric analyses for the crude extracts**

All extract samples were further analyzed using LC-MS, equipped with a diode array detector and an ion trap mass spectrometer operating with an electrospray ionization (ESI) interface. The chromatographic separation was performed using an ultra-high-performance liquid chromatography (UHPLC) system, with minor adjustments made to accommodate the narrower column dimensions and higher pressure requirements relative to conventional HPLC.

Compound identification was based on MS fragmentation patterns, which were compared against an established in-house spectral library (Table 2). Detected peaks corresponding to known compounds were assigned numerical labels, corresponding to the standard compounds in the library (Fig. 2).

**Table 2:** The fragment ions at each MS level obtained from LC-ESI-ion trap-MS spectral data of reference compounds.

| No | Compound | MS | MS2 | MS3 | MS4 | MS5 |
|---|---|---|---|---|---|---|
| 1 | Heimiol A | 471 | 453, 349, 243 | 359, 241 | 331 | - |
| 2 | Heimiol B | 471 | 453, 349, 243 | 243 | 215 | - |
| 3 | Balanocarpol | 471 | 377, 243 | 349, 243 | 173 | - |
| 4 | Copaliferol A | 681 | 587, 453, 331 | 313, 239 | - | - |
| 5 | Vaticanol A | 681 | 557, 453, 359 | 359, 265 | 265 | 239 |
| 6 | Vaticaphenol A | 907 | 813, 707, 513 | 479, 371 | 409 | - |
| 7 | Heimiol D | 907 | 813, 709, 347 | 709, 707, 625 | 689, 613, 479 | - |
| 8 | Heimiol E | 907 | 813, 719 | 719, 635 | 701, 625 | - |
| 9 | Hemsleyanol D | 907 | 813, 719 | 719, 625 | 701, 625, 515 | 607, 531, 409 |
| 10 | Hopeaphenol | 907 | 453, 359 | 341, 265 | 237 | - |
| 11 | Isohopeaphenol | 907 | 813, 453, 359 | 359, 265 | 265 | - |

**Dereplication of known oligostilbenes in the crude extracts**

LC-MS analysis of the bark extract of *Parashorea tomentella* led to the identification of seven oligostilbenes, matched against a reference compound from in-house library. All detected peaks were well-resolved, enabling confident identification based on retention time and fragmentation profiles. Notably, compound 9 was detected despite its low abundance, demonstrating the sensitivity of the method. The wood extract of *P. tomentella* exhibited a distinct oligostilbene profile compared to its bark counterpart, with differing retention times for most compounds except compound 3. Among four major peaks observed between 17.0 and 21.0 minutes, three matched known oligostilbenes. An additional peak at 17.0 minutes,

consistent with a stilbene trimer, did not correspond to any known entry in the database. Overall, the peaks displayed good resolution, facilitating dereplication.

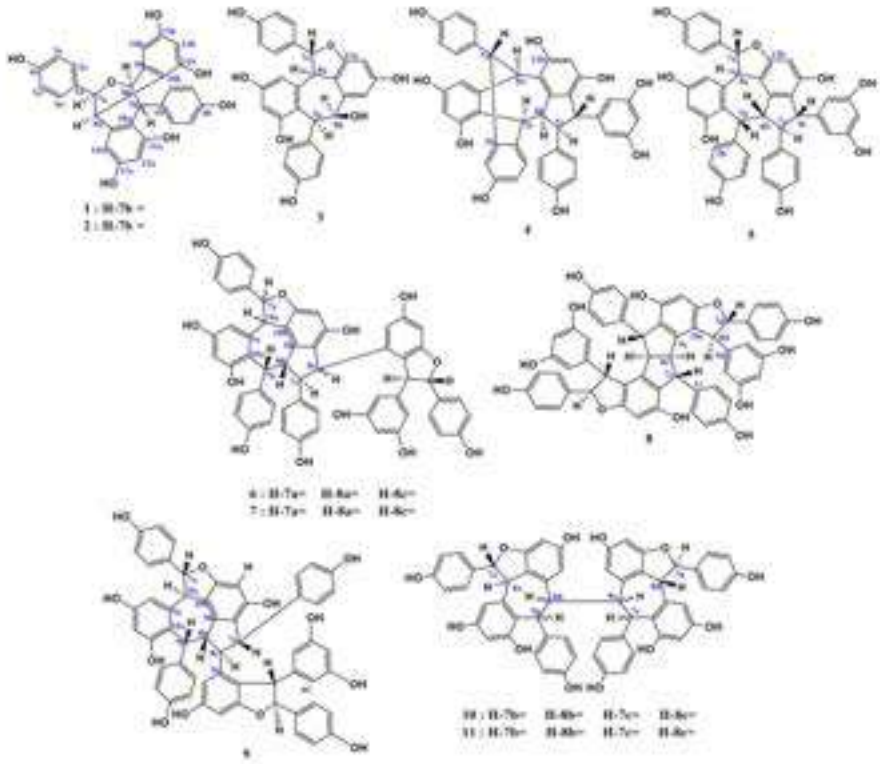

**Figure 2:** Structures of the oligostilbenes 1–11 used to generate the MS data reference library.

The methanolic bark extract of *Dryobalanops lanceolata* produced ten prominent peaks, though most eluting before 15 minutes did not correspond to known oligostilbenes. A broad, unresolved peak prior to 5.0 minutes was excluded due to incompatible fragmentation data. A minor peak at 9.8 minutes (*m/z* 502) suggested the presence of a stilbene dimer lacking one phenolic ring, possibly a degradation product. Peaks at 11.2 and 12.7 minutes were identified as stilbene dimers, while those at 14.9 and 17.2 minutes corresponded to trimeric stilbenes, although these were not present in the compound database. In total, five known compounds were identified. The chromatographic profile of the wood extract mirrored that of the bark extract, though variations in peak intensity were observed. Several unidentified peaks between 11 and 18 minutes displayed fragmentation patterns consistent with previously detected oligostilbenes. Compound 7, which was too low in abundance in the bark to be detected, was clearly observed here. Five oligostilbenes were identified based on MS fragmentation.

Analysis of the bark extract of *Dipterocarpus caudiferus* revealed a major peak at 13.4 minutes (*m/z* 681), confirmed via MS as compound 4, a stilbene trimer. Three additional peaks at 16.9, 21.2, and 23.5 minutes showed *m/z* values of 907 and were identified as compounds 6, 9, and 10, respectively. Peaks at 9.5 (*m/z* 469.2) and 10.2 (*m/z* 679.2) suggested the presence of dimeric and trimeric stilbenes, although no matches were found in the database. A broad

unresolved peak at 20.0 minutes could not be identified due to overlapping signals. The wood extract of *D. caudiferus* showed three distinct, well-resolved peaks at 13.5, 18.5, and 25.2 minutes, identified as compounds 4, 7, and 11, respectively. Compound 5, which co-eluted with several unknowns, was identified based on its intensity, *m/z* value (680), and fragmentation data as a stilbene trimer. A cluster of peaks between 22.0 and 26.0 minutes was partially resolved; however, only the peak at 24.7 minutes matched a known compound (compound 10). The remaining peaks could not be assigned due to lack of reference data.

The bark extract of *Shorea xanthophylla* displayed eight well-resolved peaks between 7.0 and 19.0 minutes. Five peaks, at 7.5, 9.1, 10.0, 15.8, and 16.9 minutes, corresponded to compounds 1, 2, 3, 5, and 6, respectively. The remaining peaks did not match any known compounds in the database. Similar to the bark extract, the wood extract of *S. xanthophylla* exhibited a series of well-defined peaks, though with a distinct chromatographic profile. A peak at 4.2 minutes was excluded due to incompatible fragmentation data. Two major peaks at 9.5 and 17.1 minutes (*m/z* 471 and 907) were identified as compounds 2 and 6, consistent with the bark extract. Additionally, peaks at 17.0, 21.9, and 23.7 minutes (all *m/z* 907) were identified as compounds 7, 9, and 10, respectively, indicative of stilbene tetramers.

The bark and wood extracts of *S. seminis* shared highly similar chromatographic profiles, with well-resolved peaks throughout. All compounds identified in the bark extract were also present in the wood extract, except for compound 5. Several minor peaks were below the detection threshold for mass spectral analysis. In total, eight oligostilbenes (compounds 1–8) were successfully identified from both extracts. The chromatograms with all identified peaks for all sample extracts are shown in Fig. 3.

The presence of broad or unresolved peaks, such as the one observed at 20.0 minutes in *Dipterocarpus caudiferus*, likely reflects the complex chemical nature of the sample matrix and the structural characteristics of oligostilbenes. One possible explanation is the presence of co-eluting isomers or oligomeric species with very similar polarity and molecular weight, which can be difficult to separate under standard chromatographic conditions. Oligostilbenes often share core structural features with subtle differences in linkage type, degree of polymerization, or hydroxylation pattern, leading to overlapping retention behaviours. Additionally, strong matrix effects may interfere with chromatographic resolution or ionization efficiency, particularly in heartwood extracts, which are known to contain dense mixtures of phenolic compounds and polymeric substances. Chemical instability, such as oxidation or partial degradation during extraction or analysis, may also contribute to peak broadening or shifting. These factors, individually or in combination, can complicate both chromatographic separation and confident spectral interpretation. Future studies may address this by incorporating sample clean-up techniques, targeted isolation, or orthogonal chromatographic methods to improve resolution and aid in the identification of such ambiguous features.

The observed variations in oligostilbene profiles between bark and wood extracts, as well as among species, appear to be primarily driven by differences in the degree of polymerization, as reflected by the distinct *m/z* values and fragmentation patterns obtained from LC-$MS^n$ analysis. Bark extracts generally exhibited a higher proportion of dimeric oligostilbenes (e.g.,

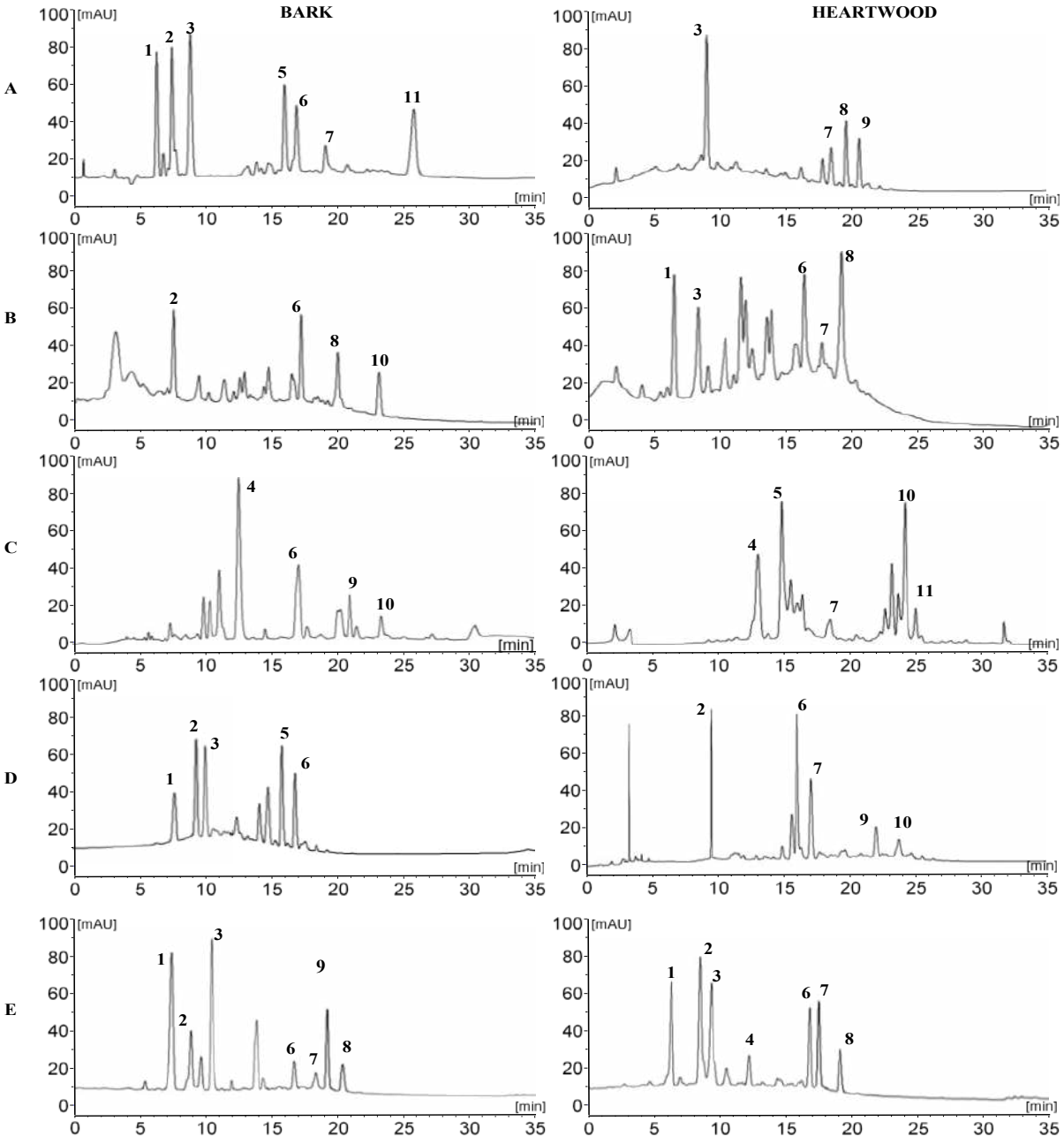


**Figure 3:** Chromatograms of crude methanolic extracts; **A.** *Parashorea tomentella*, **B.** *Dryobalanops lanceolata,* **C.** *Dipterocarpus caudiferus*, **D.** *Shorea xanthophylla, and* **E.** *Shorea seminis* recorded at 270 nm. Respective peaks are labelled with their corresponding identifying MS characteristics from the data library.

*m/z* ~470), which are likely biosynthesized rapidly in response to environmental stimuli such as UV exposure, microbial invasion, or herbivory. In contrast, wood extracts, particularly heartwood, contained a greater abundance of trimeric and tetrameric stilbenes (e.g., *m/z* ~680–907), suggesting long-term accumulation and structural reinforcement roles. Minor but consistent differences in fragmentation profiles also point to variations in interflavonoid linkage types (e.g., C–C vs. C–O–C bonds) and hydroxylation or methoxylation patterns on the aromatic rings. These structural variations not only influence chromatographic behaviour

and ionization efficiency but also likely reflect species-specific metabolic adaptations. For instance, the frequent occurrence of highly polymerized oligostilbenes in *Dipterocarpus caudiferus* wood may indicate a strategy for durable heartwood defence, while the diversity of dimers in *Parashorea tomentella* bark suggests a more dynamic defensive chemistry. Such differences underscore the chemical diversity and ecological specialization among Dipterocarpaceae species.

The chromatograms revealed that heartwood extracts exhibited a higher degree of peak co-elution and non-baseline separation compared to bark extracts. This can be attributed to the inherently more complex chemical matrix of heartwood, which tends to accumulate a broader range of secondary metabolites over time, including higher order oligostilbenes, lignans, and other phenolic polymers. These compounds often possess similar physicochemical properties, such as molecular weight, polarity, and functional group composition leading to overlapping retention times and compromised chromatographic resolution.

Additionally, the dense and lignified nature of heartwood may result in the co-extraction of structurally related but unresolved oligomeric compounds, further contributing to peak broadening and reduced separation. Matrix effects in heartwood extracts may also affect ionization efficiency, making minor components harder to detect and resolve.

To improve chromatographic clarity in future analyses, further clean-up steps such as solid-phase extraction (SPE) or liquid-liquid partitioning with more selective solvents could help reduce matrix complexity. More targeted extraction protocols focusing on specific polarity ranges may also enrich for particular classes of stilbenes. Alternatively, employing orthogonal separation techniques, such as two-dimensional chromatography or different stationary phase chemistries (e.g., phenyl-hexyl or biphenyl columns) could enhance the resolution of closely eluting compounds. Highlighting and addressing these challenges is important to fully appreciate the chemical richness of heartwood and optimize future dereplication strategies.

## DISCUSSION

This study successfully demonstrated the application of LC-MS-based dereplication for the rapid identification of oligostilbenes in bark and wood extracts from five Dipterocarpaceae species. A total of eleven known compounds were identified, revealing both species-specific and tissue-specific variations in metabolite profiles. These findings provide valuable insights into the chemotaxonomic characteristics of the family and highlight the biosynthetic diversity of oligostilbenes across different plant tissues.

*Parashorea tomentella* exhibited the most diverse oligostilbene profile among the studied species, particularly in its bark, which contained eight compounds: Heimiols A, B, and D, balanocarpol, vaticanol A, vaticaphenol A, hemsleyanol D, and isohopeaphenol. This richness suggests that the bark serves as a major site of stilbenoid biosynthesis, likely in response to environmental exposure. The wood extract showed a reduced but overlapping profile, containing balanocarpol, heimiols D and E, and hemsleyanol D. The recurrence of compounds such as balanocarpol and hemsleyanol D across both tissues suggests a core set of metabolites that are systemically distributed within the species (Lim et al., 2023).

In *Dryobalanops lanceolata*, five compounds were identified in the bark extract, including heimiols A, D, and E, balanocarpol, and vaticaphenol A. These overlap partially with the

compounds found in *P. tomentella*, indicating shared biosynthetic capabilities within the family. The wood extract of *D. lanceolata* contained copaliferol A, vaticaphenol A, hemsleyanol D, and hopeaphenol, with vaticaphenol A being the only compound common to both tissue types. The exclusive presence of copaliferol A and hopeaphenol in wood highlights tissue-specific metabolite accumulation, potentially linked to heartwood maturation and defence (Chong et al., 2009).

The bark of *Dipterocarpus caudiferus* yielded four compounds, copaliferol A, vaticaphenol A, hemsleyanol D, and hopeaphenol, while the wood extract presented a broader chemical profile. Additional compounds, including vaticanol A and isohopeaphenol, were identified only in the wood, suggesting that the heartwood may act as a reservoir for certain trimeric and tetrameric stilbenes. This extended profile may result from long-term metabolic accumulation or adaptive responses to biotic stress, such as fungal pathogens or decay (Tiwari et al., 2025).

*Shorea xanthophylla* also displayed substantial oligostilbene diversity. Its bark extract included heimiols A and B, balanocarpol, vaticanol A, and vaticaphenol A, while the wood extract contained heimiols B and D, vaticaphenol A, hemsleyanol D, and hopeaphenol. The consistent detection of vaticaphenol A in both tissues suggests a central role in the plants metabolic or defensive functions. Similarly, the presence of heimiol derivatives in both extracts reflects the continuity of stilbenoid biosynthesis across developmental stages or tissue types (Huong et al., 2025).

*Shorea seminis* demonstrated the highest degree of overlap between bark and wood extracts, with eight oligostilbenes detected in the bark and seven in the wood. Shared compounds included heimiols A, B, D, and E, balanocarpol, copaliferol A, and vaticaphenol A. Vaticanol A was identified exclusively in the bark. This high metabolite redundancy suggests that *S. seminis* is a metabolically rich species with strong potential for yielding bioactive stilbenes.

Across all species, certain compounds such as vaticaphenol A, balanocarpol, and heimiol derivatives were frequently encountered. Vaticaphenol A was especially widespread, detected in all species except in the wood of *P. tomentella*. The consistent presence of heimiol variants across genera and tissues further underscores their significance in Dipterocarpaceae secondary metabolism (Deng et al., 2017).

Interestingly, some compounds demonstrated tissue-specific distribution. Hopeaphenol, isohopeaphenol, and copaliferol A were predominantly found in wood extracts, suggesting a functional role in heartwood physiology, possibly related to structural defence or long-term storage of antimicrobial agents. In contrast, bark extracts generally exhibited broader chemical diversity, likely reflecting their direct interaction with environmental stressors, including UV radiation, pathogens, and herbivores (Mattio et al., 2020).

This study highlights the utility of LC-MS-based dereplication as a rapid and effective approach for profiling complex plant extracts. The identification of 11 known oligostilbenes across five Dipterocarpaceae species reveals distinct yet overlapping chemical signatures that are influenced by both species and tissue type. These results contribute to our understanding of the chemical ecology and taxonomic relationships within this important tropical family. Furthermore, the findings provide a foundation for future studies into the bioactivity, ecological functions, and pharmacological applications of stilbene derivatives. A summary of the oligostilbenes identified in each species and tissue type is presented in Table 3.

**Table 3:** Identified oligostilbenes in the bark and wood extracts of Dipterocarpaceae plants collected from Kadamaian, Kota Belud, Sabah.

| | **Plant (extract)** | **Compound** |
|---|---|---|
| 1 | *Parashorea tomentella* (Bark) | Heimiols A, B, D, Balanocarpol, Vaticanol A, Vaticaphenol A, Hemsleyanol D, Isohopeaphenol |
| 2 | *Parashorea tomentella* (Wood) | Balanocarpol, Heimiols D, E, Hemsleyanol D |
| 3 | *Dryobalanops lanceolata* (Bark) | Heimiols A, D, E, Balanocarpol, Vaticaphenol A |
| 4 | *Dryobalanops lanceolata* (Wood) | Copaliferol A, Vaticaphenol A, Hemsleyanol D, Hopeaphenol |
| 5 | *Dipterocarpus caudiferus* (Bark) | Copaliferol A, Vaticaphenol A, Hemsleyanol D, Hopeaphenol |
| 6 | *Dipterocarpus caudiferus* (Wood) | Copaliferol A, Vaticanol A, Heimiol D, Hopeaphenol, Isohopeaphenol |
| 7 | *Shorea xanthophylla* (Bark) | Heimiols A, B, Balanocarpol, Vaticanol A, Vaticaphenol A |
| 8 | *Shorea xanthophylla* (Wood) | Heimiols B, D, Vaticaphenol A, Hemsleyanol D, Hopeaphenol |
| 9 | *Shorea seminis* (Bark) | Heimiols A, B, D, E, Balanocarpol, Copaliferol A, Vaticanol A, Vaticaphenol A |
| 10 | *Shorea seminis* (Wood) | Heimiols A, B, D, E, Balanocarpol, Copaliferol A, Vaticaphenol A |

The application of LC-$MS^n$-based dereplication proved especially valuable in navigating the inherent chemical complexity of Dipterocarpaceae crude extracts. The ability to generate and compare multi-stage fragmentation data ($MS^2$–$MS^5$) allowed for the confident identification of closely related oligostilbenes, even when present in low abundance or embedded within dense matrices. In particular, the reproducibility of key fragmentation pathways, such as losses of phenolic groups, stilbene units, or characteristic neutral fragments, provided diagnostic clues to differentiate compounds that share similar molecular weights but differ in structural connectivity or substitution patterns. Retention time consistency, when interpreted alongside $MS^n$ fragmentation, added another layer of confidence in compound identification, especially in distinguishing positional or stereoisomers. For peaks that could not be dereplicated, the most probable reasons include low signal intensity resulting in incomplete $MS^2$ spectra, absence of distinctive fragmentation features, or the presence of oligomeric structures not yet included in the reference library, potentially representing novel stilbenoid scaffolds. These ambiguous features were acknowledged but excluded from detailed interpretation to maintain the methodological rigor of this dereplication-focused study.

Furthermore, the tissue- and species-specific distribution patterns of identified oligostilbenes appear to reflect adaptive biochemical strategies. Compounds predominantly found in bark, such as dimers and certain hydroxyl-rich stilbenes, often possess structural features (e.g., free phenolic groups, lower degrees of polymerization) associated with higher chemical reactivity and rapid mobilization in response to environmental stressors such as pathogens or UV radiation. These features support the hypothesis that bark serves as a frontline defence compartment in Dipterocarpaceae species. Conversely, heartwood extracts tended to contain more polymerized, structurally complex oligostilbenes, such as trimers and tetramers that are chemically more stable and less prone to oxidative degradation. Such compounds are likely to function as long-term protective agents, contributing to the durability and resistance of heartwood tissues against microbial decay and structural weakening. These structure-activity relationships not only help explain the observed chemical profiles but also provide insight into the ecological roles of oligostilbenes within tropical forest species.

## CONCLUSIONS

This study demonstrated the effective use of LC-ESI-$MS^n$-based dereplication for profiling oligostilbenes in the bark and heartwood extracts of five Dipterocarpaceae species: *Parashorea tomentella*, *Dryobalanops lanceolata*, *Dipterocarpus caudiferus*, *Shorea xanthophylla*, and *Shorea seminis*. Through comparison with an in-house $MS^1$–$MS^5$ data library, a total of 11 known oligostilbenes were confidently identified. The method also enabled the recognition of previously uncharacterized stilbenes based on their fragmentation patterns and condensation levels. The approach proved particularly valuable for distinguishing structurally similar compounds within complex mixtures, even at low concentrations. Unlike traditional methods that rely heavily on chromatographic conditions, the tandem MS analysis provided consistent and interpretable spectral data, reducing the dependency on precise retention times. This highlights LC-$MS^n$ as a robust tool for streamlining phytochemical workflows and minimizing unnecessary re-isolation of known metabolites. In summary, LC-MS-based dereplication not only accelerates the identification of bioactive natural products but also enhances the strategic focus of phytochemical research. The oligostilbene diversity uncovered in these Dipterocarpaceae species reinforces their significance as promising reservoirs of pharmacologically relevant compounds, supporting further investigation into their bioactivity and conservation value.

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## DECLARATIONS

**Research permit(s).** This study was conducted with the approval of the Sabah Biodiversity Council Access License Ref. - JKM/MBS.1000-2/1JLD.3.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** The authors declare that generative AI has been used in compliance with the JTBC policies, and that we have reviewed and edited the content after using this service and we take full responsibility for the content of the publication.

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**Research Article**

# Preliminary Assessment of Secondary Metabolites of Selected Pteridophytes from Jorhat, Assam, India

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## ABSTRACT

This study analyses the phytochemical constituents of seven pteridophytic species from Assam, India. The methanolic extracts of seven species: *Pyrrosia lanceolata*, *Pyrrosia nummularifolia*, *Ampheneuron opulentum*, *Sphenomeris chinensis*, *Selaginella* sp., *Diplazium esculentum*, and *Blechnum orientale* were screened for phenolic, flavonoid, and flavonol content using standard spectrophotometric methods. *Pyrrosia nummularifolia* exhibited the highest flavonoid content, while *Diplazium esculentum* had the highest flavonol concentration. These bioactive compounds, known for their antimicrobial, antioxidant, and anti-inflammatory properties, support their traditional medicinal use and highlight their potential for drug discovery. Further studies are required to isolate and identify the pharmacologically active compounds from these species.

**Keywords:** Flavonoids; flavanols; phytochemical analysis; phenolic content; pteridophytes; traditional medicine.

## INTRODUCTION

Pteridophytes, to which ferns belong, are among the earliest vascular plant groups, originating millions of years ago. These non-flowering vascular plants have played an essential role in plant evolution and ecology. Pteridophytes exhibit resistance to microbial infections, which may have contributed to their evolutionary success and longevity of over 350 million years (Sharma & Vyas, 1985). This resistance is attributed to a wide range of phytochemical constituents that play crucial roles in defence, reproduction, and growth. Essential compounds such as polysaccharides, proteins, carbohydrates, amino acids, and nucleic acids are primary metabolites that directly support plant growth and metabolism. In addition to primary metabolites, plants also produce secondary metabolites, which act as signalling molecules within the cell and are not essential for growth, development, or reproduction. Secondary metabolites, such as flavonoids, alkaloids and phenols, are synthesized through distinct metabolic pathways that diverge from the primary ones (Handique, 2024). Flavonoids, for instance, are stored in the vacuoles of plant cells as water-soluble pigments and have a fundamental C6-C3-C6 carbon framework consisting of two 6-carbon benzene rings.

With increasing research on phytochemical constituents, many pteridophyte species have been incorporated into the pharmacopoeia of various countries (Ibadullayeva et al., 2022). Among the diverse range of secondary metabolites, terpenoids are among the most prevalent bioactive compounds in ferns (Ho et al., 2010). These bioactive compounds possess a broad spectrum of medicinal properties, including anti-inflammatory, antioxidant, anti-tumour, antimicrobial, antiviral, and anti-HIV activities, and are generally regarded as safe (Proestos et al., 2005). Flavonoids, terpenoids and other secondary metabolites play a crucial role in pharmaceutical research due to their wide range of biological activities. Flavonoids, for instance, are well documented for their antioxidant, anti-inflammatory, and antimicrobial properties, making these potential candidates for drug formulations targeting oxidative stress-related diseases, infections and inflammation (Proestos et al., 2005).

Terpenoids are among the most structurally diverse classes of secondary metabolites in ferns and have demonstrated significant pharmacological potential. Some terpenoids have been shown to exhibit anticancer properties by inducing apoptosis and inhibiting tumour progression, while others function as potent antiviral or antibacterial agents (Ho et al., 2010). In recent studies, specific terpenoids from *Blechnum orientale* have displayed strong cytotoxic effects against cancer cell lines, highlighting their potential in chemotherapeutic drug development (Raja & Paul, 2019). The ability of these compounds to interact with key cellular pathways and enzymes has made them attractive candidates for new drug discovery programmes.

Therefore, this study aims to assess the antioxidant capacity of seven fern species—*Pyrrosia lanceolata*, *Pyrrosia nummularifolia*, *Ampheneuron opulentum*, *Sphenomeris chinensis*, *Selaginella* sp., *Diplazium esculentum*, and *Blechnum orientale*—collected from Assam, Northeast India. These species were selected for their traditional medicinal use and ecological relevance. This research seeks to contribute to the limited literature on fern phytochemistry and explore the pharmacological potential of these species.

Despite their widespread distribution, research on the phytochemical properties of pteridophytes remains limited. Many species contain bioactive compounds with medicinal properties, including flavonoids, alkaloids, and phenols, which contribute to their pharmacological potential. However, the specific phytochemical composition of several species from Northeast India remain unexplored. The selection of these seven species was based on their traditional medicinal uses and ecological significance in Assam. Thus, further exploration of these bioactive compounds, including their structural elucidation and bioassay-guided fractionation, is necessary to determine their full pharmacological potential and applicability in modern medicine**.**

## METHODOLOGY

### Collection of plant materials

Fresh fern specimens were collected from the Jorhat District of Assam, India (26.7509° N, 94.2037° E) in August, 2024 (Fig. 1). The specimens were authenticated and identified by the Department of Botany, Jagannath Barooah University, Jorhat, Assam, India (Table 1).

### Phytochemical screening

Preliminary phytochemical screening of the fern extracts was conducted following the standard protocol described by Handique (2024).

**Figure 1:** Map of Assam (Study area with Jorhat district highlighted).

**Table 1:** Collection Sites and GPS Coordinates of Selected Pteridophyte Species from Jorhat, Assam, India.

| Species name | Location | GPS coordinates |
|---|---|---|
| *Pyrrosia lanceolata* | J.B. College, Jorhat | 26.7506°N, 94.2033°E |
| *Pyrrosia nummularifolia* | Lachit Nagar, Jorhat | 26.7461°N, 94.2175°E |
| *Ampheneuron opulentum* | Kaliapani Reserve Forest | 26.7350°N, 94.2985°E |
| *Sphenomeris chinensis* | Rajabari, Jorhat | 26.7520°N, 94.2194°E |
| *Selaginella sp.* | Borbheta, Jorhat | 26.7254°N, 94.1937°E |
| *Diplazium esculentum* | J.B. College, Jorhat | 26.7506°N, 94.2033°E |
| *Blechnum orientale* | Kenduguri, Jorhat | 26.7602°N, 94.2221°E |

## Preparation of plant extract

An electronic balance was used to accurately weigh 0.5 g of dried fern leaves. The plant material was then transferred into a mortar, and 50 mL of 90% methanol (Merck, India) was added. Methanol was selected as the extraction solvent due to its high polarity and effectiveness in dissolving a broad range of bioactive compounds such as phenols and flavonoids (Harborne, 1998; Senguttuvan et al., 2014). The fern leaves were macerated thoroughly using a pestle to ensure complete cellular disruption and maximum extraction of secondary metabolites.

## Drying and filtration

The macerated mixture was transferred to a clean glass beaker and placed in a hot air oven at 40 °C for 20 minutes under monitored conditions in a well-ventilated laboratory environment to facilitate partial evaporation of methanol. Following this, the extract was filtered using Whatman No.1 filter paper or equivalent (HiMedia, India) to remove insoluble plant residue. The resulting crude fern extract was collected and stored in sterile, labeled containers for further phytochemical analyses.

All experiments were conducted in triplicate (n = 3) to ensure reproducibility and accuracy. For each fern species, five individual specimens were analyzed, and extractions were performed independently on three biological replicates per specimen.

**Test for phenol**

The total phenolic content (TPC) of the fern extracts was estimated using the Folin–Ciocalteu method, originally described by Singleton and Rossi (1965), with slight modifications reported by Ainsworth and Gillespie (2007) and Adusei et al., (2019) was used. In this procedure, 0.5 mL of Folin–Ciocalteu reagent (HiMedia, India), pre-diluted 1:1 with double-distilled water, was added to 0.5 mL of fern extract in a test tube. The mixture was gently shaken and incubated at room temperature (25 ± 2 °C) for 5 minutes.

Next, 2 mL of 2% sodium carbonate ($Na_2CO_3$; SRL, India) solution (prepared by dissolving 2g of $Na_2CO_3$ in 100 mL of distilled water) was added, and the tubes were incubated in the dark for 10 minutes to allow the blue chromophore to develop. Absorbance was measured at 730 nm using a UV-Visible spectrophotometer (Thermo Scientific Evolution 201). A standard calibration curve was prepared using gallic acid (SRL, India). Results were expressed as milligrams of gallic acid equivalents per gram of dry fern extract (mg GAE/g).

**Test for flavonoids**

The total flavonoid content (TFC) of the fern extracts was determined using the aluminum chloride colorimetric method, following the protocols of Goyal et al., (2010), with slight modifications reported by Seifu et al., (2017) and Ayele et al., (2022). In short, a 10 mL test tube with 2 mL of distilled water was filled with an aliquot (0.5 mL) of the extract. 0.15 mL of 5% sodium nitrite ($NaNO_2$, SRL, India) solution (prepared by dissolving 5 g of $NaNO_2$ in 100 mL of double-distilled water) was added to each test tube. After incubating for five minutes, 0.15 mL of 10% aluminium chloride, ($AlCl_3$) solution (prepared by dissolving 10 g of $AlCl_3$ (SRL, India) in 100 mL distilled water) was added. 1 mL of 1 M sodium hydroxide (NaOH, Merck, India) solution (prepared by dissolving 4 g of NaOH pellets in 100 mL distilled water) was added after 1 minute, and the volume was then adjusted with distilled water to reach 5 mL. The absorbance of the resultant solution was measured using a UV-Visible spectrophotometer (Thermo Scientific Evolution 201) at 510 nm after 10 minutes. To express the total flavonoid content of samples as mg quercetin equivalent per 100 g of sample (mg QE/100 g sample), catechin was used as a standard. Three separate analyses were performed on each sample.

**Test for flavanols**

The total flavonol concentration was quantified using the aluminum chloride colorimetric method, following the protocol of Kumaran and Karunakaran (2006) with slight modifications reported by Handique, (2024). Briefly, 2 mL of fern extract was mixed with 2% aluminium chloride ($AlCl_3$; HiMedia, India) and 5% sodium acetate ($CH_3COONa$) (prepared by dissolving 0.41 g of sodium acetate in 100 mL of distilled water). The mixture was incubated at room temperature (approximately 25°C) for 2.5 hours. Absorbance was then measured at 440 nm using a UV-visible spectrophotometer (Thermo Scientific Evolution 201).

**Statistical analysis**

All experiments were conducted in triplicates, and results are expressed as mean ± standard deviation (SD). SD was calculated using Microsoft Excel based on three independent measurements for each species. One-way ANOVA followed by Tukey's post hoc test was performed to assess significant differences ($p < 0.05$) among species.

## RESULTS

A preliminary survey of the study area revealed a high diversity of pteridophytes belonging to various families. A checklist of the recorded pteridophytes species is provided in Table 2.

**Table 2:** A checklist of pteridophytes found in Jorhat district, Assam, India.

| Sl No. | Species | Family | Habit | Reference |
|---|---|---|---|---|
| 1 | *Salvinia cucullata* Roxb. Ex Bory | Salviniaceae | Aquatic herb | (Borthakur et al., 2000) |
| 2 | *Diplazium esculentum* (Retz.) Sw. | Athyriaceae | Herb | (Borgohain & Hazarika, 2020) |
| 3 | *Lygodium japonicum* (Thunb.) Sw. | Schizaceae | Climber | |
| 4 | *Pyrrosia lanceolata* (L.) Farw. | Polypodiaceae | Climber | |
| 5 | *Pyrrosia nummularifolia* (Sw.) Ching | Polypodiaceae | Climber | |
| 6 | *Ampheneuron opulentum* (Kaulf.) Holttum | Thelypteridaceae | Terrestrial Herb | |
| 7 | *Odontosoria chinensis* (L.) J.Sm. | Lindsaeaceae | Terrestrial Herb | |
| 8 | *Selaginella* sp. | Selaginellaceae | Terrestrial Herb | |
| 9 | *Blechnum orientale* L. | Blechnaceae | Terrestrial Herb | |
| 10 | *Thelypteris namburensis* (Bedd.) C.F.Reed | Thelypteridaceae | Terrestrial Herb | (Chandra et al., 2008) |
| 11 | *Azolla pinnata* R.Br. | Salviniaceae | Aquatic Herb | (Gogoi et al., 2023) |
| 12 | *Marsilea quadrifolia* L. | Marsileaceae | Aquatic Herb | (Gogoi et al., 2023) |
| 13 | *Stenochlaena palustris* (Burm.) Bedd. | Blechnaceae | Climber | (Dutta et al., 2017) |

Phytochemical analysis of the methanolic extracts from the seven fern species revealed the presence of key secondary metabolites: phenols, flavonoids, and flavanols. *Pyrrosia lanceolata*, *Pyrrosia nummularifolia*, *Diplazium esculentum* and *Blechnum orientale* (0.331 mg/g) exhibited the highest phenol content, while *Sphenomeris chinensis* displayed the lowest (0.042 mg/g).

Flavonoid content varied among the species, with *Pyrrosia nummularifolia* having the highest level (1.732 mg/g) and *Pyrrosia lanceolata* the lowest (0.322 mg/g). For total flavanol content, *Diplazium esculentum* contained the maximum amount (8.039 mg/g), while *Pyrrosia lanceolata* (4.327 mg/g) had the minimum. These findings highlight the presence of bioactive compounds with potential medicinal properties in the fern species examined (Table 3). The identified phytochemicals have previously been associated with medicinal and physiological activities, supporting their use in the treatment of various ailments (Fig. 2, 3 and 4).

**Table 3:** Phytochemical Composition (Phenol, Flavonoid, and Flavonol Content) of the Studied Pteridophytes (Mean ± SD, n = 3).

| Specimen | Phenol Content (mg GAE/g) | Flavonoid Content (mg QE/g) | Flavonol Content (mg RE/g) |
|---|---|---|---|
| *Pyrrosia lanceolata* | 0.331 ± 0.007 | 0.322 ± 0.004 | 4.327 ± 0.020 |
| *Pyrrosia nummularifolia* | 0.331 ± 0.003 | 1.730 ± 0.006 | 7.500 ± 0.024 |
| *Ampheneuron opulentum* | 0.086 ± 0.002 | 0.487 ± 0.002 | 5.636 ± 0.015 |
| *Sphenomeris chinensis* | 0.042 ± 0.001 | 0.638 ± 0.003 | 7.250 ± 0.021 |
| *Selaginella* sp. | 0.281 ± 0.003 | 0.507 ± 0.005 | 5.145 ± 0.017 |
| *Diplazium esculentum* | 0.331 ± 0.003 | 0.388 ± 0.003 | 8.038 ± 0.028 |
| *Blechnum orientale* | 0.331 ± 0.004 | 0.572 ± 0.005 | 6.676 ± 0.023 |

One-way ANOVA showed a statistically significant difference in phenol, flavonoid, and flavanol content among species ($F = 116.65$, $p < 0.05$). However, Tukey's post hoc test did not detect significant pairwise differences ($p > 0.05$ for all comparisons), indicating that while species differ overall, no two species had significantly different flavonoid content at the 95% confidence level.

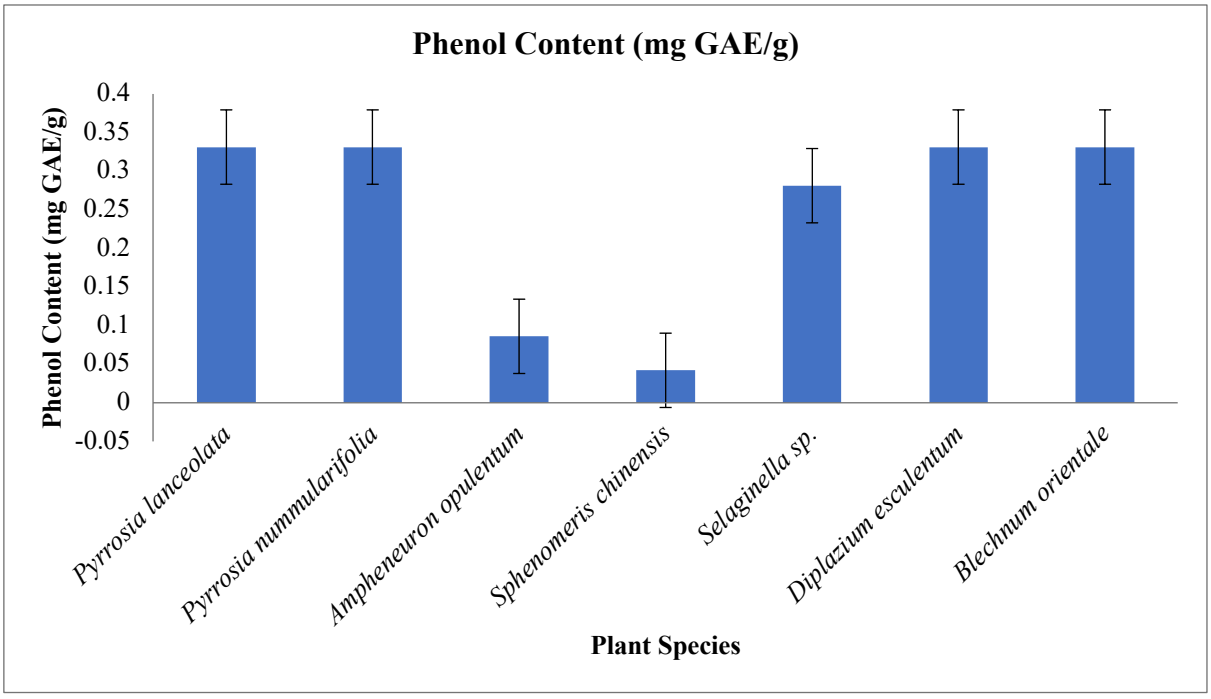


**Figure 2:** Bar Graph Showing phenol content (mg GAE/g) in the pteridophytic species.

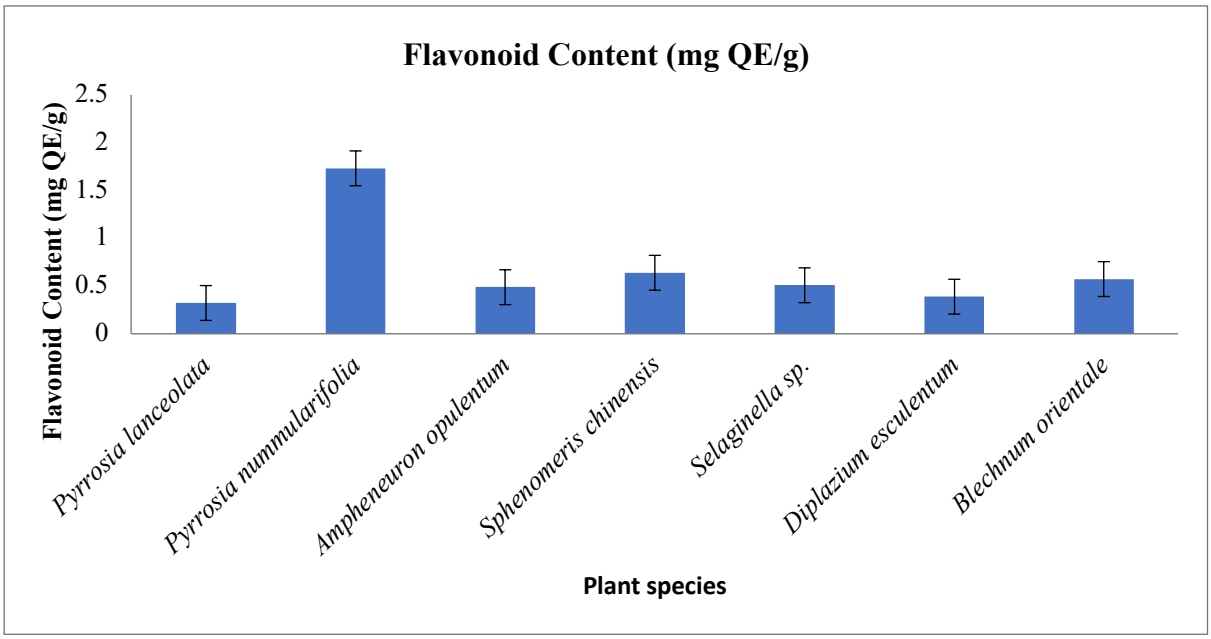


**Figure 3:** Bar Graph Showing flavonoid content (mg QE/g) in the pteridophytic species

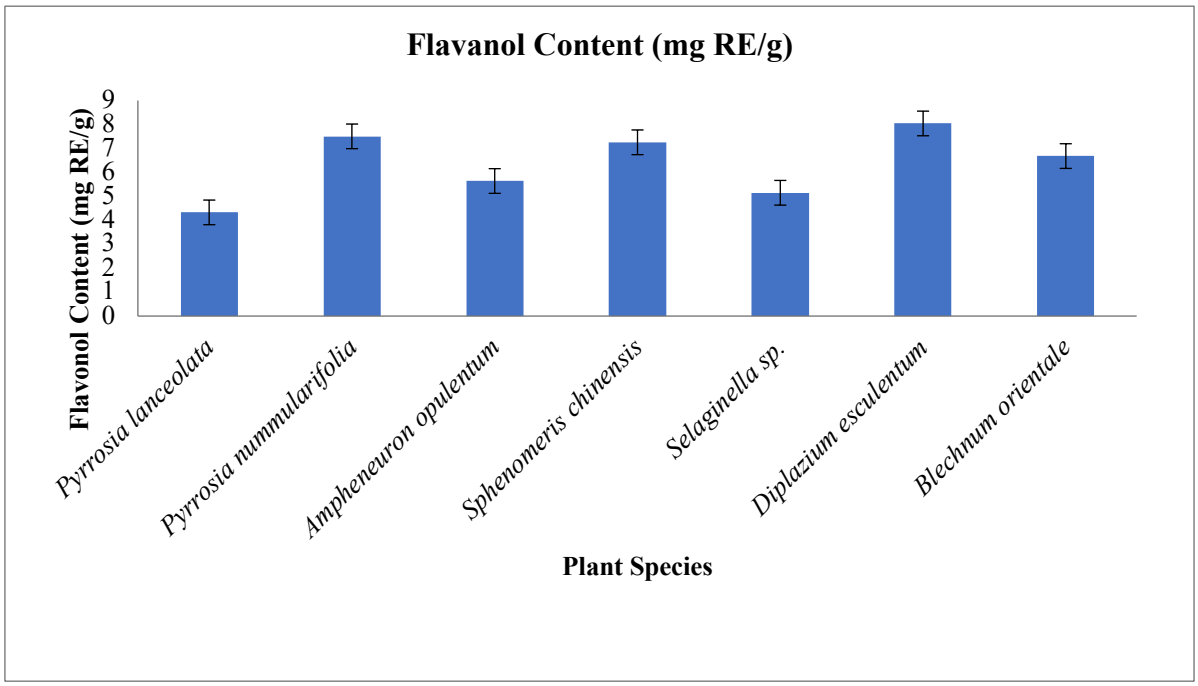


**Figure 4:** Bar Graph Showing flavanol content (mg RE/g) in the pteridophytic species

## DISCUSSION

A study conducted in Northeast India revealed that methanol extracts from three plant species contained the highest concentration of phytoconstituents, suggesting their unique physiological properties could contribute to drug discovery. Notably, *Pyrrosia lanceolata* extracts, particularly the ethyl acetate extract, exhibited strong antioxidant properties, comparable to

ascorbic acid. These benefits are likely attributed to flavonoids, a class of phenolic compounds known for their ability to neutralize free radicals (Indriani et al., 2023).

Phenolic compounds, including flavonoids, are widely recognized for their pharmacological significance, particularly their antioxidant, antimicrobial, and anti-inflammatory properties. Species with higher phenolic content, such as *Diplazium esculentum* (8.038 mg/g) and *Pyrrosia nummularifolia* (7.500 mg/g), are associated with strong antioxidant potential due to their phenolic constituents. Previous studies have reported antioxidant activity in *D. esculentum*, supporting its traditional medicinal use (Choudhury et al., 2017). Additionally, flavonoid-rich species, including *P. nummularifolia* (1.730 mg/g), have been linked to antimicrobial and anti-inflammatory effects, which may contribute to their application in wound healing and infection management. These findings reinforce the therapeutic potential of these species and provide scientific validation for their continued use in herbal medicine.

Ismiarni et al., (2015) investigated the anti-inflammatory activity of *Nephrolepsis falcata* and *Pyrrosia lanceolata*, confirming their significant anti-inflammatory effects. Fan et al., (2020) analyzed six *Pyrrosia* species and identified the presence of phytol, nonanal, and 2,4-pentadienal, which exhibited broad-spectrum antibacterial activity. These findings align with the present study, further supporting the potential of *Pyrrosia lanceolata* as a therapeutic agent. Additionally, research on *Blechnum orientale L.* identified twelve secondary metabolites, including alkaloids, anthocyanins, anthraquinones, cardiac glycosides, diterpenes, flavonoids, saponins, steroids, tannins, and triterpenoids, alongside 20 compounds detected via GC-MS analysis (Raja & Paul, 2019). A separate phytochemical study on *B. orientale* confirmed the presence of various bioactive compounds, such as alkaloids, cardioglycosides, phenols, saponins, tannins, terpenoids, quinones, flavonoids, glycosides, anthocyanins, and betacyanins. Flavonoids, tannins, and phenolic substances were found in every fern species that was examined (Borkotoky et al., 2024). *P. semipinnata* extracts in ethanol and acetone produced the highest levels of flavonoids and tannins ($302.73 \pm 0.001$ mg QE/g and $421.227 \pm 0.009$ mg GAE/g, respectively), as well as the maximum amount of extractable total phenolic components ($526.517 \pm 0.002$ mg GAE/g and $526.517 \pm 0.001$ mg GAE/g, respectively).

Additionally, GC-MS analysis of ethyl acetate and ethanol extracts identified several antibacterial compounds, further supporting the species' therapeutic potential (Amose et al., 2023). Choudhury et al., (2017) analysed the phytochemical constituents of *Diplazium esculentum*, identifying antioxidant properties and the presence of carbohydrates, saponins, phenols, flavonoids, proteins, triterpenes, and alkaloids, but excluding glycosides, Phyto steroids, tannins, amino acids, and cardiac glycosides.

## CONCLUSION

This study investigates the phytochemical composition of seven fern species from the Jorhat District of Assam, India, revealing the presence of phenols, flavonoids, and flavanols. These bioactive compounds, known for their antioxidant, antimicrobial, and anti-inflammatory properties, have traditionally been used in the treatment of wounds, cuts, tooth and gum pain, constipation, jaundice, digestive disorders, and cardiovascular ailments.

The findings highlight the potential of these species as novel therapeutic agents; however, further research is required to isolate, purify, and characterize the active compounds responsible for their medicinal effects. Additionally, comprehensive pharmacological and

toxicological studies are essential to validate their efficacy and safety for future drug development.

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## DECLARATIONS

**Research permit(s):** Not applicable.
**Ethical approval/statement:** Not applicable.
**Generative AI use:** I/we declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# *Ardisia ledangensis* (Primulaceae-Myrsinoideae), a new species from southern Peninsular Malaysia

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## ABSTRACT

*Ardisia ledangensis* Julius & Utteridge, a new species from Gunung Ledang, Johor, in the southern part of Peninsular Malaysia, is formally described and illustrated. Its placement within subgenus (§) *Crispardisia* is justified by diagnostic characters such as vascularised glandular nodules along the leaf margin and a terminal inflorescence borne on a lateral shoot, accompanied by a normal foliage leaf (rather than a reduced, bract-like leaf). The species is distinctive among Peninsular Malaysian members of the subgenus by its slender, descending habit, narrowly elliptic leaves densely covered with black glandular-dots, and unbranched, strictly terminal inflorescences. *Ardisia ledangensis* is known only from a single locality within a protected area, and its conservation status is provisionally assessed as Least Concern (LC).

**Keywords**: Conservation status; Gunung Ledang; Johor; montane; subgenus *Crispardisia*; Ericales.

## INTRODUCTION

*Ardisia* Sw. (Swartz, 1788: 48), with about 730 species distributed across the Americas, Asia, Australia, and the Pacific Islands, is one of the megadiverse and understudied understorey plant groups in the tropics (Frodin, 2004; POWO, 2025). Due to the rich biodiversity and incomplete botanical exploration across Southeast Asia, especially for many medium-sized to large genera, the ongoing discovery of new species and records is to be expected (Middleton et al., 2019). Since the last treatment in Stone's Tree Flora of Malaya (1989a), six new species have been described (Julius & Utteridge, 2012, 2021, 2022; Julius et al., 2017, 2023) from Peninsular Malaysia. In an annotated key to the genus, Stone (1989a) documented 74 species in the Tree Flora of Malaya, but full descriptions of most taxa were lacking because the majority of *Ardisia* species do not reach the size required to merit a full treatment.

Currently, the genus is classified into 17 subgenera, delineated by growth form, leaf characteristics, inflorescence position, and floral structures (see Mez, 1902; with additional subgenera published by Stone (1993), §*Scherantha*; Larsen & Hu (1995), §*Tetrardisia*; Yang & Hu (2022), §*Odontophylla*). Eleven of these subgenera are present in the Malesian region (see Stone (1982) for a discussion and key to the groups in Malesia; Larsen & Hu (1995) for §*Tetrardisia*; and Utteridge et al. (2023) for an updated discussion and key to the groups in Borneo). Whilst the definition of the genus is potentially problematic as it may need to be either expanded or restricted (see Larson et al., 2023), the subgenera, with re-evaluation of some species placements, are useful for classification and identification (Julius et al., 2021; Utteridge et al., 2023). All eleven Malesian subgenera are also present in Peninsular Malaysia, including §*Crispardisia*, to which the new species belongs. This subgenus is characterised by crenate or crenulate leaves with distinct dark or swollen nodulations, usually located in each marginal sinus or at the apex of the teeth, and rarely on the upper or lower surfaces near the leaf margin (e.g., *Ardisia caloneura* C.M.Hu & J.E.Vidal and *A. prolifera* C.M.Hu & J.E.Vidal from Laos). The inflorescences are terminal or lateral (axillary); when terminal, they are on a lateral branch, usually subtended by one to several leaves, rarely without leaves. Additionally, many species, including the taxon described here and other members of the subgenus in Peninsular Malaysia, are characterised by numerous leaves along the lateral branch, rarely 2–3 leaves, as observed in *A. recurvipetala* Julius, Siti-Munirah & Utteridge from Terengganu and *A. filipendula* C.M.Hu & J.E.Vidal from Laos.

Fieldwork conducted in the southern part of Johor in 2015 and 2022, aimed at obtaining new collections of Primulaceae for the family revision of the Flora of Peninsular Malaysia, led to the discovery of an unidentified taxon of *Ardisia* from Gunung Ledang. Fruiting material was collected during the 2015 fieldwork, while only sterile individuals were observed in the field during the 2022 survey. Examination of herbarium specimens of §*Crispardisia* at KEP, K, and SING revealed additional specimens of the same taxon, including flowering material housed at KEP. Access to both flowering and fruiting specimens enabled a detailed comparison with existing taxa and facilitated a formal description of the new species. Thus, *Ardisia ledangensis* Julius & Utteridge is formally described and illustrated here as a new endemic species to Peninsular Malaysia.

## METHODOLOGY

Field observations of living specimens were undertaken by the first author in 2015 and again in 2022. These were complemented by a comparative examination of herbarium collections of other *Crispardisia* taxa housed at KEP, K, and SING. To support identification and description, key taxonomic references (e.g., Stone, 1982; Larsen & Hu, 1991; Chen & Pipoly, 1996; Hu & Vidal, 2004) were reviewed, and digital specimen images were consulted via JSTOR Global Plants (http://plants.jstor.org/), Plants of the World Online (POWO: http://www.plantsoftheworldonline.org/), and the Naturalis Biodiversity Center BioPortal (http://bioportal.naturalis.nl/). Herbarium acronyms follow Index Herbariorum (Thiers, 2024). Morphological assessments were made using both fresh and herbarium specimens, including rehydrated floral parts. Descriptive terms for shapes follow the conventions of the Systematics Association Committee (1962). Collections with flowers and fruits are denoted as 'fl.' and 'fr.', respectively, while the subgenus is marked as '§'. The conservation status was evaluated according to IUCN Red List criteria (IUCN 2012; IUCN Standards and Petitions Subcommittee, 2024).

## RESULTS

### Taxonomic treatment

***Ardisia ledangensis*** Julius & Utteridge **sp. nov.** (§*Crispardisia*) (Figs. 1–2)

**Diagnosis.** Distinguished from other species in §*Crispardisia* by the slender, descending woody stem; subcoriaceous, narrowly elliptic leaves measuring 3–10.5 cm long with dense black glandular-dots on both surfaces; unbranched inflorescences borne strictly at the terminal of lateral branches; and glabrous floral parts (excluding pedicels) that are also marked with dense, black glandular-dots.

**Type: —** MALAYSIA. Peninsular Malaysia: Johor, Gunung Ledang, 2° 22' N, 102° 37' E, 1160 m elevation, lower montane, 18 October 1994 (fl.), *Mat Asri Ngah Sanah FRI 38678* (holotype KEP!).


**Figure 1:** *Ardisia ledangensis* sp. nov. **A.** Habit. **B.** The young leaves [Photos by Avelinah Julius].

**Description.** Shrub, 1.5–2.5 m tall, with slender stems descending, except erect in the new shoots, *c.* ≤ 1 cm in diam. *Indumentum* of simple, short, white hairs on the vegetative parts

when young. *Leaves* spirally arranged, petioles brown-red, (2–)4–10 mm long, slender, winged, sparsely hairy abaxially when young, glabrous when mature; lamina brownish red to dark green with pinkish margin when young, completely green except dark brown-red base when mature, subcoriaceous, narrowly elliptic, 3–10.5 × 0.6–2.5 cm, densely covered with prominent glandular-dots on both surfaces, base cuneate–attenuate, margins crenulate with 4–8 crenulations on each side with vascularised glandulars present on sinuses between crenatures, revolute, apex acute to acuminate, sometimes slightly caudate, with acumen 3–8 mm long, glabrous except in young leaves which are sparsely hairy beneath; midrib flat above, raised beneath; lateral veins distinct above, prominent below, 7–13 pairs, arching and joining towards margins to form intramarginal veins; intercostal veins obscure. *Inflorescences* subsessile, strictly terminal on lateral leafy branches, condensed, unbranched, racemose. *Flowers* 5–merous; pedicels 1.8–3 cm long, slender, glabrous; calyx lobes ovate, 1.8–2 × 1.2–1.5 mm, recurved downward in fruits, margins irregular, not ciliate, slightly overlapping at the base, glabrous but densely covered with black glandular-dots on both surfaces; corolla lobes lanceolate-ovate, *c*. 5 × 3 mm, margin hyaline, apex acute, glabrous but densely covered with black glandular-dots on both surfaces; stamens 5, subsessile, anthers lanceolate, *c*. 3 × 1 mm, connectives mucronate, thecae not locellate, dehiscent by longitudinal slits, densely covered with black glandular-dots abaxially; ovary subglobose, 1.3–1.5 × 1–1.3 mm, glabrous but covered with dense, black glandular-dots, ovules 2 arranged in 1-series, style 1.2–1.5 mm long with slender stigma. *Fruits* red (ripe), sub-globose, *c*. 1 × 1 cm, glabrous but rough, densely covered with black glandular-dots on the surface.

**Phenology: —** Based on the two available specimens, this species was observed in fruit in September, and in flower in October. The apparent sequence of fruiting prior to flowering may reflect incomplete sampling rather than the true phenological pattern. Additional collections will be necessary to clarify the flowering and fruiting chronology of this species

**Habitat: —** Lower montane forest on rocky soils. On Gunung Ledang, the plant was found near the summit, in a cool, humid, and shaded area.

**Etymology: —** *Ardisia ledangensis* is a highly localised species, currently only known from Gunung Ledang, which is the origin of its epithet.

**Provisional conservation status: —** Least Concern (LC). No threats have been identified for either the species or the habitat, i.e., there is no continuing decline in the extent of occurrence, area of occurrence, or habitat quality of this species. Additionally, Gunung Ledang is a National Park, a protected area. There is currently no information on the population size of this species as no species-specific surveys have been conducted in the area. Thus, the species is given a provisional assessment of Least Concern.

**Additional specimen examined: —** MALAYSIA. Peninsular Malaysia: Johor, Muar, Gunung Ledang FR, on the way to summit, 2° 22’ N, 102° 37’ E, 1234 m elevation, 9 September 2015 (fr.), *Julius et al. FRI 64039* (K; KEP!).

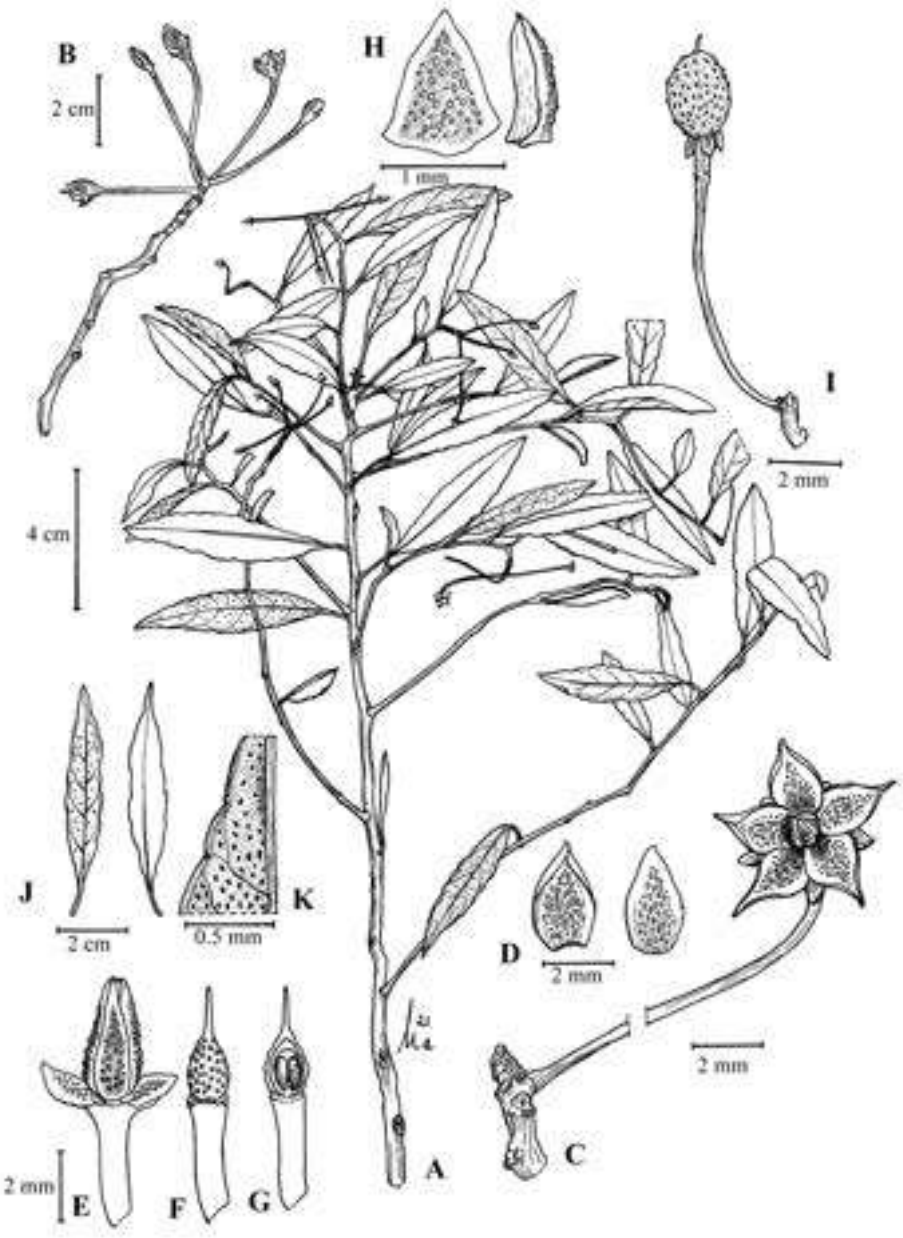


**Figure 2:** *Ardisia ledangensis.* **A.** Habit. **B.** Inflorescence. **C.** Flower with peduncle shown partially truncated to fit the plate layout. **D.** Corolla lobe, adaxial view (left), abaxial view (right). **E.** Stamen with corolla removed. **F.** Ovary. **G.** Ovary section showing the ovules. **H.** Calyx lobe, abaxial view (left), side-view (right) Vascularised glandulars on leaf margin. **I.** Fruit (young). **J.** Leaf, adaxial view (left), abaxial view (right). **K.** Lamina close-up showing leaf margin with vascularised glandulars and glandular-dots. [Drawn by Mohd Aidil Nordin].

## DISCUSSION

In Peninsular Malaysia, members of §*Crispardisia* can be classified into two informal groups based on the inflorescence position (Stone, 1989a, 1989b; Julius & Utteridge, 2021). The first group has inflorescences strictly terminal on lateral (axillary) branches, while the second group is characterised by inflorescences that are lateral and/or terminal on the main stem or lateral branches. *Ardisia ledangensis* falls into the first group because its inflorescences are strictly

terminal on lateral (axillary) branches. Within this group, *Ardisia ledangensis* is morphologically similar to the montane species *A. rosea* King & Gamble, with both having smaller leaves less than 11 cm long (rarely up to approximately 13 cm long in the latter). However, the new species differs in several characters: the stem grows descending (vs. erect), the lamina is subcoriaceous and narrowly elliptic (vs. coriaceous, rarely subcoriaceous with ovate to elliptic lamina), the petiole is subsessile, rarely up to 1 cm long (vs. distinctly petiolate, 0.8–1 cm long), and the inflorescence is unbranched (vs. 1–2 branched).

The new species also resembles *Ardisia vidalii* C.M.Hu var. *vidalii* in the shrubby habit, glabrous mature leaves, and dense black glandular-dots on the lamina and floral parts. However, when compared to *A. vidalii* var. *vidalii*, which is a small, erect shrub (0.7–1 m tall) with glabrous vegetative parts, the new species is a taller shrub (1.5–2.5 m tall) with simple white hairs on young vegetative parts. The new species also differs in the subsessile, condensed, unbranched racemes with pedicels 1.8–3 cm long, compared to the short-pedicellate (5–6 mm), simple subumbels of *A. vidalii* var. *vidalii*; in addition, only 2 ovules per ovary are seen in the new species, whereas they are 7–8 in *A. vidalii* var. *vidalii*.

The new species also slightly resembles *Ardisia corymbifera* Mez in its shrubby habit, narrowly elliptic, and glabrous mature leaves. However, *A. ledangensis* differs in several characters: its leaves are much smaller (3–10.5 × 0.6–2.5 cm), subcoriaceous, and the lamina is densely covered with prominent black glandular-dots on both surfaces, whereas those of *A. corymbifera* are larger (*c.* 14 × 3 cm), thinly chartaceous, and the lamina is sparsely covered with glandular-dots. The inflorescences of *A. ledangensis* are subsessile, condensed, unbranched racemes with long slender pedicels (1.8–3 cm), while *A. corymbifera* bears nodding, branched to 2-order, umbellate-corymbose inflorescences with shorter, stout pedicels (1–1.3 cm long). In addition, the ovary and fruit of *A. ledangensis* are rough, covered with dense, glandular-dots, while those of *A. corymbifera* are smooth and lacking glandular-dots.

Specimens of §*Crispardisia* are often misidentified as the common and weedy species *Ardisia crenata* Sims, particularly by collectors unfamiliar with the group. As a result, the folder of *A. crenata* at KEP contains a mix of material, including a flowering specimen of the new species described here. This could be attributed to the elliptic leaf shape exhibited by both *A. ledangensis* and *A. crenata*. However, the leaf lamina of the new species is densely covered with prominent, glandular-dots on both surfaces (rather than smooth in *A. crenata*), with few tiny vascularised glandular areas on the sinuses visible to the naked eye (compared to many, larger, and more obvious glandular areas in the latter), secondary veins that are laxly spaced, arching and joining towards the margins to form intramarginal veins (vs. closely spaced and spreading towards the margins in the latter), and rough fruits covered with dense black glandular-dots (compared to smooth fruits without glandular-dots in the latter). Through these comparisons, *A. ledangensis* could not be matched to any existing species known in Peninsular Malaysia and adjacent areas, hence it is described as a new species here.

*Ardisia* is recognised as a significant indicator of the quality of tropical and subtropical forests due to the specific habitats some species occupy (Julius et al., 2021). For example, *A. recurvipetala* is currently only found in unlogged areas within Taman Negeri Kenyir, Terengganu, indicating its role as a marker of primary forest within the reserve. Similarly, the newly identified *A. ledangensis* serves as an indicator of intact habitats in the upper montane forest of Gunung Ledang. Uncovering and formally describing a new species is vital for cataloguing local biodiversity, advancing knowledge on species distributions, and offering

indicators of ecosystem integrity, especially within the context of the ongoing revision of the family Primulaceae in the Flora of Peninsular Malaysia project.

This latest discovery brings the number of §*Crispardisia* species native to Peninsular Malaysia to 12. The key to the species of this subgenus for Peninsular Malaysia is provided below. Of these, five species, including the new one, are endemic to Peninsular Malaysia: *Ardisia lankawiensis* King & Gamble, *A. ledangensis*, *A. minor* King & Gamble, *A. recurvipetala*, and *A. recurvisepala* Julius & Utteridge. Gunung Ledang, also known as Mount Ophir, is one of the key habitats for such endemic species and is considered among the most well-documented botanical sites in Peninsular Malaysia (Ridley, 1901) . However, despite its extensive historical collections, new plant species continue to be discovered, highlighting the importance of ongoing fieldwork. These discoveries not only enrich our understanding of the flora in this region but also underscore the need for continued documentation of Malaysia's plant diversity (Ridley, 1901; Kiew, 2018; Nordin et al., 2021). Gunung Ledang is home to several other endemic species, including *Cycas cantafolia* Jutta, K.L.Chew & Saw, *Fordia ophirensis* Ridl., *Garcinia montana* Ridl., and *Jasminum ledangense* Kiew, further emphasising that even well-studied areas can still yield surprises.

**Key to *Ardisia* §*Crispardisia* in Peninsular Malaysia** (updated from Julius & Utteridge 2021)

1. Inflorescences strictly terminal on lateral leafy branches .....2
- Inflorescences lateral (axillary) and/or terminal on main stem or lateral branches .....6

2*. Lamina coriaceous or/rarely subcoriaceous, relatively small 2.5–10.5 cm long, rarely up to *c*. 13 cm long; ……..... 3
- Lamina chartaceous, rarely subcoriaceous, larger (7–)9–16(–18) cm long .....4

3. Lamina coriaceous, rarely subcoriaceous, ovate to elliptic, 2.5–6.5(–9) cm long, rarely up to *c*. 13 cm long, covered with dense black glandular-dots on both surfaces, prominent above, obscure beneath; inflorescences branched ..... **Ardisia rosea**
- Lamina subcoriaceous, narrowly elliptic, 3–10.5 cm long, covered with dense and prominent black glandular-dots on both surfaces; inflorescence unbranched…….**Ardisia ledangensis sp.nov.**

4.Inflorescences much branched, peduncle longer (1.5–)5 cm long…….....**Ardisia polysticta** Miq. (Miquel 1861: 576)
- Inflorescences usually unbranched, and if only 1–2 with peduncle(s), then these are less than 1.5 cm long .....5

5. Leaf apex obtuse, sometimes acute, secondary veins closely spaced …..... **Ardisia crenata**
- Leaf apex long acuminate to acuminate-caudate, secondary veins laxly spaced ..... **Ardisia ridleyi** King & Gamble (1906: 146)

6. Inflorescence strictly lateral (axillary) on main stem .....7
- Inflorescence lateral (axillary) and/or terminal on main stem or lateral branches …….....8

7. Lamina oblong-lanceolate, subcoriaceous, apex usually shortly acuminate rarely long and slightly caudate, 0.5–1(–2) cm long; pedicels longer and slender ..... **Ardisia sphenobasis** Scheff. (Scheffer 1867: 65)

- Lamina elliptic, coriaceous, apex long acuminate-caudate, 2–2.5 cm long; pedicels shorter and thicker ..... **Ardisia minor**

8. Inflorescence lateral (axillary) and terminal on lateral branches .....9
- Inflorescence lateral (axillary) on main stem and/or terminal on lateral branches …….....10

9. Leaf margin with vascularized glandulars without pustule-like structures along the crenations; corolla lobes spreading……..……..……. **Ardisia lankawiensis**
- Leaf margin with vascularized glandulars and pustule-like structures along the crenations; corolla lobes recurved downward…….……**Ardisia recurvipetala**

10. Lamina oblanceolate-elliptic, apex acuminate, densely, villous-pilose hairs on both surfaces; inflorescences terminal on lateral branches; hairs white ..... **Ardisia villosa**
- Lamina narrowly elliptic to oblong-elliptic, apex acuminate-caudate, lamina soon glabrescent except the midrib; inflorescences lateral (axillary) on main stem and terminal on short lateral branch; hairs rust coloured .....**Ardisia recurvisepala**

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## DECLARATIONS

**Research permit(s).** This study was conducted with approval from the Johor National Parks Corporation [Research Permit Ref. no: Penyelidikan/INV/2022/00005].
**Ethical approval/statement**. Not applicable.
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Total Phenolic, Total Flavonoid and Antioxidant Activities of *Durio graveolens* Becc. from Sabah, Malaysia

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## ABSTRACT

Sabah is home to diverse wild durian species, including the orange-fleshed durian (*Durio graveolens* Becc.), locally known as "dalit." Despite its prevalence, scientific data on this wild durian remains limited. This study aimed to characterise the phytochemical composition and antioxidant potential of *D. graveolens* fruit parts (flesh, seed, mesocarp, and exocarp). Freeze-dried samples were extracted using 80% methanol and 60% acetone, followed by qualitative phytochemical screening. Total phenolic and total flavonoid contents were quantified via the Folin-Ciocalteu and aluminium chloride colourimetric methods, respectively. Antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging assays, and ferric reducing antioxidant power (FRAP) assay. The 60% acetone extracts demonstrated superior phytochemical content and antioxidant activity compared to methanolic extracts. The mesocarp exhibited the highest total phenolic (76.64 ± 1.21 mg GAE/g, $p < 0.01$) and flavonoid (69.30 ± 0.69 mg CE/g, $p < 0.01$) contents, along with the strongest antioxidant activity (DPPH $IC_{50}$ = 70 µg/ml, $p < 0.01$; ABTS $IC_{50}$ = 50 µg/ml, $p < 0.01$; FRAP = 71.15 mg TE/g, $p < 0.01$). These findings highlight the mesocarp's potential as a natural antioxidant source with promising pharmaceutical applications.

**Keywords**: Antioxidant properties; phytochemical content; dalit; orange-fleshed durian.

## INTRODUCTION

Borneo is recognised as a biodiversity hotspot for the genus *Durio*. The most famous durian species is *Durio zibethinus* Murr., known for commercially cultivated and widely consumed fruits in Southeast Asia (Maninang et al., 2011). Due to its popularity, researchers are interested in exploring the properties of this species and its potential applications. For example, the volatile composition of several varieties of *D. zibethinus*' pulp was reported to contain compounds of esters, alcohols, a few aldehydes and sulphurs (Chawengkijwanich et al. 2008; Chin et al. 2007a; Chin et al. 2008b; Voon et al. 2007). Other researches include the antioxidant studies (Ashraf et al. 2010; Chingsuwanrote et al. 2016; Evary & Nur, 2018), anti-inflammatory (Chingsuwanrote et al., 2016), and several applications of *D. zibethinus* such as the potential of its seed as stabiliser in juice production (Herlina et al. 2016) and durian peels as new insulating particleboards in building insulation (Hirunlabh et al., 2003).

In Sabah, approximately 14 species out of 27 have been reported, including those that are popular edible types and lesser-known wild durian which are endemic to the region (Soegeng-Reksodihardjo, 1962; Nyffeler & Baum, 2001; Mursyidin et al. 2024). The most notable wild durian is *Durio graveolens* Becc., known locally in Sabah as 'Durian dalit' and commonly found in the local markets. It grows wild in Borneo, the Malay Peninsula, and Sumatra. Morphologically, it is smaller than the common *D. zibethinus*, with a thin to thick, vividly coloured pulp ranging from red to orange, and the fruit naturally opens when ripe (Soegeng-Reksodihardjo, 1962). This durian has a unique cheese-like texture with a sweet flavour (Sunaryo et al., 2016). Despite its ecological and economic value, scientific information on *D. graveolens* remains scarce (Nasaruddin et al. 2013; Sunaryo et al. 2016; Gaber et al. 2025). Existing studies have reported on fruit performance and its nutritional properties (Sunaryo et al., 2016), phylogenetic relationships in several *Durio* species (Kanzaki et al., 1998), proximate and fatty acid content (Nasaruddin, 2013), and antimicrobial properties against gram-negative bacteria (Gaber et al. 2025).

The consumption of fruits and vegetables has numerous health benefits due to the source of phytochemicals associated with a reduced risk of oxidative stress-related diseases (Kubola et al. 2011 & Chingsuwanrote et al. 2016). Antioxidants in fruits are generally linked to phenolic and flavonoid content, associated with its ability to scavenge radicals. Gorinstein et al. (2011) reported that some exotic Thai fruits exhibited high antioxidant properties in DPPH assay, strongly correlated with the high total polyphenolic content in the fruits. Similarly, several underutilised Malaysian fruits were also reported to possess remarkable radical scavenging activity due to high phenolic constituents (Ikram et al., 2009).

The present study aimed to evaluate phytochemical content by quantifying the total phenolic and flavonoid contents of 80% methanol and 60% acetone extracts of different parts of durian fruit. Antioxidant potential of orange-fleshed *D. graveolens* were evaluated through three assays: 2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH), 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation assay (ABTS) and ferric reducing antioxidant power assay (FRAP). This research contributes new insights into antioxidant potential of *D. graveolens* as valuable natural sources of bioactive compounds with their antioxidant capabilities. Furthermore, this research enhances the scientific understanding of this wild durian species and lays the foundation for its sustainable use, conservation, and potential application in future nutraceutical or propagation efforts.

## METHODOLOGY

### Sample collection and preparation

The orange-fleshed *D. graveolens* was bought from the local market at Tamparuli, Sabah (6°8'3"N, 116°16'4"E). The sample selection was based on the colour of the fruit, size consistency, shape and flesh colour with preference given to fruits exhibiting minimal natural opening upon ripening (Fig. 1). The sample was verified by Mr Joel bin Dawat from the Systematic Botanic Section, Sepilok Forest Research Centre, Sabah (5° 52' 26.3" N, 117° 56' 59.1" E). The herbarium specimen (BORH 3011) was deposited in the BORNEENSIS, Herbarium, Universiti Malaysia Sabah. The samples were cleaned and separated into flesh, seed, mesocarp and exocarp. The sample parts were stored at −80 °C before being freeze-dried for five days. The freeze-dried samples were ground into a fine powder using a Waring blender (Waring, Japan) and stored at −80 °C until further use.

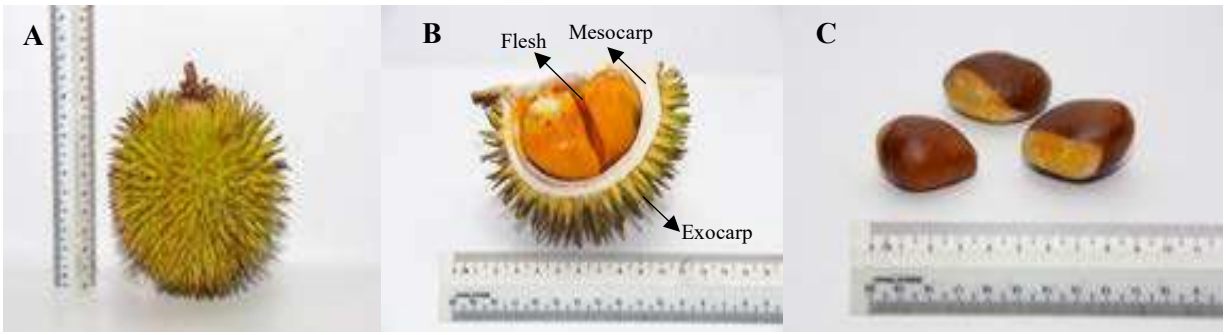


**Figure 1:** *Durio graveolens*. **A.** Irregular round-shaped with green to yellowish spine, 9–12 cm long. **B.** Durian slice with 10–15 cm wide covering the orange-coloured pulp with labelled flesh, mesocarp and exocarp. **C.** Glossy, dark brown seeds with 3–5 cm length.

### Sample extraction

The flesh, seed, mesocarp and exocarp were extracted using 80% methanol and 60% acetone in the ratio of 1:20 (w/v). The vials were agitated for two hours at 200 rpm using an orbital shaker. The solution was filtered through Whatman No. 5 filter paper, and the supernatant was collected in a 20 ml vial wrapped in aluminium and stored at −80°C. Phytochemical contents and antioxidant activity assays were determined in the extracts.

### Total phenolic content (TPC)

The Folin-Ciocalteu method from Muhtadi and Ningrum (2019) was used to measure total phenolics, with some modifications in the sample-to-reagent ratio. The sample extracts (10 µl, 50 mg/mL) were added into a 96-well plate containing 75 µl Folin-Ciocalteu reagent (10%) and incubated in a microplate reader (Thermo Scientific, USA) for 5 minutes before the addition of 75 µl of sodium carbonate solution ($Na_2CO_3$, 6%) into the well plate. The well plate was incubated in the dark for 90 minutes and the absorbance of TPC was measured at 725 nm using a microplate reader (Thermo Scientific, USA). Gallic acid, in the concentration range from 0.05 to 0.25 mg/ml was used as a reference standard. The TPC was expressed as milligrams of gallic acid equivalent (mg GAE) per gram of dry sample, as indicated in equation (1).

$$\text{Gallic acid equivalent (mg/g)} = C1 \times V/m \quad (1)$$

Where C1 represents concentration from gallic acid standard curve (mg/ml), V is the extract volume used in this assay (ml), and m is the dry weight of extract (g).

**Total flavonoid content (TFC)**

The TFC was determined based on the colourimetric method with a slight modification in the sample-to-reagent ratio (Muhtadi & Ningrum, 2019). In this assay, 100 µl of the sample extract (50 mg/ml) was mixed with 400 µl distilled water in a 2 ml centrifuge tube. Subsequently, 60 µl of 5% sodium nitrite ($NaNO_2$), 30 µl of 10% aluminium chloride ($AlCl_3$), and 200 µl of 1 M sodium hydroxide (NaOH) were added sequentially and the tube was shaken for 5 minutes after adding each reagent. The tube was incubated in the dark for 30 minutes to allow colour development. A total of 200 µl was added into 96-well plate and the absorbance of TFC was measured at 420 nm using a microplate reader (Thermo Scientific, USA). Catechin was used as the reference standard with a calibration curve prepared using a concentration range of 0.05–0.4 mg/ml. The results were expressed as mg catechin equivalent per gram of dry sample, based on equation (1).

**2,2-diphenyl-1-picrylhydrazyl radical assay (DPPH)**

The method outlined by Wang and Li (2014) was used to test the extracts' scavenging ability against the DPPH radical. The extract was serially diluted to concentrations of 7.8, 15.6, 31.25, 62.5, 125, 250, 500 and 1000 µg/ml. A total of 100 µl of each sample extract was mixed with 100 µl of 0.1 mM DPPH working solution in a 96-well plate and incubated in the dark for 30 minutes. The absorbance was measured at 519 nm using a microplate reader (Thermo Scientific, USA). The DPPH scavenging percentage was calculated according to equation (2), and results were expressed in $IC_{50}$ value (concentration of sample able to scavenge 50% of the DPPH free radical). Trolox was used as positive control in this assay.

$$DPPH\ scavenging\ activity\ (\%) = 1 - (\frac{Sample\ reading - Empty\ sample\ reading}{DPPH\ reading - Blank\ reading})\ X\ 100 \qquad (2)$$

**2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation assay**

ABTS assay was carried out using the method outlined by Wang and Li (2014). The ABTS reagent was prepared by reacting 15 ml of ABTS solution (7.4 mM) with 264 µl potassium persulphate ($K_2S_2O_8$, 2.6 mM). The solution was kept at room temperature for 24 hours in the dark. The working solution was diluted, and absorbance was measured to obtain a value of 0.70 ± 0.02. The ABTS working solution (100 µl) was added to each well containing serially diluted sample extracts (7.8–1000 µg/ml) and incubated in the dark for 30 minutes. A microplate reader (Thermo Scientific, US) was used to measure the absorbance at 734 nm. The results were expressed in $IC_{50}$ (concentration of the extracts capable of scavenging 50% of the ABTS radical). Trolox was used as positive controls in this assay.

**Ferric reducing antioxidant power assay (FRAP)**

This procedure was performed according to Abu Bakar et al. (2009). The FRAP reagent was freshly prepared by mixing acetate buffer (300 mM, pH 3.6), 2,4,6-tris(2-pyridyl)-1,3,5-triazine solution (TPTZ, 10 mM), and ferric chloride hexahydrate ($FeCl_3.6H_2O$, 20 mM). The sample extracts (50 mg/ml, 20 µl) were added into the 96-well plate containing 180 µl FRAP reagent and incubated for 30 minutes in the dark. The absorbance was measured at 593 nm, and the results were expressed as milligrams of Trolox equivalent per gram of dried sample (mg TE/g), based on equation (3).

FRAP values (C) = C1 x v/ M (3)

Where C = total FRAP content in mg TE/g, C1 = concentration of trolox obtained from the calibration curve in mg/ml, V= volume of extract in ml, and m = the weight of the sample in g.

**Statistical analysis**

All the experiments were carried out in triplicate. The mean data were displayed as means ± standard deviations and statistically assessed using multiple variance analysis (two-way ANOVA) using Tukey's test in SPSS version 20.0 to evaluate the effects of sample part and solvent system, as well as their interaction ($p < 0.01$). Pearson's correlation coefficients were used to analyse the associations between the antioxidant activities of the three independent tests (DPPH, ABTS, and FRAP) and phytochemical content (TPC and TFC).

## RESULTS

**Total phenolic content (TPC)**

The TPC of the extracts exhibited significant variation, ranging from 0.96 to 43.10 mg GAE/g for 80% methanolic extracts and 1.33 to 76.64 mg GAE/g for 60% acetone extracts (Fig. 2). The non-edible parts of both solvent extractions displayed higher TPC than the flesh part. The 60% acetone extracts of seed, mesocarp and the exocarp exhibited higher TPC values compared to the 80% methanolic extracts ($p < 0.01$). The highest TPC value was demonstrated by the mesocarp extracted using 60% acetone ($p < 0.01$), followed by the 80% methanol extracts with 76.64 ± 1.21 and 43.10 ± 0.9 mg GAE/g dried sample, respectively. The two-way ANOVA revealed significant effects of the sample parts and the solvent extractions on total phenolic content.

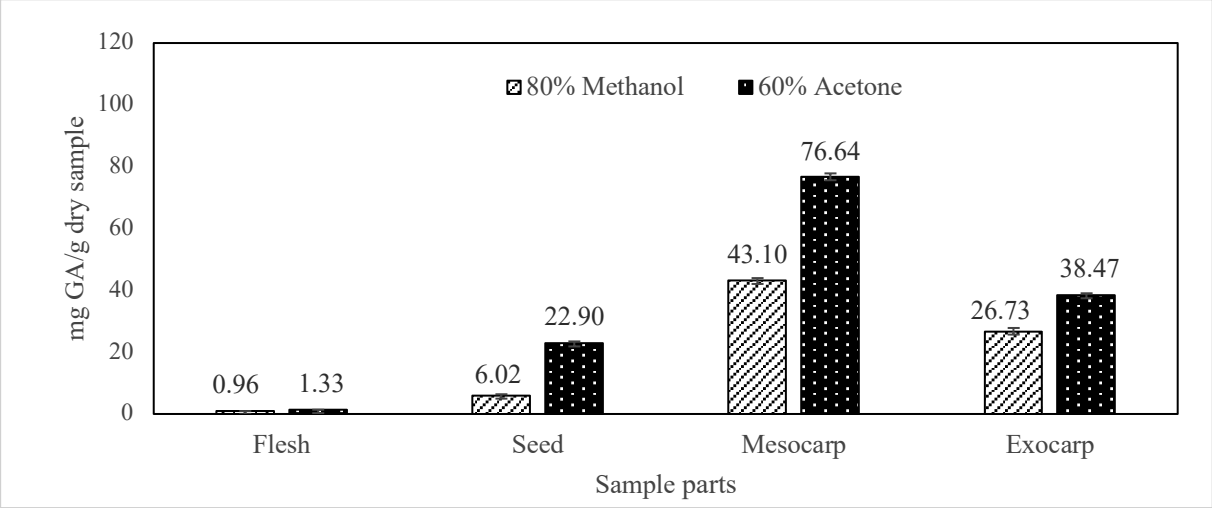


**Figure 2:** Total phenolic content in orange-fleshed *D. graveolens* (mg Gallic acid in 1 gram of dry sample). The data analysis involved using a two-way ANOVA with two factors: four sample parts and the different solvent extraction methods. (All significant at p <0.01 level).

**Total flavonoid content (TFC)**

The total flavonoid content (TFC) of *D. graveolens* extracts varied significantly among fruit parts, ranging from 0.06 to 42.90 mg CE/g for 80% methanol extracts and 0.56 to 69.30 mg CE/g for 60% acetone extracts (Fig. 3). Consistent with the total phenolic content results, the

mesocarp exhibited the highest flavonoid content (69.30 ± 0.69 mg CE/g in 60% acetone; 42.90 ± 1.93 mg CE/g in 80% methanol, $p < 0.01$), followed by exocarp > seed > flesh. A two-way ANOVA demonstrated significant main effects of fruit part and extraction solvent on the total flavonoid content ($p < 0.01$), with notable differences among the flesh, seed, mesocarp and exocarp, as well as between 80% methanol and 60% acetone extracts.

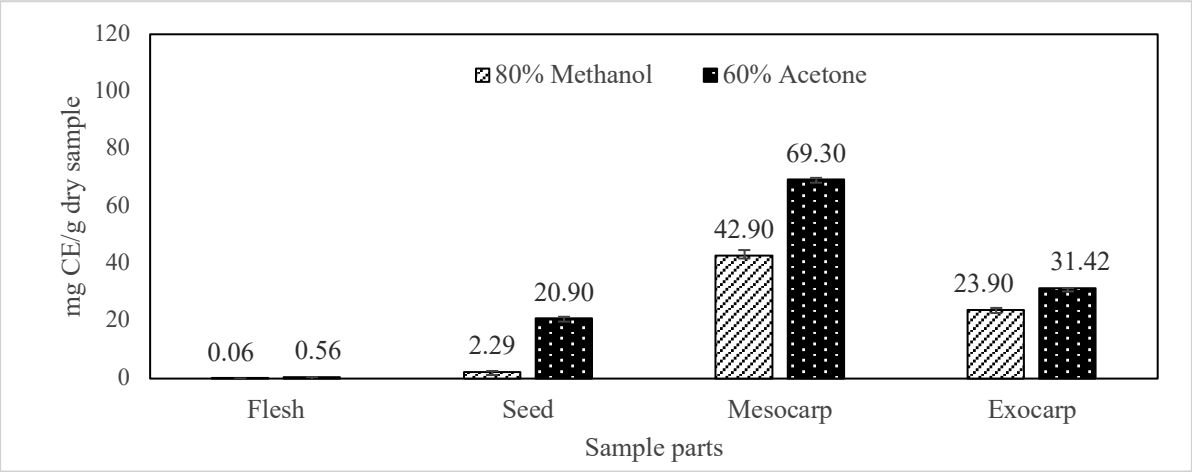


**Figure 3:** Total flavonoid content in orange-fleshed *D. graveolens* (mg Catechin in 1 gram of dry sample). The data analysis involved using a two-way ANOVA with two factors: four sample parts and the different solvent extraction methods. (All significant at p <0.01 level).

## Antioxidant activity (DPPH and ABTS assay)

The antioxidant capacity of *D. graveolens* extracts was quantified through DPPH and ABTS radical scavenging assays, expressed as $IC_{50}$ values (concentration required to inhibit 50% of radicals; Table 1). The $IC_{50}$ values ranged from 70.4 to 2511.1 µg/ml for DPPH and 50.0 to 2228.4 µg/ml for ABTS assay across all sample extracts. The flesh part exhibited the highest $IC_{50}$ compared to the non-edible parts, with mesocarp displayed the lowest values in both solvent extractions. The 60% acetone extract of the mesocarp demonstrated the strongest antioxidant capacity, with 70.4 ± 1.6 µg/ml and 50.0 ± 1.3 µg/ml in the DPPH and ABTS assays, respectively ($p$ <0.01).

**Table 1:** $IC_{50}$ values of 80% methanol and 60% acetone extracts from different fruit parts of *Durio graveolens* evaluated using DPPH and ABTS radical scavenging assays.

| Solvent extraction | Fruit' part | $IC_{50}$ DPPHc(µg/ml) | $IC_{50}$ ABTS (µg/ml) |
|---|---|---|---|
| 80% Methanol | Flesh | 2511.1 ± 79.8 | 2228.4 ± 38.6 |
| | Seed | 662.2 ± 120.7 | 543.9 ± 80.4 |
| | Mesocarp | 116.8 ± 10.7 | 66.3 ± 1.2 |
| | Exocarp | 196.5 ± 12.4 | 133.2 ± 11.0 |
| 60% Acetone | Flesh | 2034.8 ± 234.1 | 1753.2 ± 61.0 |
| | Seed | 218.8 ± 14.7 | 195.3 ± 23.2 |
| | Mesocarp | 70.4 ± 1.6 | 50.0 ± 1.3 |
| | Exocarp | 175.9 ± 6.8 | 106.5 ± 7.8 |
| Trolox | Trolox | 5.2 ± 0.6 | 5.5 ± 0.1 |

*Notes: Data represent mean ± standard deviation (n=3). Trolox was used as the positive control. The data analysis involved using a two-way ANOVA with two factors: four sample parts and the different solvent extraction methods. (All significant at p <0.01 level).

**Ferric reducing antioxidant power assay (FRAP)**

The reducing capacity of *D. graveolens* extracts, as determined by FRAP assay, demonstrated significant variation among fruit parts (Fig. 4). The 60% acetone extracts exhibited FRAP values ranging from 1.72 to 71.15 mg TE/g, while the 80% methanolic extracts showed values between 1.50 and 59.96 mg TE/g. The non-edible parts exhibited higher FRAP values as compared to the flesh parts in 80% methanol and 60% acetone extracts. The mesocarp displayed highest FRAP values, suggesting strongest antioxidant activity in 60% acetone (71.15 ± 0.41, $p < 0.01$) and 80% methanol extracts (59.96 ± 1.03 mg TE/g, $p < 0.01$), respectively.

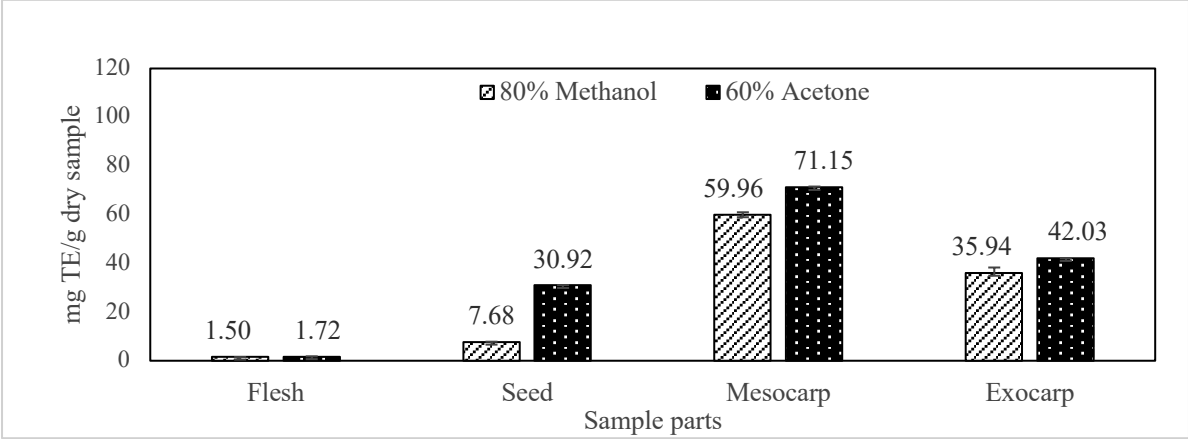


**Figure 4:** Ferric reducing antioxidant power assay in orange-fleshed *D. graveolens* (mg Trolox per 1 gram of dry sample). The data analysis involved using a two-way ANOVA with two factors: four sample parts and the different solvent extraction methods. (All significant at p <0.01 level).

**Correlation between phytochemical content and antioxidant activities**

Pearson correlation analysis revealed significant relationships between phytochemical composition and antioxidant capacity (Table 2). Total phenolic content (TPC) and total flavonoid content (TFC) both showed very strong correlations with FRAP (r = 0.987 and r = 0.983, respectively; $p < 0.01$). In contrast, significant inverse correlations were observed between TPC and TFC with $IC_{50}$ values of DPPH and ABTS assays ($r < -0.90$, $p < 0.01$).

**Table 2:** Pearson's correlation coefficients of TPC and TFC versus DPPH, ABTS and FRAP assay.

| Phytochemical content | DPPH [2] | ABTS | FRAP |
|---|---|---|---|
| TPC [1] | -0.990* | -0.992* | 0.987* |
| TFC | -0.987* | -0.989* | 0.983* |

[1] TPC; Total phenolic content, TFC; total flavonoid content.
[2] DPPH; 2,2-diphenyl-1-picrylhydrazyl radical assay, ABTS; 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) radical cation assay, FRAP; Ferric reducing antioxidant power assay.
*Correlation is significant at the 0.01 level (2-tailed).

## DISCUSSION

**Phytochemical content of *D. graveolens* (TPC and TFC)**

The 60% acetone extraction yielded significantly higher TPC values in the non-edible parts (seed, mesocarp, and exocarp) compared to 80% methanolic extracts. This finding aligns with

previous studies demonstrating the higher extraction efficiency of acetone/water mixtures for phenolic compounds in various plant materials, such as in *Macadamia tetraphylla* L.A.S. Johnson (Dailey & Vuong, 2015), *Lippia javanica* Spreng. (Bhebhe et al., 2016), *Eucalyptus* leaves (Nasr et al., 2019), and brewer's spent grains (Meneses et al., 2013). The higher extraction efficiency observed with acetone may result from its intermediate polarity, as it allows for more effective extraction of varied phenolic constituents than methanol.

The flesh extracts showed consistently low TPC values (2.1–3.8 mg GAE/g) between solvent extraction. Consistent with our findings, Abu Bakar et al. (2015) reported that phenolic compounds tend to accumulate in the outer parts as a defence mechanism against pathogens and predators in *Artocarpus* species. Low TPC values have also been observed in the flesh parts of several *Artocarpus* species (Abu Bakar et al., 2015) and Ceri Terengganu (Looi et al., 2020) when compared to their non-edible parts. The differential phenolic distribution can be explained by their physiological roles in plant defence mechanisms. Phenolic compounds serve as both natural pesticides and protective agents against oxidative stress induced by UV radiation (Osorio-Esquivel et al., 2011). Furthermore, they contribute to structural integrity in plant cell walls. As the outer parts are directly exposed to environmental stressors including sunlight, pathogen attack, and physical damage, they typically exhibit higher phenolic biosynthesis compared to the protected inner flesh (Abu Bakar et al., 2015; Looi et al. 2020). This defence-related metabolic investment explains the significantly higher phenolic content observed in the non-edible portions of *D. graveolens*.

The mesocarp (inner peel) of durian exhibited higher phytochemical content than the exocarp. This may be due to the exposure of the outer peel (exocarp) to direct sunlight, temperature fluctuations, and mechanical injury, which disrupt cellular integrity and promote the degradation of bioactive compounds (Feng et al., 2022). Another factor is prolonged post harvest exposure, which can accelerate oxidative loss of phenolics and other secondary metabolites, while the surface is highly susceptible to microbial colonisation that can metabolise or transform native phytochemicals (Narra et al., 2023; ShivShankar et al., 2024; Rawson et al., 2011). In contrast, the mesocarp, being more shielded from light and microbial attack, can better preserve its phenolics and flavonoids, as also observed in Malaysian *Durio zibethinus* mesocarp (Noorhashim et al., 2025).

The 60% acetone extraction demonstrated superior efficacy for flavonoid recovery compared to 80% methanol, consistent with previous reports for brewer's spent grains (Meneses et al., 2013) and *Scurrula ferruginea* (Roxb. ex Jack) Danser leaves (Justine et al., 2019). This enhanced extraction efficiency likely stems from acetone's intermediate polarity, which facilitates solubilisation of diverse flavonoid compounds. Notably, the flesh portion showed consistently low flavonoid content (0.56–2.15 mg CE/g) regardless of solvent system. These findings suggest that flavonoid accumulation patterns in fruit tissues are conserved across species, with protective outer tissues typically containing higher concentrations than edible flesh portions.

**Antioxidant activities (DPPH, ABTS and FRAP assays)**

The mesocarp with 60% acetone extract demonstrated the strongest activity, with $IC_{50}$ values of 70.4 ± 1.6 µg/ml (DPPH) and 50.0 ± 1.3 µg/ml (ABTS), consistent with its high phenolic and flavonoid contents. This enhanced antioxidant capacity likely results from synergistic interactions among its phytochemical constituents. Comparative analysis revealed superior radical scavenging activity in our samples relative to other *Durio* species. The ethyl acetate extract of *D. kutejensis* (Hassk.) Becc. fruit showed higher $IC_{50}$ values (97.4 µg/ml DPPH;

100.8 µg/ml ABTS) (Arung et al., 2015). Similarly, methanol extracts of *D. zibethinus* peel exhibited reduced activity ($IC_{50}$ = 102.37 ± 1.98 µg/ml) despite containing 33.77 ± 1.77 mg GAE/g phenolics (Wang & Li, 2014). Ethanol extracts of *D. zibethinus* cultivars (Medan and Monthong) displayed intermediate activity (78.83 ± 1.67 and 72.77 ± 6.60 µg/ml, respectively; Muhtadi & Ningrum, 2019).

Consistent with our DPPH and ABTS results, the mesocarp displayed the highest ferric reducing antioxidant power (71.15 ± 0.40 mg TE/g in acetone; 59.96 ± 1.03 mg TE/g in methanol, $p < 0.01$), followed by exocarp > seed > flesh. This tissue-specific pattern correlates with the observed phenolic and flavonoid distribution, explaining the notably lower antioxidant capacity in the flesh portion. These findings align with previous reports on *Myristica fragrans* Houtt., where the seed demonstrated superior reducing activity compared to the flesh (Assa et al., 2014). The redox properties of phenolic compounds, as described by Rice-Evans et al. (1997), provide a mechanistic basis for these observations. Phenolics function as effective antioxidants through multiple pathways: (1) serving as reducing agents, (2) donating hydrogen atoms, and (3) quenching reactive oxygen species. The variation in FRAP values across fruit parts reflects differences in both the concentration and redox potential of their constituent phytochemicals (Nasr et al. 2019).

**Influence of total phenolic and total flavonoid content on antioxidant performance**

Strong associations were observed between total phenolic and flavonoid content and ferric reducing antioxidant power, indicating that samples richer in phenolics exhibited greater reducing capacity. These findings align with previous studies reporting similar correlations in *Lepidium meyenii* Walp. (r = 0.941, $p < 0.01$; Gan et al., 2017) and *Eucalyptus camaldulensis* Dehnh. (r = 0.985, $p < 0.01$; Nasr et al., 2019) extracts. Additionally, an inverse relationship was noted between phytochemical content and $IC_{50}$ values in both DPPH and ABTS assays. These results demonstrate that extracts with higher phenolic and flavonoid concentrations require lower doses to achieve 50% radical scavenging, consistent with the findings of Evary et al. (2019). The observed patterns support the established mechanism wherein antioxidant efficacy is directly proportional to phenolic concentration, which function as hydrogen donors to neutralise free radicals and mitigate oxidative stress (Evary et al. 2019).

## CONCLUSIONS

This study evaluated the phytochemical composition and antioxidant potential of 80% methanolic and 60% acetone extracts from different parts of *D. graveolens* fruit. The findings showed that the mesocarp contained significantly higher levels of total phenolic and flavonoid compounds and exhibited stronger antioxidant activity in FRAP, DPPH, and ABTS assays compared to other parts of the fruit. A strong correlation between these compounds and antioxidant activity suggests they are the main contributors to the observed effects. The high antioxidant capacity of the mesocarp extracts points to their potential as natural sources for pharmaceutical, nutraceutical, and cosmetic applications. These results also support the traditional use of *D. graveolens* and provide useful information for developing value-added products from this under-utilised durian species. Further research should aim to isolate the key active compounds, examine their bioavailability, and investigate their health benefits through *in vivo* studies.

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## DECLARATIONS

**Research permit(s).** JKM/MBS.1000-2/2 JLD.10-45. Sabah Biodiversity Council.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** The authors declare that generative AI has been used in compliance with the JTBC policies, and that we have reviewed and edited the content after using this tool/service and we take full responsibility for the content of the publication.

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**Short Note**

# Differences in Seed Germination and Seedling Survival of Selected Dipterocarpaceae Species Collected from Contrasting Forest Types in Brunei Darussalam

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**Keywords**: Borneo; endemic; *ex-situ* conservation; heath forest; Kerangas; lowland rain forests.

The Dipterocarpaceae family is prevalent in the lowland forests of Borneo, accounting for 21.9% of all tree species in these forests (Slik et al., 2003; Atmoko et al., 2025). This species-rich family occurs as trees that dominate the canopy and emergent layers (Ghazoul, 2016) and are important to the ecological functioning of Bornean forests. One key characteristic of the Dipterocarpaceae family is mass flowering and mast fruiting, which occurs at irregular intervals ranging from two to ten years (Appanah, 1993). During these events, Dipterocarps produce seeds that are predominantly gravity-dispersed, resulting in short seed dispersal distances, usually near parent trees (Osada et al., 2001). The seeds of the Dipterocarpaceae family are recalcitrant (Ekasari & Oktaviani, 2024), able to germinate quickly after dispersal, but are typically not viable for long periods in the seed bank due to their high-water content and sensitivity to desiccation (Tweddle et al., 2003). The biological traits of Dipterocarpaceae, including irregular fruiting, short seed dispersal distances, and recalcitrant seeds, limit their natural regeneration potential. When compounded by external threats such as logging and deforestation, populations may fail to recover adequately, leading to long-term declines (Pang et al., 2021).

Of the 162 Dipterocarpaceae species native to Borneo, 99 species are designated as either Vulnerable, Endangered, or Critically Endangered in the IUCN Red List (2025). To date, only 32 threatened Dipterocarpaceae family species are protected *ex-situ* (Bartholomew et al., 2021). *Ex-situ* conservation of Dipterocarpaceae species is done by collecting recalcitrant seeds and seedlings from forests after masting events and growing them outside of their natural habitats (Ghazoul, 2016). This strategy helps protect threatened tree species by minimizing the environmental impacts caused by overexploitation and loss of their natural habitat (Susilowati et al., 2021). Successful *ex-situ* conservation programs can also produce seedlings or saplings that can eventually be replanted into natural habitats to reforest degraded areas (Mestanza-Ramón et al., 2020). However, successful *ex-situ* conservation efforts require information on seed and seedling growth to enable appropriate treatments to be developed.

The present study focuses on the assessment of seeds and seedlings survival of selected Dipterocarpaceae species in Brunei Darussalam, Northwest Borneo. The lowland forests in Brunei Darussalam are dominated by the Dipterocarpaceae family (Sukri et al., 2012) with 153 species (Ashton, 1964). The country retains a high percentage of intact forests, particularly lowland mixed dipterocarp forests (Bryan et al., 2013). This provides an ideal setting to collect seeds and seedlings by the Botanical Research Centre, Universiti Brunei Darussalam (UBD BRC) for *ex-situ* conservation efforts. This pilot study aims to evaluate *ex-situ* seed germination and seedling survival of selected Dipterocarpaceae species collected from three contrasting forest types, i.e. mixed dipterocarp, heath and peat swamp forests, in Brunei Darussalam. We chose the Dipterocarpaceae family due to its ecological importance and diversity in Bornean forests, where it is also facing increased anthropogenic threats that necessitates urgent conservation action. Our primary research question was how do seed germination and seedling survival rates vary among selected Dipterocarpaceae species and across different forest types in Brunei Darussalam? We hypothesised that (1) species-specific differences in seed germination and seedling survival will be recorded, and (2) seedling survival will differ between the three forest types. Knowledge gaps in seed germination and seedling survival of these high conservation value species to enhance their *ex-situ* conservation strategies still exist. Through this pilot study, our goal is to present much needed species-specific information to better tailor species recovery and forest restoration efforts across the region.

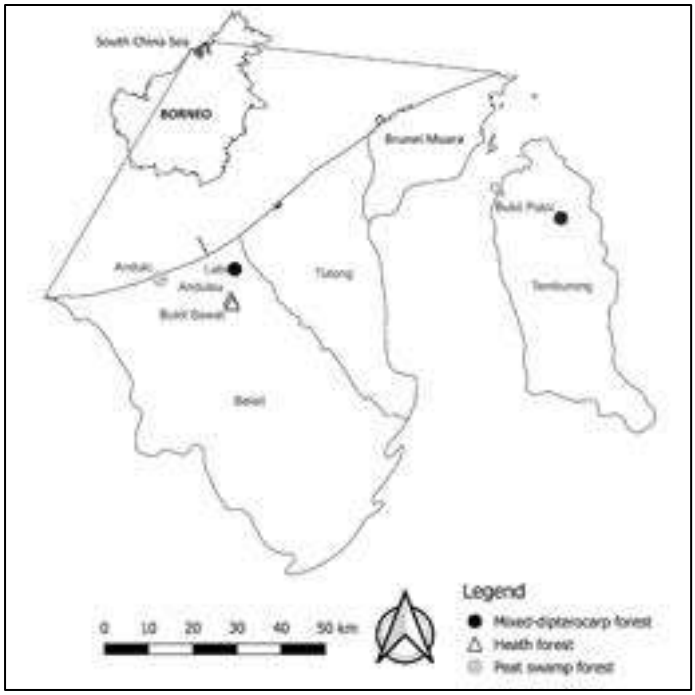


**Figure 1:** Location of the study sites consisting of five different locations in three forest types within the Belait and Temburong districts, Brunei Darussalam.

The study was conducted in five locations within the Belait and Temburong districts of Brunei Darussalam, encompassing three distinct forest types: mixed dipterocarp forest (MDF), heath forest (HF), and peat swamp forest (PSF; Fig. 1). All locations consisted of intact, primary forests: Bukit Patoi (4°45'0.60"N 115°11'0.04"E) in Temburong and Labi

(4°27'34.13"N 114°28'36.20"E) in Belait are MDFs, Andulau (4°36'18.69"N 114°30'30.41"E) and Bukit Sawat (4°34'18.10"N 114°30'42.91"E) are both HFs, and Anduki (4°37'32.57"N 114°21'53.45"E) is a PSF site.

At each of the five study locations, intensive surveys for Dipterocarpaceae seeds and seedlings were conducted along an accessible forest trail covering a distance of 2.0 km per location throughout May 2021 during a localised masting episode. Surveys were conducted with the assistance of botanists from the Brunei National Herbarium (BRUN).

From the surveys, two Dipterocarpaceae species were recorded as seeds: *Dipterocarpus borneensis* Slooten and *Dryobalanops rappa* Becc., and five species were recorded as seedlings: *Cotylelobium burckii* (F.Heim) F.Heim, *Hopea pentanervia* Symington ex G.H.S.Wood, *Hopea vacciniifolia* Ridl. Ex P.S.Ashton, *Richetia laxa* (Slooten) P.S.Ashton & J.Heck., and *Rubroshorea scaberrima* (Burck) P.S.Ashton & J.Heck. Field identification of these species was conducted by locating and identifying the parent trees, with the support of botanists from BRUN. Voucher specimens from the parent trees were also collected, and taxonomic identification was confirmed by cross-checking with BRUN collections, including type specimens where available. Representative photographs of all the seven species collected are provided in Fig. 2.

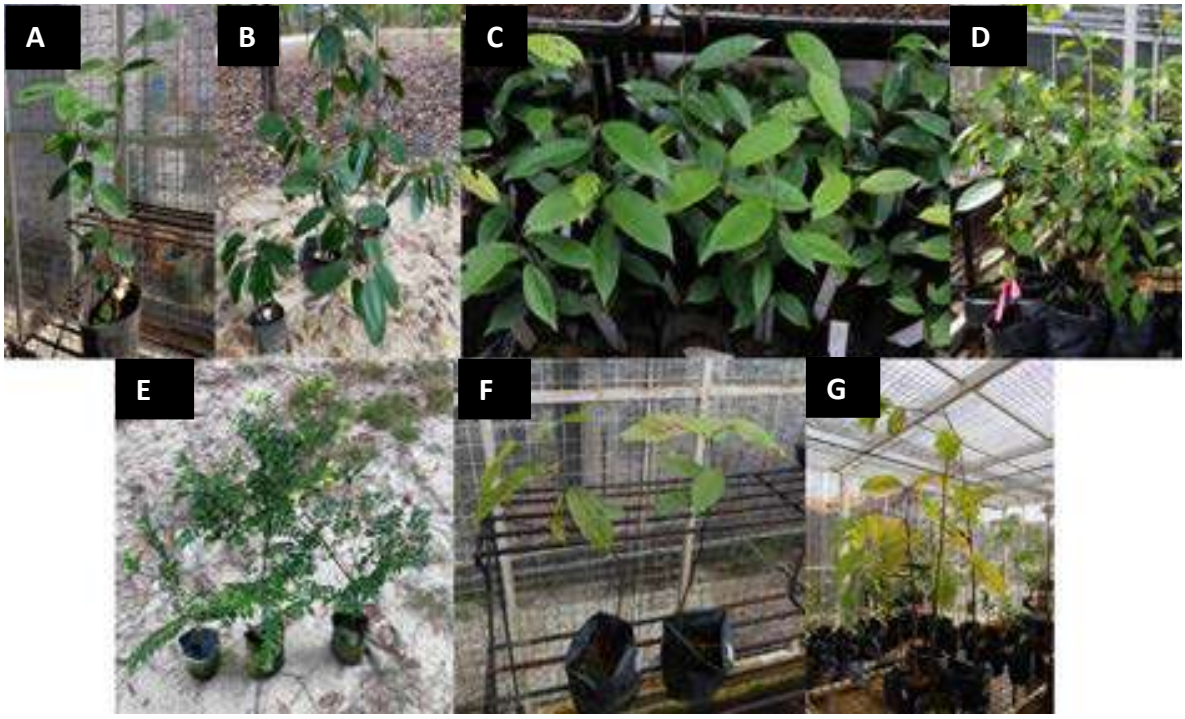


**Figure 2:** Representative photographs of the seven Dipterocarpaceae species collected in this study. The images show seedlings of: **A.** *Cotylelobium burckii*, **B.** *Dipterocarpus borneensis*, **C.** *Dryobalanops rappa*, **D.** *Hopea pentanervia*, **E.** *Hopea vacciniifolia*, **F.** *Richetia laxa*, and **G.** *Rubroshorea scaberrima.*

Seeds of *D. borneensis* and *D. rappa* were collected from among undamaged and non-germinating winged seeds on the forest floor within an area of 4 $m^2$ under at least three parent trees. This standardised plot size was chosen to ensure consistency in sampling effort and spatial coverage across all sites. Falling seeds were also obtained by shaking the tree branches following Velazco et al. (2018). Seedlings (height of 10 – 80 cm only) of *C. burckii*,

*H. pentanervia*, H. *vacciniifolia*, *R. laxa* and *R. scaberrima* were collected within an area of 4 $m^2$ of at least three parent trees. Seedling heights were decided based on advice from the Brunei Forestry Department and was chosen to target healthy, naturally regenerating individuals that had progressed beyond the earliest seedling stage but had yet to reach the sapling phase. Selecting seedlings within this range ensured they were robust enough to tolerate transplanting stress during *ex-situ* relocation, while still representing the early growth stages appropriate for survival monitoring. Individual seedlings were extracted with care from the forest floor, ensuring the root system and root ball remained intact, following Susilowati et al. (2021).

The collected seed count comprised 153 seeds of *D. borneensis* from Labi (MDF) site and 306 seeds of *D. borneensis* from Bukit Sawat (HF) site, and 231 seeds of *D. rappa* collected from Anduki (PSF) site. For seedlings, a total of 40 *C. burckii* and 80 *H. vacciniifolia* were collected from Labi (MDF) site, 44 *R. laxa* were collected from Bukit Patoi (MDF) site, 51 *H. pentanervia* were collected from Bukit Sawat (HF) site, and 43 *R. scaberimma* were collected from Andulau (HF) site.

The collected seeds and seedlings were sorted according to species and carefully placed in labelled, resealable bags with moisture to preserve their condition. Seedlings were tagged with labels attached to their stems, indicating their species codes and respective collection sites. To minimize transpiration, about two-thirds of each leaf on all seedlings was carefully trimmed (Papuangan et al., 2014). Seeds and seedling collections were conducted in the mornings (from 8.00 am to 11.00 am) and all collected seeds and seedlings were transported on the same day to the UBD shade house for planting and re-potting.

For re-potting purposes in the shade house, forest soils from the same location as the parent tree species were also collected. General soil characteristics of the three forest types are summarised in Table 1 to provide environmental context for the forest soils from each forest type. Approximately 50 kg of forest soil within an area of 4 $m^2$ from each parent tree per location was collected using shovels. Forest floor litter was first removed by hand and an area of 1 $m^2$ was excavated for soil collection to a depth of 15 cm from the topsoil. Collected soils were then placed into appropriately labelled large bags, corresponding to their respective collection site.

**Table 1:** General soil characteristics of the three forest types studied in Brunei Darussalam (adapted from Jaafar et al., 2016).

| Forest type | Soil Texture | pH Range | Nutrient Availability | Water-Holding Capacity |
|---|---|---|---|---|
| Mixed dipterocarp forest (MDF) | Well-drained | 5.0 – 5.5 | Moderate | Good |
| Heath forest (HF) | Sandy | ~4.0 – 4.5 | Low | Low |
| Peat swamp forest (PSF) | Organic-rich | ~3.5 – 4.5 | Low | High |

Prior to sowing, a simple water flotation test was used to assess seed viability (Tiansawat et al., 2016). Collected seeds were placed in a container of water; seeds that sank were considered viable, while floating seeds were considered as non-viable and discarded. All viable seeds were cleaned with tap water, and their wings were manually removed. De-winged seeds were then sown into individual planting cells (depth of 5 cm, a top diameter of 5.8 cm, and a bottom diameter of 4.3 cm, containing soil mixture) within a black plastic tray (50 × 30 cm). All cells contained a mixture of the forest soil from the collection site that

corresponds to the planted species and commercial potting soil (Bio-Root Medium; K.N. Nursery (B) Sdn Bhd, Brunei Darussalam) in a 1:1 ratio. Individual cells were labelled with the corresponding specimen code, name, number and collection site.

Individual seedlings were re-potted in individual polybags (0.7 L to 1.2 L volume). All polybags contained a mixture of the forest soil from the collection site that corresponds to the planted species and commercial potting soil (Bio-Root Medium; K.N. Nursery (B) Sdn Bhd, Brunei Darussalam) in a 1:1 ratio. Individual seedlings were labelled with the corresponding specimen code, number and collection site. All seeds and seedlings were maintained in the UBD BRC shade house under controlled conditions throughout the monitoring period. The shade house was covered with 50% black shade netting to reduce light intensity and simulate tropical understory conditions. An automated misting system was programmed to release fine water mist for 10 minutes at 7:00 am, 12:00 pm, and 5:00 pm daily. Environmental conditions were monitored throughout the study, with temperature ranging from 26°C to 33°C and relative humidity ranging from 75% to 95%, representing typical lowland tropical conditions.

After one month following the field collections, all planted seeds (n = 690 seeds; 2 species) were censused for seed germination, while all planted seedlings (n = 258 seedlings; 5 species) were censused for survival. Seed germination was characterized by signs of radical development and the appearance of cotyledons on the soil surface (Sasaki, 2008). Seedling death was confirmed by gently scraping stems of withering plants to determine whether plant tissue had died. The census was conducted monthly over a seven-month period, until December 2021.

All the statistical analyses were conducted in R 4.3.1 (R Core Team, 2023). Differences in final mean percentage seed germination at the end of the census period (December 2021) between forest types were determined using one-way ANOVA. In a separate analysis, final mean percentage seedling survival at the end of the census period (December 2021) was initially subjected to two-way analysis of variance (ANOVA) tests to determine differences between species, between forest types (MDF or HF), and species by forest type interaction. The two-way ANOVA however did not record any significant interactions between species and forest type. Therefore, two separate one-way ANOVA tests were conducted for the final mean percentage seedling survival to determine between-species differences and between-forest type differences. Multiple comparisons using Tukey's HSD test were also conducted. Data for the mean percentage seed germination and mean percentage seedling survival were arcsine-transformed after checking their normality of residuals and homogeneity of variances. Differences were considered significant when $P < 0.05$.

Seeds were observed to begin germinating within the first two months after planting. *Dryobalanops rappa* exhibited the most significant monthly increase in seed germination percentage, reaching a peak of 47.2% in July 2021. Conversely, *D. borneensis* from both MDF and HF collection sites consistently displayed lower monthly germination percentages (< 10%) throughout the census period.

At the end of the census period (December 2021), *D. rappa* seeds collected from the PSF recorded significantly higher final mean percentage germination (21.5 ± 2.70%) compared to *D. borneensis* seeds collected from HF (3.27 ± 1.02%) and MDF (3.92 ± 1.57%; $P < 0.001$; F-value: 62.96; df: 2, 687; Fig. 3). However, the final mean percentage survival of *D. borneensis* seeds did not significantly differ between HF and MDF collection sites.

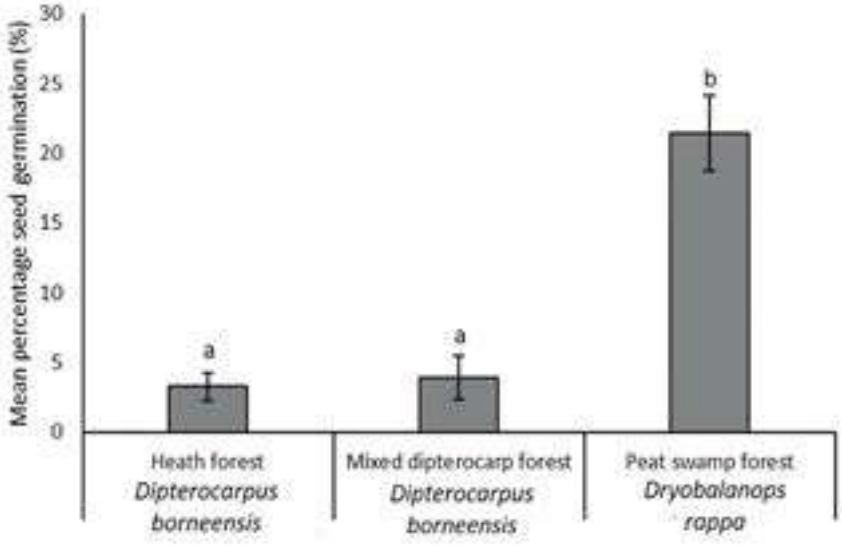


**Figure 3:** Final mean percentage seed germination at the end of the census period (December 2021) for *Dipterocarpus borneensis* seeds collected from the heath forest in Bukit Sawat and mixed dipterocarp forest in Labi, and *Dryobalanops rappa* seeds collected from the peat swamp forest in Anduki. Data values for mean percentage seed germination were arcsine-transformed, but untransformed data of mean percentage and error bars representing standard errors (SE) were used in the presentation. Different letters within a panel indicate significant differences at $P < 0.05$ as obtained from Tukey's HSD after one-way analysis of variance (ANOVA).

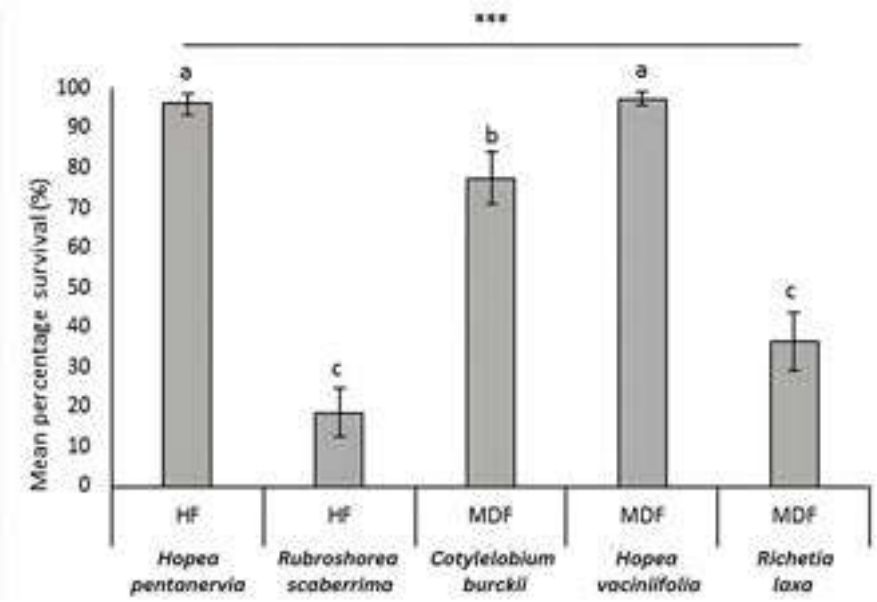


**Figure 4:** Final mean percentage seedling survival at the end of the census period (December 2021) for *Hopea pentanervia* and *Rubroshorea scaberrima* seedlings collected from heath forest sites and *Cotylelobium burckii*, *Hopea vaciniifolia* and *Richetia laxa* seedlings collected from mixed dipterocarp forest sites. Data values for mean percentage survival were arcsine-transformed, but untransformed data of mean percentage and error bars representing standard errors (SE) were used in the presentation. Different letters within a panel indicate significant differences at $P < 0.05$ as obtained from Tukey's HSD after one-way analysis of variance (ANOVA). Significant differences between forest types were detected after a two-way analysis of variance (ANOVA) test at $\alpha = 0.05$ level (* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

The two-way ANOVA test revealed significant differences in final mean percentage seeding survival between the five study species and significant differences between the two forest types ($P < 0.001$; Fig. 4).

No significant interactions were detected between species and forest types for final mean percentage seeding survival. Seedlings of *H. vaciniifolia* collected from the MDF and *H. pentanervia* collected from the HF recorded significantly highest mean percentage survival (97.5 ± 1.76% and 96.1 ± 2.75%, respectively), while seedlings of *R. laxa* from MDF and *R. scaberrima* from HF recorded significantly lowest mean percentage survival (36.4 ± 7.34% and 18.6 ± 6.00%, respectively; $P < 0.001$; Fig. 4). Regardless of species, seedlings collected from MDF recorded significantly higher mean percentage survival compared to seedlings collected from HF (MDF: 75.6 ± 3.36%; HF: 60.6 ± 5.07%; $P < 0.001$; Fig. 4).

At the end of the monitoring period, *D. rappa* recorded significantly higher percentage seed germination than *D. borneensis*. This is consistent with field observations by Din et al. (2018) that *D. rappa* seedlings were 10 times more abundant than *Rubroshora albida*, suggesting that *D. rappa* had a greater chance of survival after successful mast fruiting. In contrast, the percentage of seed germination of *D. borneensis* collected at both HF and MDF sites was much lower. As *D. borneensis* is well-known as a generalist species (Bell & Sultan, 1999), we had expected higher germination success. Our results may be partly due to differences in seed viability between these two species (Naito et al., 2008). Seeds of *D. rappa* seeds were much larger in length and width than those of *D. borneensis*, and larger seeds often have longer seed viability and thus higher germination success (Daws et al., 2008). The comparatively smaller seeds of *D. borneensis* may be more prone to drying, thus lowering their viability and hampering seed germination (Appanah & Turnbull, 1998).

Our findings have shown that three Dipterocarpaceae species, *H. vaciniifolia*, *H. pentanervia* and *C. burckii*, recorded the highest percentage seedling survival. Seedlings of Dipterocarpaceae species are known to prefer varying light intensities (Paine et al., 2012; Widiyatno et al., 2020). *Hopea vacciniifolia* are treelets and mid-storey trees, while *H. pentanervia* and *C. burckii* are classified as canopy and mid-storey trees (Coode et al., 1996), and each species likely show different degrees of shade tolerance throughout their early growth stages. In contrast, *Richetia* and *Rubroshorea* species are less shade tolerant and more light-demanding in their early growth stages (Appanah & Weinland, 1993). Both *R. laxa* and *R. scabberima* seedlings exhibited larger leaf areas and thinner leaves than *H. vacciniifolia*, *H. pentanervia* and *C. burckii* (Fig. 2) which could have resulted in greater transpiration and leaf wilting affecting seedling growth of *R. laxa* and *R. scaberrima*.

Our pilot study revealed species-specific differences in seed germination and seedling survival of selected Dipterocarpaceae species collected from three contrasting forest types in Brunei Darussalam. Notably, *D. rappa* exhibited higher seed germination rates, while *H. vacciniifolia*, *H. pentanervia*, and *C. burckii* showed high seedling survival over a seven-month period. These results indicate that these species hold strong potential as candidates for *ex-situ* conservation. In contrast, lower germination in *D. borneensis* and reduced seedling survival in *R. scaberrima* and *R. laxa* suggest that additional treatments or alternative approaches such as targeted specimen collection or *in-situ* conservation interventions, may be required to support their conservation.

One limitation of the study was the relatively small sample size for species collected as seedlings. Our field collection was conducted during a localised Dipterocarpaceae masting

episode and initially focused on seed collections. However, the presence of naturally regenerating individuals resulted in opportunistic collections of available Dipterocarpaceae seedlings for our study. Limited availability of seedlings during these field collections and a deliberate effort to minimise disturbance to forest ecosystems necessitated a lower sample size. Notably, rare or threatened species were encountered infrequently, making it difficult to obtain larger, more representative samples. Nevertheless, the data provide valuable baseline insights, and future studies should expand sampling across multiple sites and time periods to enhance statistical robustness.

Another key limitation is the relatively short monitoring period of seven months, which likely did not fully capture long-term survival trajectories, especially for slow-growing tropical tree species. While our study provides insights into early-stage establishment under *ex-situ* conditions, longer-term monitoring is essential to assess seedling growth into saplings, evaluate their developmental success, and refine conservation protocols. Future research should include extended observation periods to better determine species-specific growth responses and the sustainability of *ex-situ* conservation efforts.

In addition, this pilot study focused on quantifying differences in *ex-situ* seed germination and seedling survival across species and forest types. Experimentally assessing the factors, both biotic and abiotic, that directly influence seed germination and seedling survival was beyond the scope of our work. A critical extension to our study should therefore focus on these underlying factors, through controlled modifications of *ex-situ* environmental conditions and quantification of biotic factors, such as signs of seed damage and seedling herbivory, that are complimented by field assessments of *in-situ* environmental conditions and biotic influences.

Our results focus on *ex-situ* performance of the study species, to better inform ongoing conservation practices at the UBD BRC. A parallel comparison with *in-situ* monitoring of seed germination and seedling survival, although not logistically feasible for our pilot study, would have enabled an evaluation of the extent to which *ex-situ* conditions replicate natural environments in the species' habitats. This approach would help identify potential mismatches in environmental cues or resource availability that can then be modified to improve seed germination and seedling survival under controlled nursery conditions.

Despite a focus on *ex-situ* seed germination and seedling survival, we highlight the important practical implications of our findings upon *in-situ* conservation planning. Species that performed well under *ex-situ* conditions, such as *D. rappa*, *H. vacciniifolia*, and *H. pentanervia*, may exhibit strong natural regeneration potential and can be prioritised for restoration projects in degraded areas and enrichment planting in forest plantations or secondary forest areas. Meanwhile, species with lower performance may benefit from targeted *in-situ* conservation measures such as habitat protection, population reinforcement, or assisted natural regeneration. These insights can inform species selection and site-specific planning for forest restoration initiatives across Borneo and the broader Southeast Asian region, where Dipterocarpaceae forests face increasing ecological pressure.

## ACKNOWLEDGEMENTS

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## DECLARATIONS

**Research permit(s).** Permit no.: JPH/UND/17 [JPH/IND/SPL/FORM7/2025/002]. Agency name: Forestry Department, Ministry of Primary Resources and Tourism, Bandar Seri Begawan BB3910 Brunei Darussalam.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# A New Variety of *Capparis* (Capparaceae) from Northern Peninsular Malaysia

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## ABSTRACT

Here we describe and illustrate *Capparis scortechinii* var. *setiuensis*, a new variety of Capparaceae, which is endemic to Terengganu, from the northern region of Peninsular Malaysia. This taxon is placed within the section *Monosticocalyx* characterised by its persistent leaves, free and dimorphic sepals in bud, with the outer pair notably larger and enclosing the inner sepals. It resembles *C. scortechinii* var. *scortechinii* in the inflorescence characteristics, where each flower is subtended by a prominent leaf-like bract and arranged in a dense racemose formation along the rachis, which is densely covered with rusty-brown hairs. However, this variety is easily recognised by its pinkish to greenish leaf-like bract with brown venation, which is glabrous and caducous soon after. Additionally, this variety can be distinguished by its completely glabrous filaments and gynophores, in contrast to var. *scortechinii*, which has these parts that are densely hairy at their base. An updated identification key for Peninsular Malaysian species of *Capparis*, along with the provisional conservation status of this taxon, is also presented.

**Keywords**: BRIS soil; conservation status; *Capparis* sect. *Monostichocalyx*; Setiu; Terengganu.

## INTRODUCTION

*Capparis* L., a genus of approximately 146 species of small trees, bushy or scandent shrubs, and woody climbers (POWO, 2025), is the largest genus in the family Capparaceae. This Old World tropical and subtropical genus is characterised by simple, persistent or caducous leaves and stipular thorns, which are usually persistent but occasionally absent. *Capparis* exhibit zygomorphic flowers borne in various inflorescence types, including solitary arrangements, racemes, umbels, sub-umbels, fascicles, and supra-axillary rows. The flowers possess four sepals (either free or with the outer pair connate in bud), four asymmetrical petals (the two dorsal petals erect and connate at the base, the two lateral petals free and spreading), six to numerous stamens, a prominently extended gynophore, and a 1–6-locular ovary that develops into either a berry-like fruit or a pepo (Kers, 2002). Morphologically, *Capparis* is divided into four sections: *Capparis* sect. *Capparis*, sect. *Busbeckea* (Endl.) Benth. & Hook.f. (Bentham & Hooker 1862:109), sect. *Monostichocalyx* Radlk. (Radlkofer, 1839: 101) and sect. *Sodada* (Forsk.) Endl. (Endlicher, 1839: 893). Peninsular Malaysian *Capparis* species that belongs to sect. *Monostichocalyx*, is characterized by well-developed, persistent leaves and free sepals (Jacobs, 1960). Nine species of *Capparis* from Peninsular Malaysia were treated in the Flora Malesiana account (Jacobs, 1960), and an additional 2 species were added since then (Julius, 2022: 1 newly described species; Kiew & Rafidah, 2008: 1 newly recorded species). These recent findings suggest a high probability of undescribed taxa waiting to be discovered in Peninsular Malaysia.

A *Capparis* species initially observed by the second author in 2018 at a locality in Setiu, Terengganu, was recently rediscovered during a flora survey of Beach Ridges Interspersed with Swales (BRIS) soil vegetation led by the third author. To confirm its identity, flowering and fruiting specimens were recollected from the same locality and deposited in the Kepong Herbarium (KEP). Superficially, this new material resembles *C. scortechinii* King due to the dense, rusty hairs on the inflorescence rachis and flower bud enveloped by leaf-like bracts. However, detailed examination and comparison with *C. scortechinii* specimens, along with consultation of relevant literature (e.g., Gardner & Hall, 2015; Jacobs, 1965; Maurya et al., 2021), revealed that it represents an undescribed taxon, which is formally described below. A key to the species of *Capparis* in Peninsular Malaysia is also provided to aid in identification.

## METHODOLOGY

This research was conducted through field observations of living plants, with supplementary examination of both fresh and herbarium-preserved specimens deposited at KEP. Identification and comparison were carried out by reviewing relevant taxonomic publications (e.g., Chayamarit, 1991; Jacobs, 1965; Julius, 2022); in addition, specimen images from Global Plants JSTOR (http://plants.jstor.org/), Kew Herbarium Catalogue (http://apps.kew.org/herbcat/gotoHomePage.do), and Plants of the World Online (POWO: http://www.plantsoftheworldonline.org/) were consulted. Vegetative and reproductive parts measurements were taken from fresh, wet, and dried herbarium specimens. Flowering and fruiting materials are indicated by ‘fl.’ and ‘fr.’, respectively. The conservation assessment of the species was undertaken using IUCN categories of threat (IUCN, 2012; IUCN Standards and Petitions Subcommittee, 2024).

## RESULTS

### Taxonomic treatment

### Key to Peninsular Malaysian species of *Capparis*
[modified after Julius (2022)]

1 Flowers arranged in a series along the twig just above the leaf axil, with up to 6 flowers…….....**2**
– Flowers arranged in a short fascicle, umbels to subumbels, racemes or paniculate, either terminal on the main stem or lateral leafy twigs in the leaf axil……...……...**5**
2 Stipular thorns absent……..…….. ***C. acutifolia***
– Stipular thorns present…….……**3**
3 Leaf apex long acuminate, 10–13 mm long. Petals broadly elliptic. Fruit torulose…….....***C. cucurbitina***
– Leaf apex mucronate or shortly acuminate, 4–6 mm long. Petals oblanceolate or obovate to elliptic. Fruit ellipsoid or oblong……..…**4**
4 Leaf subcoriaceous to chartaceous, base cordate, apex mucronate. Petals up to 1.6 cm long. Stamens 15–18…….……***C. micracantha* subsp. *micracantha***
– Leaf coriaceous, base cuneate, apex shortly acuminate. Petals up to 2.4 cm long, stamens > 18……..……***C. micracantha* subsp. *korthalsiana***
5 Inflorescences in axillary fascicles……..…***C. pubiflora***
– Inflorescences racemes, paniculate, umbels or sub-umbels……..**6**
6 Inflorescences paniculate……***C. erycibe***
– Inflorescences racemose, umbels or subumbels……..……..…**7**
7 Inflorescences strictly racemose, flowers densely or loosely arranged in raceme……....**8**
– Inflorescences umbellate, sub-umbellate and/or flowers arranged in racemose and becoming crowded at the distal part of the inflorescence……..……**11**
8 Flowers loosely arranged in racemes with early caducous leaf-like bracts. Lamina surface bullate……... ***C. kenaboiensis***
– Flowers are densely arranged in racemes and subtended by persistent and conspicuous leaf-like bracts. Lamina surface smooth …….……....**9**
9 Leaf-like bracts smaller, narrowly elliptic, (1.0–)1.3–2.5 × (0.1–)0.2–0.8 cm, ……... ***C. scortechinii* var. *ruthiae***
– Leaf-like bracts larger, boat-shaped to elliptic, 2.0–3.0 × 0.9–1.6 cm …….....**10**
10 Leaf-like bracts thick, elliptic, 2.5–3.0 × 1.0–1.3 cm, densely hairy with velvety, shiny and rusty hairs abaxially, glabrous adaxially, persistent before anthesis. The gynophore is densely hairy at the base.....***C. scortechinii* var. *scortechinii***
– Leaf-like bracts thin, boat-shaped, but elliptic-shaped when spreading, 2–2.5 × 0.9–1.6 cm, pinkish to greenish with brownish venation, shortly pubescent but glabrous with the naked eye on both surfaces, caducous before anthesis. The gynophore glabrous throughout ……***C. scortechinii* var. *setiuensis* var. nov.**
11 Inflorescences in racemes and/or subumbels, terminal sometimes axillary, sepals hairy.....**12**
– Inflorescences in umbels, lateral or axillary, sometimes terminal, sepals glabrous.....**13**
12 Lamina subcoriaceous to coriaceous, broadly ovate sometimes ovate-elliptic, 13–16 × 5.5–8.5 cm, drying leaves reddish brown rarely pale green with pale yellow rarely dark red venation on both surfaces, intercostal veins obscure. Inflorescence terminal with flowers arranged in racemose and becoming crowded at the distal part of the inflorescence, stamens

30–40.....***C. trinervia* var. *chungiana***

– Lamina chartaceous, oblong-elliptic or broadly lanceolate, (5–)10–14(–19) × (2–)3.5–8.5 cm, drying leaves dull green with brownish main nerves on both surfaces, intercostal veins irregularly reticulate, and distinct. Inflorescence terminal with flowers arranged racemosely and becoming crowded at the distal part of the inflorescence, sometimes subumbellate on 3–4 cm long peduncles in the axils of the uppermost leaves, stamens (30–)60–70 .....***C. trinervia* var. *trinervia***

13 Twigs flexuous ..... ……. ***C. sepiaria***

– Twigs ± straight ..... ……...**14**

14 Leaf < 5 cm long, margin revolute, lamina coriaceous to subcoriaceous, apex obtuse or retuse. Umbels pedunculate, 1–4-flowered, axillary and/or terminal ……..……. ***C. versicolor***

– Leaf > 5 cm long, margin flat at the edge and not revolute, lamina chartaceous, apex usually obtuse, sometimes acute, with acumen 5–10 mm long. Umbels sessile, 3–5-flowered with 1–2 small leaves, sometimes a few umbels united to a small panicle, terminal or lateral on small twigs……...…….***C. diffusa***

**New Taxon**

***Capparis scortechinii* var. *setiuensis*** Julius, Dome & Jamilah MS sp. nov., Fig. 1

**Diagnosis.** This new variety of *Capparis* is similar to *Capparis scortechinii*, particularly var. *scortechinii*, in its leaf venation and hairiness, as well as its inflorescence pattern. The inflorescence consists of a single flower subtended by a large, leaf-like bract, arranged in a compact racemose along the inflorescence rachis, which is covered by rusty-brown hairs. However, this variety is easily recognised by its chartaceous, pinkish to greenish leaf-like bract with brown venation, which is shortly pubescent but glabrous to the naked eye and caducous soon after (in contrast to the coriaceous and velvety, rusty-brown hairs that persist in var. *scortechinii*). Additionally, var. *setiuensis* has entirely glabrous filaments and gynophore, unlike var. *scortechinii*, which is densely hairy at the base.

**Type.** MALAYSIA. Peninsular Malaysia: Terengganu, Setiu District, Kampung Pandan Jaya, Rhu Sepuluh, grows on lowland, BRIS soil vegetation, on private land and near housing area, 5°34'24.8"N 102°50'47.6"E, 3 m elev., 3 November 2022 (fl., fr.), *A. Julius et al.*, *FRI100802* (holotype: KEP!)]

**Description.** A shrub *c.* 2.5 m tall, with long, scrambling and wiry woody branches. ***Indumentum*** of simple, rusty brown hairs on branches, petioles and peduncles, minutely pubescent on sepals, white hairs on petals. ***Twigs*** straight, terete, young pubescent with rusty brown hairs, mature glabrous and green. ***Stipular thorns*** retrorse, (2–)3–5 mm long, young pubescent with rusty brown hairs, mature glabrous, flanked the leaves. *Leaves* spirally arranged; petiole thick, 1–1.5 cm long, young pubescent with rusty brown hairs, mature glabrous and green; lamina coriaceous to subcoriaceous, 10–13.5 × 4–6 cm, young pubescent with white hairs above, rusty brown hairs beneath, mature glabrous on both surfaces, base cuneate to cuneate-rounded, margin ciliate with white hairs, apex acuminate, with acumen 4–6 mm long, recurved downward; midrib flat or slightly sunken above, raised beneath, brownish turning green with age, young pubescent with white hairs above, densely rusty hairs beneath, mature glabrous on both surfaces; *lateral veins* 4–6 pairs, looping and joining towards margin, young brownish covered with rusty hairs beneath, mature glabrous and green, distinct above,

prominent beneath; intercostal veins reticulate, distinct above, prominent beneath. ***Inflorescences*** terminal on a robust, lateral specialized flowering branches and leafy twigs, rachis stout, young densely rusty brown, mature green and glabrous; bracts leaf-liked, pinkish to greenish with brownish venation, chartaceous, boat-shaped, but elliptic-shaped when spreading, 2–2.5 × 0.9–1.6 cm, apex acuminate and recurved, enveloping young bud, caducous before anthesis, shortly pubescent but glabrous with naked eye on both surfaces. ***Flowers*** many, in compact racemes, buds globose, 7–10 × 6–14 mm, pinkish; pedicels greenish, 7–12 mm long, pubescent with white hairs; sepals 4, pinkish except whitish margin, outer-pair imbricate, orbicular, larger one *c*. 1.2 × 1.5 cm, smaller one *c*. 1.4 × 1 cm, inner pair elliptic, 1.2–1.3 × 0.6–0.7 cm; petals 4, pale pink with white margin, venation on surfaces pinkish, dorsal pair obovate to ovate, *c*. 1.4 × 1 cm, connate at base, glabrous except wooly at base abaxially, wooly except near apex adaxially; ventral pair oblanceolate, 2.3–2.5 × 1 cm, wooly abaxially, glabrous adaxially; stamens 48–55, unequal length, filament 2.5–4 cm long, white but pink on upper half, anther narrowly sagittate, 2–3 mm long, basifixed, dark pink abaxially, white adaxially; ovary pinkish to brownish, ovoid with pointed tip, 3–4 × 1–3 mm, on gynophore 2.5–4 cm long, pinkish, entirely glabrous, stigma obscure. ***Fruits*** only 1–2 fully developed per infructescence, young green turning yellow to brown when ripe, globose to sub-globose on stout gynophore, 6.0–6.5 × 5 cm. ***Seeds*** a few; embryo with thin cotyledons tightly coiled into a small bundle and a long, thick radicle coiled several times around the cotyledon.

**Distribution.** Endemic and rare in Peninsular Malaysia, Terengganu, known only from Pantai Rhu 10 and Merang at Setiu District.

**Ecology and phenology.** Occurring within lowland habitats on BRIS soils, typically in open, sun-exposed areas. Flowering specimens were collected in March and November, with additional flowering observed in July and December. Fruiting material was observed and collected in July and November.

**Etymology.** The varietal epithet is named after the locality where this taxon was first discovered.

**Provisional conservation status.** Critically Endangered (CR) B2ab (ii, iii, iv). *Capparis scortechinii* var. *setiuensis* is currently known only from two localities in Setiu, Terengganu, Peninsular Malaysia, and inhabits lowland coastal vegetation outside protected areas. There are currently only six mature individuals known, with fruiting observed once or twice during the study period. However, no seedlings or saplings were found in the vicinity of the parent trees. The area of occupancy (AOO) is estimated to be 8 km², calculated using a 2 × 2 km grid as per IUCN guidelines. This variety is highly localised and has a restricted distribution. As observed during recent field surveys, both known populations are exposed to threats from habitat disturbance and land-use changes, particularly those associated with coastal development and vegetation clearance. Based on its very limited AOO of 8 km², occurrence at fewer than five locations, and the continuing decline in the area of occupancy, quality of habitat, and number of locations, this variety is assessed as Critically Endangered B2ab (ii, iii, iv)**,** following the IUCN Red List Categories and Criteria (IUCN, 2012; IUCN Standards and Petitions Subcommittee, 2024).

**Additional specimen examined.** MALAYSIA. Peninsular Malaysia: Terengganu, Setiu District, Merang, grows on lowland, BRIS soil vegetation, at the edge of equine track and nearby construction area of PERKESO Rehabilitation Center, 5°31' 0.8508" N 102° 58' 7.392" E, 8 m elev., 25 July 2024 (fl., fr.), *Dome Nikong FRI107943* (KEP!)]

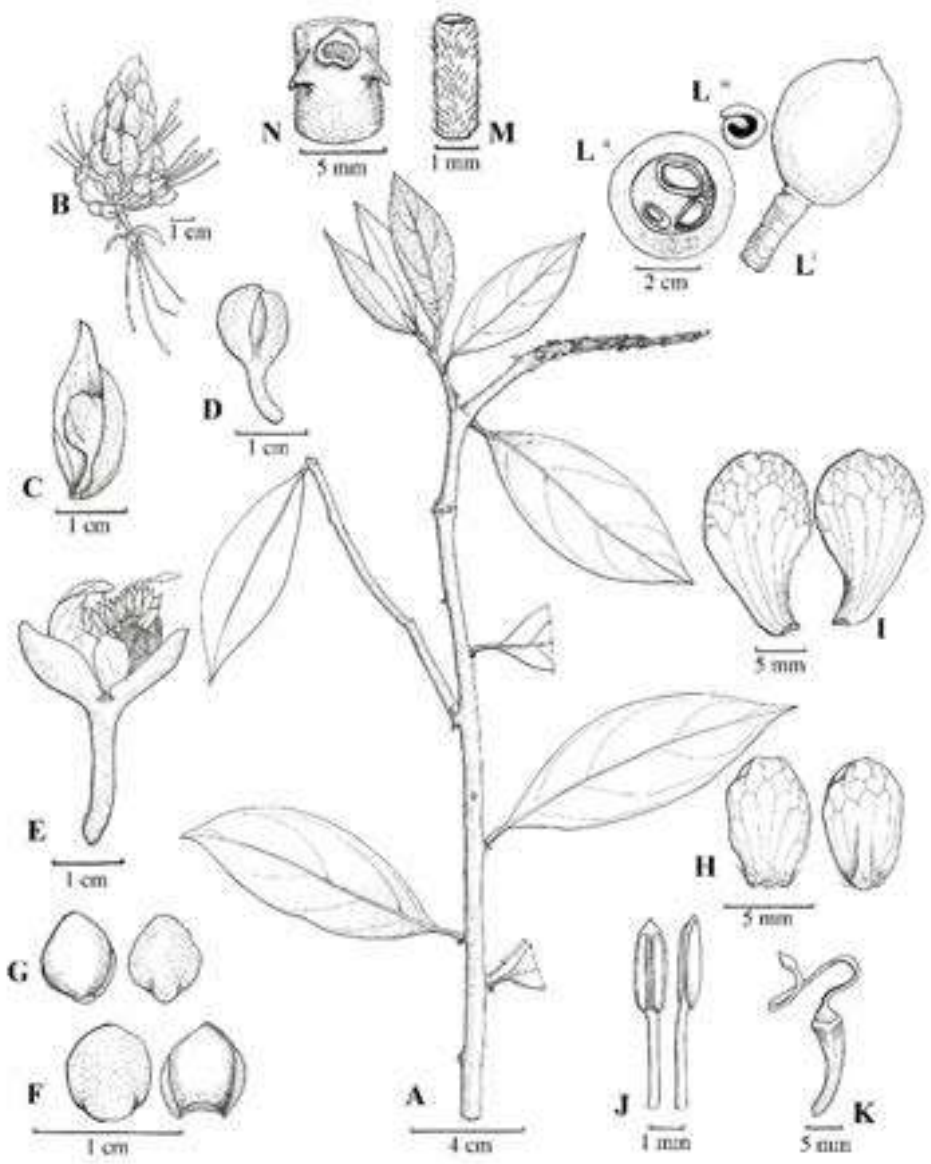


**Figure 1:** *Capparis scortechinii* var. *setiuensis* Julius, Dome & Jamilah var. nov. **A.** Flowering branches, **B.** Inflorescence, **C.** Flower bud enclosed by the leaf-like bract, **D.** Flower bud, **E.** Opening flowers, **F.** Sepals, outer pair, **G.** Sepals, inner pair, **H.** Petals, dorsal pair, **I.** Petals, lateral pair, **J.** Stamen, adaxial view (left), abaxial view (right), **K.** Pistil, **L.** Fruit (i), with cross section showing seeds (ii) and coiled cotyledon (iii), **M.** Indumentum, **N.** Stipular thorns.

## DISCUSSION

Among the Peninsular Malaysian taxa of the genus *Capparis*, *C. scortechinii* is easily recognised by its compact inflorescence, with each flower subtended by a large, leaf-liked bract. However, the branching pattern of its inflorescence has not been fully understood due to the limited material available at KEP and on loan from SING and BKF—until the discovery of this new variety.


**Figure 2:** *Capparis scortechinii* var. *setiuensis*. **A.** Habit, **B**. Flowering branch, **C**. Inflorescence, **D.** Inflorescence on main and lateral branches, **E**. Inflorescence, close up, **F**. Infructescence and inflorescence. [Photos by DN (A-B, E-F) and AJ (C-D)].

Julius (2022) recently noted that var. *scortechinii* has both terminal and axillary inflorescences, whereas var. *ruthiae* Julius has only terminal inflorescences. For var. *scortechinii*, its inflorescence is described as axillary because leaves were not observed below the flowers, though scars— possibly from fallen leaves or bracts— were present. Some of these scars are

flanked by rudimentary stipular thorns, which are also observed in the new variety, suggesting that both varieties share the unique characteristic of leaf-like bracts flanked by stipular thorns. However, field observations of the new variety revealed that a few young leaves were present below the inflorescence on lateral twigs but fell off soon before anthesis (Fig. 2, B). In the case of var. *ruthiae*, it is possible that its inflorescences also occur terminally on young, new lateral twigs, but such occurrences have not yet been observed or collected. Thus, it is assumed that all varieties of *C. scortechinii* bear terminal inflorescences on both main leafy branches and lateral twigs.

Compared to var. *scortechinii*, which inhabits lowland rainforests, and var. *ruthiae*, which is frequently found at forest margins, or in gaps, or along roadsides from hill slopes up to 1,280 m, var. *setiuensis* is the only variety found growing on BRIS soil (Fig. 2, A)— a substrate characterised by its sandy texture and low nutrient retention, making it a challenging environment for plant growth. Due to its restricted distribution, with only one to two individuals found at each site, and its ability to adapt and survive in nutrient-poor soils, this new variety may be worth propagating as part of an ex-situ conservation program. Attempts to find wildlings after the fruiting season so far have been futile. Seeds are pseudo-viviparous, and the survival of the seedlings after germination is low. Moreover, var. *setiuensis* is rare and at risk of extinction due to ongoing housing development and other threats related to land use changes.

## CONCLUSION

*Capparis scortechinii* var. *setiuensis* is confirmed as a distinct and narrowly distributed variety restricted to BRIS soil habitats in Setiu, Terengganu. The variety clearly differs from the other varieties of *C. scortechinii*, and its occurrence within privately owned and rapidly developing housing areas, combined with a small number of known individuals, indicates a high risk of extinction. These findings emphasis the urgency of targeted conservation efforts for this variety, particularly through cooperation with landowners, awareness initiatives, and *ex-situ* propagation. Further research is urgently needed to systematically document its phenology, fruit set, and seed biology to support conservation efforts. This study also highlights that unique endemic taxa can still occur even in marginal or human-modified habitats, emphasising the importance of continued botanical documentation and conservation planning in such landscapes.

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## DECLARATIONS

**Research permit(s).** This study was conducted on private land, and consent from the owner was obtained verbally prior to the research being conducted.
**Ethical approval/statement**. Not applicable.
**Generative AI use.** We declare that generative AI was not used in this study nor in the writing of this article.

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**Research Article**

# Bird Diversity and Functional Guilds in Sungai Talibu Forest Reserve, Sabah, Malaysia

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## ABSTRACT

A rapid avifaunal assessment was conducted in Sungai Talibu Forest Reserve, a secondary mixed dipterocarp forest in Telupid, Sabah, Malaysia, to investigate its avian community and ecology as a baseline for future forest management initiatives. The assessment was part of a multi-disciplinary scientific expedition by the Sabah Forestry Department. Using a modified MacKinnon List method, the four-day survey recorded 15 lists, detecting 391 individuals. A total of 107 species from 40 families were identified, with a Shannon Diversity Index of H= 3.88 and Evenness $E_H$= 0.65. Chao1 species richness, estimated via SuperDuplicates®, suggested approximately 138 species, with 31 undetected. The effective number of species ($Num_{eff}$) was 48. Eight Bornean endemics were recorded, including the Borneo Ground Cuckoo (*Carpococcyx radiceus*). The survey also detected species of high conservation concern: Helmeted Hornbill (*Rhinoplax vigil*) and Greater Green Leafbird (*Chloropsis sonnerati*). Pycnonotidae was the most abundant family, with 45 individuals (11.5%) across 10 species. Insectivores dominated feeding guilds, with 174 individuals (44.6%) from 55 species and 20 families, followed by frugivores with 146 individuals (37.3%) from 30 species and 13 families.

**Keywords:** Avifaunal survey; MacKinnon List method; Sungai Talibu Forest Reserve; frugivores; insectivores.

## INTRODUCTION

In 2004, the Sabah Forestry Department (SFD) conducted a forest inventory exercise to ascertain tree density and structure, used to estimate standing timber and forest recovery capacity of Forest Management Unit (FMU) 17A, which includes the Sungai Pinangah Forest Reserve (Class II), Telupid, Sabah, Malaysia. The results were documented in a 10-year forest management plan (SFD, 2009). The first forest management plan was approved by the SFD in 2008. A portion of the Sungai Pinangah FR (Class II) was later degazetted and reconstituted as the Sungai Talibu Forest Reserve (STFR), a Protection Forest Reserve (Class I). From 13–18 May 2024, the SFD, through its Research and Development arm at the Forest Research Centre, Sepilok, conducted a multi-disciplinary scientific expedition to the STFR to study in-depth its ecology and social ecosystem services. The expedition base camp was at the Kun-Kun Riverside Park, situated in the northeast corner of the STFR, accessible from the main gravelled Tangkulap-Deramakot road. This paper documents the outcome of the avifaunal survey conducted during the expedition. The main objective of this survey was to investigate the avian community and ecology within the forest reserve as a baseline for future forest management initiatives.

## METHODS

### Site description

The STFR was gazetted as a forest reserve on 24 December 2014, with an area of 20,881 ha. It was previously gazetted in 1962 as part of Tangkulap Forest Reserve (Class II). As a commercial reserve, it was logged between the early 1970s and 2003. During that time, the issuance of short-term logging licenses at short intervals, as well as poor logging practices, resulted in excessive logging in some areas of the reserve. Currently, the STFR is part of the Tangkulap-Sungai Talibu FMU No. 17A and is managed by the SFD (SFD, 2009). The main focus of the management plan was to intensively restore severely degraded areas. The FMU 17A was certified as well-managed in 2016 by SCS Global Services in accordance with the Forest Stewardship Council's Principles and Criteria (FSC, 2023).

The natural vegetation of the northern portion of the STFR consists of mainly lowland and upland ultramafic forests over the Bidu-Bidu soil association (see Figs. 1 and 2). In the lowland ultramafic forest, the most common tree family is the Dipterocarpaceae, whereas in the upland ultramafic forest non-dipterocarps contribute most of the main canopy layer, namely, *Gymnostoma sumatranum* (Casuarinaceae), *Calophyllum* sp. (Guttiferae) and *Swintonia* cf. *acuta* (Anacardiaceae). In the upland kerangas forest, Dipterocarpaceae are also very common although shorter in stature compared to those growing in lowland mixed dipterocarp forests in the southeast of the reserve (Bower et al., 1975). As mentioned earlier, most of the lowland areas were logged in the past.

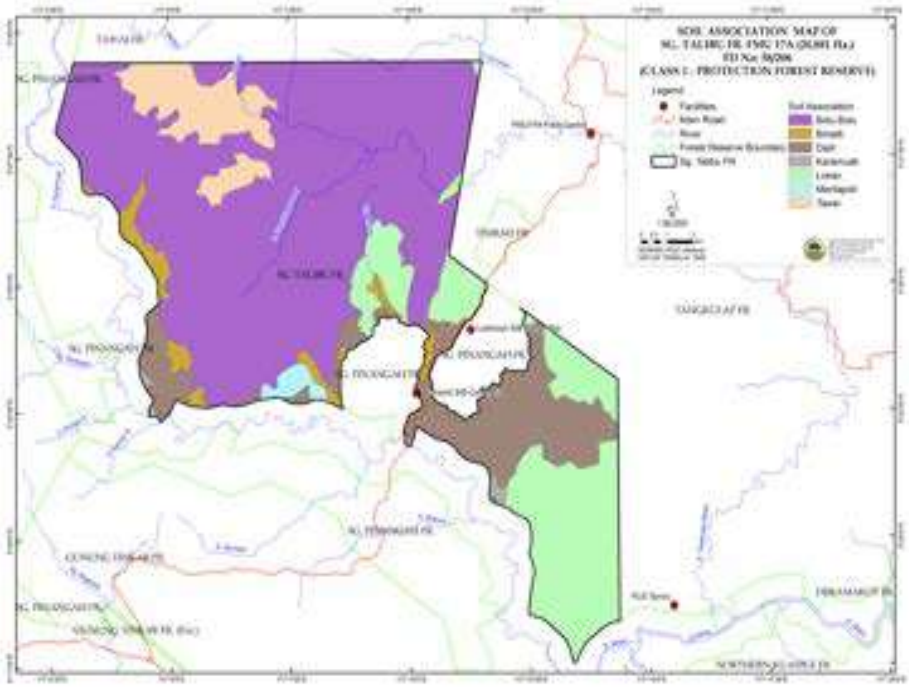

**Figure 2:** Soil association map of Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

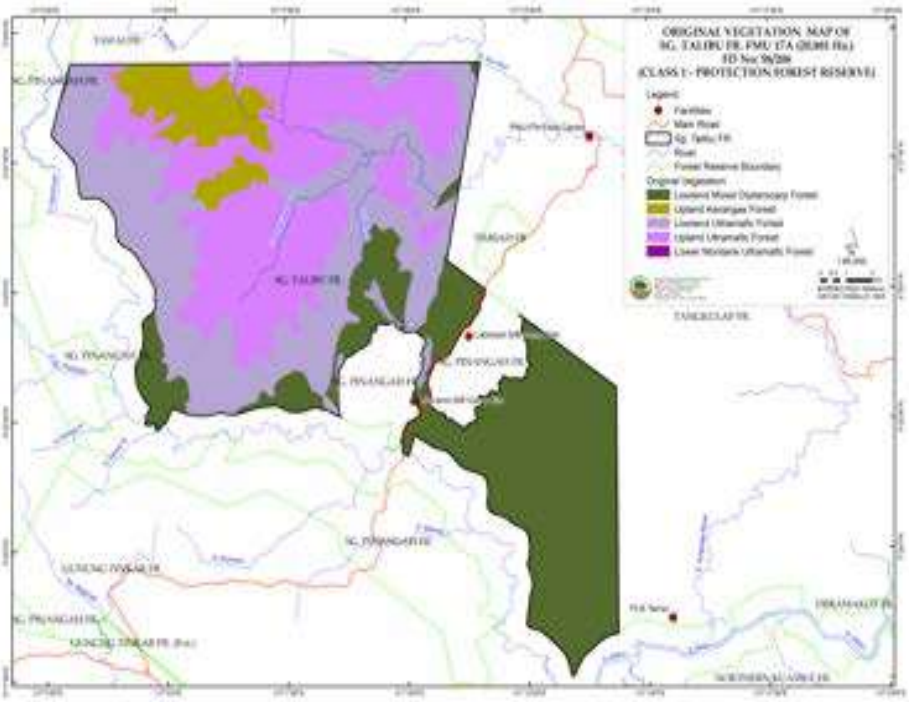

**Figure 1:** Natural vegetation of Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

**Modified MacKinnon List method**

The MacKinnon List (ML) method, introduced by MacKinnon and Phillipps (1993), is a practical and widely used approach for the rapid assessment of species-rich environments, especially when time, manpower, or funding is limited. Unlike more resource-intensive methods such as point counts or mist-netting, ML offers a flexible and non-invasive alternative. It is widely adopted by government agencies, NGOs, and citizen science projects for its simplicity and adaptability to varying observer expertise and field conditions (Poulsen et al., 1997; Yong et al., 2011). MacLeod et al. (2011) demonstrated that species abundance rankings derived from ML closely match those obtained from fixed-radius point counts, while being more robust to variation in detectability and effort. The method's independence from fixed spatial or temporal units also allows for meaningful comparisons across surveys conducted under different conditions (Herzog et al., 2002). Its versatility has extended to other taxa, including mammals and fish (Bach et al., 2020).

In standard ML practice, observers compile sequential lists of species based on the order in which they are detected—visually or aurally—during a survey. Each list contains a fixed number of different species, commonly ten, although Herzog et al. (2002) recommend longer lists (e.g., 15 species) for species-rich tropical systems. Once a species appears in a list, it is excluded from that list and re-enters only when the next list begins. This generates a set of species samples independent of time or space, allowing for a straightforward species accumulation curve.

As with all our assessments, we used a modified version of the ML method developed by our team, in which the number of individuals per species was also recorded within each 15-species list. This adjustment, informed by Herzog et al. (2002) and MacLeod et al. (2011), helps reduce the chance of double-counting and supports more robust estimates of diversity and abundance metrics—such as Shannon diversity (H′), Chao1 richness, and the Effective Number of Species ($Num_{eff}$). Given that our surveys typically span only 3–4 days, reflecting the logistical constraints of rapid assessments by the SFD, this modification improves accuracy for abundance-based comparisons. A species accumulation curve was also generated post-survey to assess sampling completeness. An asymptote in this curve suggests that most of the site's bird diversity was likely detected.

**Survey methods**

Surveys were conducted for 4 days (14–17 May 2024), beginning at 6:30 am and ending after 4 hours. Approximately 5.5 km of the main and secondary earth roads leading into the central portion of the STFR with ultramafic forests, were surveyed. Night surveys to detect nocturnal birds were conducted, weather-permitting, for two hours within 2 km from the base camp as elephants posed a safety risk along the roads.

Every observer had a pair of Nikon binoculars (8 × 42). The reference field guides were Phillipps and Phillipps (2014) and the Merlin Bird ID application (Cornell Lab of Ornithology, 2025). The latest taxonomic changes were determined from the IOC World Bird List (Gill et al., 2025). Whenever possible, a Nikon P1000 mega-zoom camera (3,000 mm equivalent) was used to photograph unfamiliar birds and confirm their identities.

One team member recorded all observations, ensuring no intra-list and inter-list double-counts. Given that approximately half of the individuals were detected aurally, as is standard in tropical forest surveys where dense canopy limits visibility—most birds were first heard and, if the call

was unfamiliar, were then visually sought for confirmation; very common species recognised by call were recorded immediately. Identification by bird vocalisations is widely accepted as equivalent to sight records in established protocols (Ralph et al. 1995; Bibby et al. 2000) and performs comparably to visual counts in estimating richness and composition (Haselmayer & Quinn 2000; Anderson et al. 2015). Our experienced team verified uncertain calls against reference archives (e.g. Xeno-canto Foundation, 2025) to ensure accuracy and consistency. As such, strict care was applied to ascertain the uniqueness of individual bird records, particularly when inputting abundance data within a single 15-species list. The delineation of distinct individuals of the same species hinged upon several criteria: a) the directional origin of their vocalisations; b) the sequential occurrence of two or more calls emanating from a comparable direction to a previously documented individual; and c) the perceived distance from a previously logged individual was deemed sufficient to warrant the classification of a call as originating from a separate individual. This distance refers to the point at which we were confident that the bird could be reliably identified. For common and well-known species, this could be determined quickly through calls alone, whereas for less familiar or uncertain detections, we sought visual confirmation whenever possible. The survey team consisted of experienced birders with over 55 years of cumulative field experience in bird identification in Sabah, ensuring that such judgments were made consistently and with a high degree of reliability. Moreover, in cases where bird species were observed in flocks, photographs were taken and reviewed to estimate the flock's size, with attention given to avoiding double-counting instances. When surveying a secondary road (i.e., smaller branch earth roads that extend from the main road), only bird species not previously recorded along the stretch were documented when returning to the main road.

**Analyses**

From the survey data, we derived basic diversity information, including species richness, number of families, the most speciose family, and the most abundant species. Latest updates on Bornean endemics were obtained from Gill et al. (2025) while conservation status followed the IUCN Red List (IUCN 2025). A species accumulation curve was generated by adding those species not recorded on any of the previous lists to the total species number, which was then plotted as a function of the list number.

For diversity indices, we calculated Shannon diversity index (H'), the effective number of species (exponential of H'), $Num_{eff}$, and Chao1 Estimate. The formula for $Num_{eff}$ is as follows:

$$Num_eff = exp\{-\sum_{i=1}^{s} p_i \ ln(p\_i)\}$$

$Num_{eff}$ accounts for species richness and evenness (Jost, 2006). Chao1 estimates 'true' species richness, including the number of unobserved species. It is calculated using the SuperDuplicates® online calculator (https://chao.shinyapps.io/SuperDuplicates/), developed by Chao et al. (2017). The calculator only requires the total number of species observed and the number of species observed only once, with data type listed as 'abundance data'.

In addition to these metrics, feeding guilds data were used to provide information on how the bird community used specific forest resources (fruits, insects, arthropods, seeds, other resources). Such information may indicate the condition or ecological health of the forest ecosystem at STFR, as

the relative representation of different feeding guilds may reflect habitat quality and resource availability. Species were categorised according to six feeding guilds based on their preferred diet; carnivores, frugivores, insectivores, nectarivores, granivores and generalists. Species were considered generalists if they were known to consume roughly similar amounts of animal- and plant-based food resources. Guild information was determined mainly from Phillipps and Phillipps (2014) and Wells (1999 & 2007). Feeding guilds were then compared among habitat types (e.g., forest, forest edge and open areas) to examine the relative importance of these habitats to different guilds.

## RESULTS

### Avifaunal composition and species richness

The four survey days yielded 15 lists and a total of 391 individuals from a total of 20 survey hours. The avifauna of the STFR was represented by 107 species from 40 families, with a Shannon Diversity Index of H=3.88 and an Evenness, $E_H$=0.65.

The survey documented eight Bornean endemic species (Table 1), of which the Bornean Ground Cuckoo (*Carpococcyx radiceusis*) is the only one classified as Vulnerable on the IUCN Red List, while the Bornean Bristlehead (*Pityriasis gymnocephala*) and Charlotte's Bulbul (*Iole charlottae*) are categorised as Near Threatened. With the exception of the Dusky Munia (*Lonchura fuscans*), all endemic species were strictly dependent on forest habitats. Beyond the endemics, the survey also recorded species with critical conservation statuses: the Helmeted Hornbill (*Rhinoplax vigila*) as Critically Endangered and the Greater Green Leafbird (*Chloropsis sonnerati*) as Endangered. Additionally, five species were identified as Vulnerable: the Bornean Ground Cuckoo (*Carpococcyx radiceusis*), Rhinoceros Hornbill (*Buceros rhinoceros*), Long-tailed Parakeet (*Psittacula longicauda*), Javan Myna (*Acridotheres javanicus*), and Great Slaty Woodpecker (*Mulleripicus pulverulentus*).

**Table 1:** The IUCN conservation statuses of Bornean endemic bird species detected in May 2024 at the Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia (IUCN, 2025). LC: Least Concern, NT: Near Threatened, VU: Vulenerable.

| Common name | Family | IUCN status |
|---|---|---|
| Bornean Black-capped Babbler | Pellorneidae | LC |
| Bornean Bristlehead | Pityriasidae | NT |
| Bornean Brown Barbet | Megalaimidae | LC |
| Bornean Ground Cuckoo | Cuculidae | VU |
| Charlotte's Bulbul | Pycnonotidae | NT |
| Cream-eyed Bulbul | Pycnonotidae | LC |
| Dusky Munia | Estrildidae | LC |
| White-crowned Shama | Muscicapidae | LC |

Table 2 is a summary of the most commonly represented families and species. The bird family whose members were most commonly encountered in the STFR was Pycnonotidae, with 45 individuals recorded. This family also had the highest species richness with 10 species recorded. The Nectariniidae was the second most common, with 41 individuals and 8 species detected. Other

notable families included Cuculidae and Timaliidae, both contributing 22 individuals, while Pellorneidae exhibited a species richness of seven. Among individual species, the Bold-striped Tit-babbler (*Mixornis bornensis*) and Little Spiderhunter (*Arachnothera longirostra*) were the most frequently observed, with 16 individuals each, followed by the Blue-crowned Hanging Parrot (*Loriculus galgulus*) with 15 individuals. The complete species and family lists are provided in Appendices I and II, respectively.

**Table 2:** Most commonly represented families and species of birds recorded in May 2024 at the Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

| Rank | Family (no. of individuals) | Family (no. of species) | Species (no. of individuals) |
|---|---|---|---|
| 1 | Pycnonotidae (45) | Pycnonotidae (10) | Bold-striped Tit-babbler/ Little Spiderhunter (16) |
| 2 | Nectariniidae (41) | Cuculidae/Nectariniidae (8) | Blue-crowned Hanging Parrot (15) |
| 3 | Cuculidae/Timaliidae (22) | Pellorneidae (7) | Green Iora/ Orange-bellied Flowerpecker (13) |
| 4 | Psittaculidae (19) | Megalaimidae/Picidae (5) | Greater Green Leafbird/ Purple-naped Spiderhunter (12) |
| 5 | Chloropseidae (18) | Alcediniidae/Cisticolidae/ Timaliidae/Muscicapidae (4) | Cream-vented Bulbul/ Spectacled Bulbul (11) |

Apart from observed species richness (107), $Num_{eff}$ was approximately 48 species, and the SuperDuplicates® online calculator estimated the Chao1 estimate there to be approximately 138 total species (Table 3). Thus, the calculator estimated that approximately 31 species were undetected, i.e., the survey detected about 77.5% of the total species in the area. The number of doubletons (species detected only twice) was estimated to be 16, less than the actual number of 27 obtained from the survey. As shown in Figure 3, the species accumulation curve had not reached a plateau, suggesting that additional surveys would likely yield further species. Based on the linear regression line, it estimated that another two lists, or an extra four-survey days, were needed to detect the estimated 138 species of birds predicted by SuperDuplicates®, particularly in the upland ultramafic forests and the logged-over mixed dipterocarp forests in the southeast of the reserve that were not covered during the survey.

**Table 3:** Results from SuperDuplicates®– Chao1 estimation of the bird species recorded in May 2024 at the Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

| Estimated no. of doubletons | Estimated species richness | Standard error | 95% C.I. lower | 95% C.I. upper | No. of undetected species | Undetected percentage (%) |
|---|---|---|---|---|---|---|
| 16.39 | 138.01 | 8.32 | 125.5 | 158.97 | 31.01 | 22.47 |

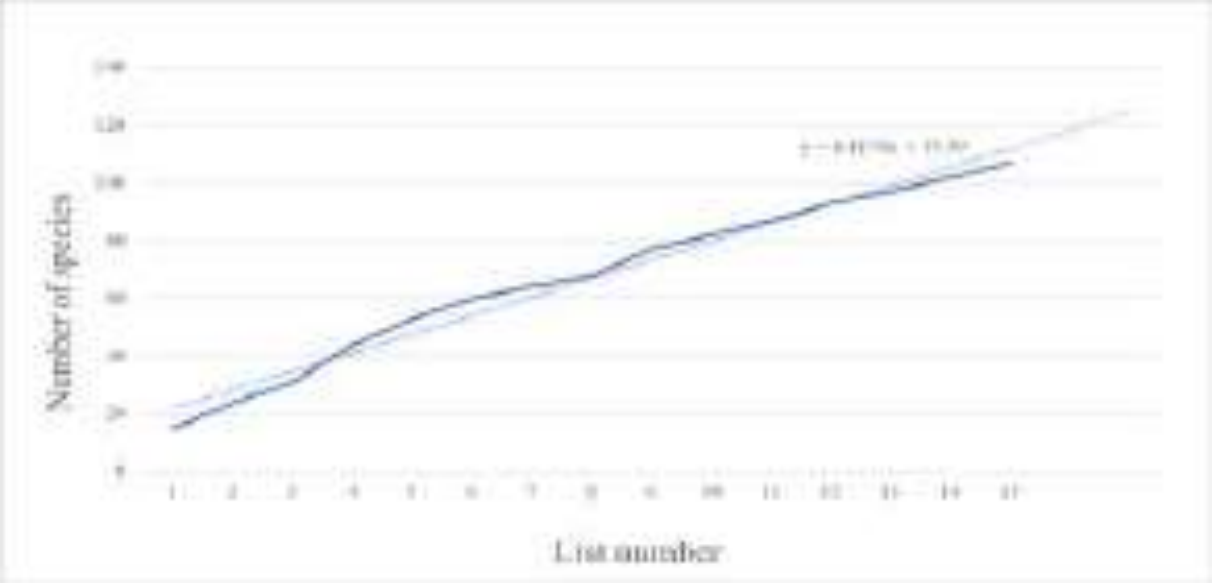


**Figure 3:** Species accumulation curve and linear regression line of birds recorded in May 2024 at the Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

## Preferred habitat types

Species were categorised based on their preferred habitats (e.g., forest, forest edge, open areas) and feeding guilds (Fig. 4). Of the 107 species detected, 101 (94.4%) were forest-dependent, with 81 (75.7%) classified as strictly forest species. In contrast, only six open-area species (20 individuals) were recorded, mostly near the expedition base camp at Kun-Kun Riverside Park. This low count of open-country birds is likely due to the area's relative isolation; the nearest oil palm plantations lie approximately 2.6 km to the north, limiting edge effects and habitat encroachment from surrounding land use.

## Feeding guilds

Insectivores and frugivores comprised 80.0 % of the total individuals detected, with insectivores being the most abundant at 174 individuals (44.5%). Among the 55 insectivorous species (spanning 21 families), 45 were strictly forest specialists (Fig. 4). The Bold Striped Tit-babbler and the Green Iora (*Aegithina viridissima*) were the most abundant insectivores detected with 16 and 13 individuals, respectively.

The second most dominant feeding guild was the frugivores, with 146 individuals (37.3%) detected, representing 30 species across 13 families. Among them, the bulbuls (Pycnonotidae) were the most frequently recorded family, with 45 individuals. Both the Cream-vented Bulbul (*Pycnonotus simplex*) and the Spectacled Bulbul (*Ixodia erythropthalmos*) were the most commonly recorded species, each with 11 individuals, followed by Charlotte's Bulbul with seven individuals.

**Figure 4:** Number of bird species according to habitat types and feeding guilds, recorded in May 2024 at the Sungai Talibu Forest Reserve, Telupid, Sabah, Malaysia.

## DISCUSSION

The avifaunal survey conducted at the STFR reveals a robust diversity of bird species, with a Shannon Diversity Index of H=3.88 and a species richness of 107 species across 40 families, reflecting an above-average avifaunal composition for a forest reserve of its type. The large number of forest specialists (101) suggests ecological integrity for the STFR's forest habitats, despite its proximity to disturbance in the surrounding forest reserves and oil palm estates. Notably, the presence of nine Bornean endemics, including the Vulnerable Bornean Ground Cuckoo and the Near Threatened Bornean Bristlehead and Charlotte's Bulbul, highlights the conservation significance of the STFR within the Bornean biodiversity hotspot. The detection of Critically Endangered (Helmeted Hornbill) and Endangered (Greater Green Leafbird) species further elevates the reserve's importance as a refuge for globally threatened avifauna.

However, the species accumulation curve derived from our MacKinnon Lists (Fig. 3) has not reached a plateau, indicating that we conducted too few surveys to truly estimate the number of forest species. This underestimate was suggested by the 31 undetected species predicted by the Chao1 estimate of 138 species. Additional surveys, particularly in the upland ultramafic forests and logged-over mixed dipterocarp forests, could refine our understanding of the reserve's total species pool.

Drawing on a series of surveys conducted by the authors using a consistent modified ML method, we compared bird diversity to other forest reserves that, like the STFR, have experienced varying degrees of logging or disturbance (see Appendix III for a full summary of species and family richness across 22 forest reserves in Sabah). Among those on ultramafic soils, Sapagaya Forest Reserve recorded 114 species from 43 families (Joeman et al., 2020a), while Bukit Hampuan yielded 71 species from 33 families (Petol et al., 2021a). Both sites differ from the STFR in elevation: Sapagaya spans lowland dipterocarp to upland ultramafic forest (~880 m a.s.l.), whereas Bukit Hampuan is a lower montane reserve with surveys reaching 1,100 m a.s.l. Despite being

surveyed primarily in lowland to upland forest, the STFR's richness (107 species, 40 families) closely approaches that of Sapagaya and exceeds that of Bukit Hampuan. The STFR also compares favourably with non-ultramafic reserves. Mensalong, for example, recorded 108 species from 42 families (Joeman et al., 2020b), while Mengilan reported 106 species from 41 families (Petol et al., 2021b). In contrast, lower richness was documented in Mount Mandalom (91 species, 38 families; Joeman et al., 2024) and Sungai Tongod (96 species, 38 families; Joeman et al., 2023). These comparisons suggest that the STFR, despite a history of logging and limited elevational coverage, supports avifaunal diversity on par with Sabah's better-known forest reserves.

These comparisons provide a meaningful reference point for evaluating the STFR's bird diversity. To place these findings in a broader context, species richness at more intensively studied sites such as Danum Valley and Deramakot can be considered. Danum Valley, a largely undisturbed lowland dipterocarp forest on fertile sedimentary soils, has recorded over 275 bird species based on formal research (Adam & Omar, 2002; Sheldon et al., 2001, 2014). In contrast, citizen science platforms report higher numbers, with the Cornell Lab of Ornithology's eBird listing 341 species (Cornell Lab of Ornithology, 2024) and Avibase's Bird Checklist of the World reporting 388 species (Lepage, 2025). Deramakot, a selectively logged but well-managed lowland forest on similar sedimentary substrates, supports over 147 species from formal surveys (Bili, 2013), including all eight Bornean hornbills and the Bornean Bristlehead while eBird lists 296 species (Cornell Lab of Ornithology, 2024). Although these sites differ from STFR in terms of soil type and forest management history, their species richness values, derived from both structured research and birdwatching platforms, provide useful upper-bound benchmarks. These figures highlight the conservation value of STFR's avifauna within the broader context of lowland forest diversity in Sabah.

The prevalence of Pycnonotidae and Nectariniidae as the most abundant and species-rich families aligns with patterns observed in other forest reserves, likely reflecting their vagility and adaptability to a range of forest conditions and resource availability. Feeding guild analysis reveals a clear predominance of insectivores and frugivores, comprising 81.84% of species, a figure consistent with the mean of 77.59±6.15% across surveyed reserves (Table 4). This stability in guild proportions, evidenced by the low coefficient of variation (CV=7.92%) for combined insectivorous and frugivorous species, suggests a predictable ecological structure across forest reserves, irrespective of degradation levels or abiotic variables.

Insectivores, represented by 174 individuals across 55 species, likely benefit from the relatively abundant arthropod prey in the STFR's forest understory. However, only the generalist Malaysian Blue Flycatcher (*Cyornis turcosus*) was detected, while more forest-dependent species such as the Sunda Blue (*C. banyumas*) and Bornean Blue Flycatcher (*C. superbus*) were notably absent. This absence aligns with observations by Wong (1986) that specialist blue flycatchers tend to decline in disturbed or regenerating forests. Similarly, although four species of Timaliidae were recorded, none were strict forest specialists. Wong's study highlighted that habitat disturbance disproportionately affects such understory insectivores, particularly among the babbler guild. The persistence of generalist species, such as the Bold-striped Tit-babbler, coupled with the limited presence of microhabitat specialists, suggests that while the STFR retains some key structural features, it may not yet provide the habitat complexity required to support the full complement of specialist insectivores.

In contrast, the Pellorneidae were better represented in the present study, with seven species (15 individuals), including Bornean Black-capped Babbler (*Pellorneum capistratoides*), Bornean Swamp Babbler (*P. macropterum*), Ferruginous Babbler (*P. bicolor*), and Mourning Babbler (*P. malaccense*). Although specific Pellorneidae taxa were not enumerated by Wong (1986), his study highlighted the vulnerability of ground- and understorey-foraging insectivores to habitat disturbance. The presence of multiple Pellorneidae species in the STFR suggests that certain aspects of forest floor and understorey structure, such as leaf litter and dense vegetation, have been retained or have sufficiently recovered. Nonetheless, the absence of more microhabitat-sensitive Timaliidae and specialist flycatchers further underscores that the STFR may still lack the full structural and microclimatic complexity required to support the most disturbance-sensitive components of the insectivore guild.

Frugivores were the second most prevalent feeding guild in the STFR, with 146 individuals across 30 species and 13 families, markedly led by bulbuls (Pycnonotidae; 45 individuals), especially the Cream-vented and Spectacled Bulbuls (11 each), followed by Charlotte's Bulbul (7 individuals). This pattern is consistent with other studies in Malaysian dipterocarp forests, where Pycnonotidae frequently dominate frugivore assemblages in both primary and logged habitats (Shafie et al*.,* 2023). Seasonal studies on Mount Kinabalu further demonstrated that frugivorous bird activity closely follow fruiting phenology, with fluctuations in abundance corresponding to temporal peaks in fruit availability (Kimura et al*.,* 2001). Although neither Wong (1986) nor Pollock et al*.* (2022) systematically addressed frugivores, other regional studies have noted a similar trend: generalist frugivores such as bulbuls tend to persist in regenerating or structurally simplified forests, while more specialised canopy or fig-associated frugivores may decline.

The presence of additional frugivorous taxa in the STFR—including two species of hornbills (seven individuals), five species of barbets (16 individuals), the Bornean Bristlehead (five individuals), two species of flowerpeckers (17 individuals), and two species of leafbirds (18 individuals)—further supports the conclusion that the forest's vertical vegetation structure and fruit resource availability have recovered to a functionally meaningful extent. While some canopy frugivore specialists may still be underrepresented, the observed diversity and abundance of small- to large-bodied frugivores suggest that the STFR is capable of supporting a broad spectrum of frugivore guilds, especially those associated with fruiting shrubs, mid-storey trees, and light-gap canopy species.

Open-area species were scarce, with only six species and 20 individuals recorded. These included the Barn Swallow (*Hirundo rustica*), Pacific Swallow (*Hirundo tahitica*), Javan Myna (*Acridotheres javanicus*), Dusky Munia (*Lonchura fuscans*), Brown-throated Sunbird (*Anthreptes malacensis borneensis*), and Yellow-bellied Prinia (*Prinia flaviventris latrunculus*)—all typical of disturbed or open habitats. In the STFR, the swallows were observed sallying for insects along gravel and earth roads, while the remaining species were confined to grassy verges along these roads—microhabitats that mimic open-country conditions. Their limited distribution supports the view that the STFR's interior remains largely unaffected by edge effects, likely due to its isolation from nearby oil palm estates.

**Table 4:** Comparison of insectivores/frugivores in Sungai Talibu Forest Reserve and in other selected forest reserves (in alphabetical order).

| Forest reserve/ Site | Total no. of species, S | % of insectivorous + frugivorous species | Insectivores | | | | | Frugivores | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | % of total individuals | No. of species | % of total species | No. of families | Biomass (g) | % of total individuals | No. of species | % of total species | No. of families | Biomass (g) |
| Balingkadus | 86 | 82.56 | 53.87 | 42 | 48.84 | 22 | 5009.72 | 26.29 | 29 | 32.56 | 10 | 15337.11 |
| Bkt. Gemok | 69 | 72.46 | 47.83 | 33 | 47.83 | 19 | 4641.50 | 24.64 | 17 | 24.64 | 10 | 19178.22 |
| Bkt. Hampuan | 71 | 81.69 | 53.19 | 36 | 50.70 | 21 | 4717.83 | 15.17 | 22 | 28.17 | 8 | 6931.63 |
| Bkt. Mentapok & Bkt. Monkobo | 89 | 76.40 | 48.10 | 40 | 44.94 | 19 | 6772.69 | 35.31 | 28 | 31.46 | 12 | 40155.60 |
| Gn. Tingkar | 114 | 81.58 | 55.81 | 67 | 58.77 | 23 | 11099.95 | 29.66 | 26 | 22.81 | 11 | 29489.30 |
| Kabili-Sepilok | 159 | 72.96 | 57.27 | 77 | 48.43 | 28 | 36673.21 | 25.57 | 39 | 24.53 | 12 | 92924.35 |
| Kawang | 47 | 76.60 | 38.67 | 22 | 46.81 | 15 | 2215.17 | 28.89 | 14 | 29.79 | 9 | 7791.81 |
| Meliau Range | 90 | 68.89 | 47.83 | 42 | 46.67 | 20 | 4382.02 | 25.47 | 20 | 22.22 | 10 | 15169.52 |
| Menghilan | 75 | 85.33 | 54.73 | 40 | 53.33 | 18 | 6947.05 | 25.72 | 24 | 32.00 | 12 | 30802.57 |
| Mensalong | 101 | 81.19 | 42.76 | 48 | 47.52 | 21 | 5988.00 | 43.46 | 34 | 33.66 | 12 | 48088.58 |
| Mt. Mandalom | 92 | 75.00 | 45.65 | 42 | 45.65 | 21 | 3428.49 | 29.35 | 27 | 29.35 | 11 | 16960.58 |
| Nuluhon-Trusmadi (2023 & 2024) | 124 | 83.06 | 49.89 | 76 | 61.29 | 26 | 12347.45 | 24.31 | 27 | 21.77 | 11 | 47149.66 |
| Ranforest Discovery Centre, Sepilok | 154 | 68.18 | 50.09 | 68 | 44.16 | 25 | 54770.35 | 24.44 | 37 | 24.03 | 12 | 133951.61 |
| Sepagaya | 116 | 80.17 | 61.40 | 60 | 51.72 | 26 | 12696.75 | 25.80 | 33 | 28.45 | 13 | 32466.92 |
| Sg. Pin CA | 65 | 60.00 | 46.25 | 26 | 40.00 | 17 | 5168.70 | 21.32 | 13 | 20.00 | 7 | 61184.81 |

*(Continued to next page)*

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| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Sg. Rawog CA (2018) | 120 | 82.50 | 52.50 | 63 | 52.50 | 21 | 19340.52 | 37.13 | 36 | 30.00 | 12 | 22118.96 |
| Sg. Rawog CA (2023) | 88 | 80.68 | 52.56 | 41 | 46.59 | 16 | 5201.18 | 34.88 | 30 | 34.09 | 11 | 53841.33 |
| Sg. Talibu | 107 | 79.44 | 42.70 | 55 | 51.40 | 20 | 6591.83 | 37.30 | 30 | 28.04 | 13 | 40093.34 |
| Sg. Tindikon & Sg. Tikolod | 79 | 79.75 | 57.19 | 39 | 49.37 | 22 | 4237.60 | 21.10 | 24 | 30.38 | 10 | 20690.51 |
| Sg. Tongod | 85 | 80.00 | 54.68 | 44 | 51.76 | 22 | 4389.91 | 34.21 | 24 | 28.24 | 10 | 41824.19 |
| Tawai | 68 | 80.88 | 54.43 | 38 | 55.88 | 17 | 3411.02 | 32.91 | 17 | 25.00 | 9 | 17570.34 |
| **Mean, μ** | 95.19 | 77.59 | 50.83 | 47.57 | 49.72 | 20.90 | 10477.66 | 28.71 | 26.24 | 27.68 | 10.71 | 37796.24 |
| **SD** | 28.43 | 6.15 | 5.64 | 15.51 | 4.95 | 3.42 | 12710.37 | 6.66 | 7.37 | 4.13 | 1.59 | 30044.75 |
| **CV (%)** | **29.87** | **7.92** | **11.09** | **32.60** | **9.95** | **16.36** | **121.31** | **23.19** | **28.11** | **14.94** | **14.80** | **79.49** |

Table 4 compares insectivore and frugivore figures between STFR and other selected forest reserves surveyed by the authors using the ML method. Notably, the percentage of total insectivorous and frugivorous species across all forest reserves showed the least variation (CV = 7.92%), suggesting that this characteristic remains highly consistent and predictable, regardless of factors such as forest quality, past management history, or abiotic influences like precipitation, elevation, and soil types. However, understanding how these dominant feeding guilds interact with other ecological factors in degraded forest reserves to maintain this stability is beyond the scope of these surveys.

Insectivorous and frugivorous birds were the dominant feeding guilds across all sites. At the STFR, they made up 79.4% of the total species recorded, closely matching the overall mean of 77.6% (±6.2%) across all forest reserves, and just below the peak value of 85.3% observed at Mengilan FR. When examined separately, the proportion of insectivores and frugivores—by species count and individual numbers—varied moderately among sites. However, the number of families within each guild was remarkably consistent. Insectivores averaged 20.9 families per site (±3.4; CV = 16.4%), while frugivores averaged 10.7 families (±1.6; CV = 14.8%). These low coefficients of variation suggest a stable taxonomic breadth across sites, despite differences in overall species richness and forest conditions.

Interestingly, we recorded the Scarlet-breasted Flowerpecker (*Prionochilus thoracicus*) but not the Grey-breasted Babbler (*Malacopteron albogulare*), both of which are rare in Sabah and typically associated with ultramafic or peat swamp forests (Davies & Payne, 1982; Sheldon et al., 2009, 2014). The presence of *P. thoracicus* may reflect the distinctive floristic composition of STFR, which is underlain by ultramafic soils, whereas the absence of *M. albogulare* aligns with its generally low detectability and narrower microhabitat requirements. Both species were previously recorded in the peat swamp forest of Klias Forest Reserve, reinforcing their shared affinity for structurally complex and edaphically distinct habitats. Although ultramafic forests are often floristically simple, they provide critical refugia for habitat specialists and disturbance-sensitive birds. The occurrence of *P. thoracicus* in STFR underscores the conservation value of such forests in maintaining regional avifaunal diversity, while the absence of *M. albogulare* may reflect either its naturally low density, patchy distribution, or more likely, its low detectability due to its skulking behaviour, low vocal activity, and preference for dense understory.

## CONCLUSIONS

The avifaunal survey of STFR reveals a relatively rich and diverse bird community (107 species), comparable to other forest reserves surveyed using the ML method, as discussed above. The high proportion of forest-dependent species, along with the presence of habitat-restricted and rare taxa such as the Scarlet-breasted Flowerpecker, Bornean Ground Cuckoo, and Bornean Bristlehead, underscores the reserve's conservation importance. The dominance of families such as Pycnonotidae and Nectariniidae, and the prevalence of insectivorous and frugivorous guilds, reflect a structurally intact forest consistent with patterns observed in other high-integrity reserves. While the survey accounted for 77.5% of the estimated species pool, additional richness is likely in unsampled microhabitats, suggesting that true avifaunal value of the STFR remains

underrepresented. These results support the case for sustained protection and monitoring to safeguard the ecological integrity of the STFR as a stronghold for Sabah unique ultramafic birdlife.

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## DECLARATIONS

**Research permit(s).** Not applicable because the study was conducted as part of an expedition conducted by the Forest Research Centre, Sabah Forestry Department and all authors are staff of the Centre.
**Ethical approval/statement.** Not applicable.
**Generative AI use.** We declare that Grammarly was used to edit this manuscript prior to submission, and was used in compliance with the JTBC policies. We have reviewed and edited the content after using this tool/service, and I/we take(s) full responsibility for the content of the publication.

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## APPENDIX I

### Complete list of bird species recorded in May 2024 at the Sungai Talibu Forest Reserve (Class I), Telupid, Sabah, Malaysia.

Common names are sorted alphabetically. Names in bold denote Bornean endemics. All taxa are according to the classification in Gill et al. (2025) and conservation status is according to IUCN Red List (2025).

| Common name | Species | Family |
|---|---|---|
| Ashy Tailorbird | *Orthotomus ruficeps borneoensis* | Cisticolidae |
| Asian Fairy-bluebird | *Irena puella* | Irenidae |
| Asian Red-eyed Bulbul | *Pycnonotus brunneus brunneus* | Pycnonotidae |
| Banded Bay Cuckoo | *Cacomantis sonneratii* | Cuculidae |
| Banded Broadbill | *Eurylaimus javanicus* | Eurylaimidae |
| Barn Swallow | *Hirundo rustica* | Hirundinidae |
| Black-and-red Broadbill | *Cynbirhynchus macrorhynhos macrorhychus* | Eurylaimidae |
| Black-and-yellow Broadbill | *Eurylaimus ochromalus* | Eurylaimidae |
| Black-bellied Malkoha | *Phaenicophaeus diardi* | Cuculidae |
| Black-eared Barbet | *Psilopogon duvaucelii* | Megalaimidae |
| Black-headed Bulbul | *Brachypodius melanocephalos* | Pycnonotidae |
| Black-naped Monarch | *Hypothymis azurea* | Monarchidae |
| Blue-crowned Hanging Parrot | *Loriculus galgulus* | Psittaculidae |
| Blue-eared Kingfisher | *Alcedo meninting meninting* | Alcedinidae |
| Blyth's Paradise Flycatcher | *Terpsiphone affinis borneensis* | Monarchidae |
| Bold-striped Tit-babbler | *Mixornis bornensis* | Timaliidae |
| **Bornean Black-capped Babbler** | *Pellorneum capistratoides* | Pellorneidae |
| **Bornean Bristlehead** | *Pityriasis gymnocephala* | Pityriasidae |
| **Bornean Brown Barbet** | *Calorhamphus fuliginosus* | Megalaimidae |
| **Bornean Ground Cuckoo** | *Carpococcyx radiceus* | Cuculidae |
| Brown Boobook | *Ninox scutulata borneensis* | Strigidae |
| Brown Fulvetta | *Alcippe brunneicauda* | Alcippeidae |
| Brown-backed Needletail | *Hirundapus giganteus giganteus* | Apodidae |
| Brown-throated Sunbird | *Anthreptes malacensis bornensis* | Nectariniidae |
| Buff-rumped Woodpecker | *Meiglyptes grammithorax* | Picidae |
| Buffy Fish Owl | *Ketupa ketupu pageli* | Strigidae |
| Changeable Hawk-eagle | *Nisaetus cirrhatus* | Accipitridae |
| **Charlotte's Bulbul** | *Iole charlotte* | Pycnonotidae |
| Common Hill Myna | *Gracula religiosa religiosa* | Sturnidae |
| **Cream-eyed Bulbul** | *Pycnonotus pseudosimplex* | Pycnonotidae |
| Cream-vented Bulbul | *Pycnonotus simplex perplexus* | Pycnonotidae |
| Crested Serpent Eagle | *Spilornis cheela* | Accipitridae |
| Dark-necked Tailorbird | *Orthotomus atrogularis humphreysi* | Cisticolidae |
| Diard's Trogon | *Harpactes diardii* | Trogonidae |
| **Dusky Munia** | *Lonchura fuscans* | Estrildidae |
| Everett's White-eye | *Zosterops everetti* | Zosteropidae |
| Ferruginous Babbler | *Pellorneum bicolor* | Pellorneidae |
| Finsch's Bulbul | *Iole finschii* | Pycnonotidae |
| Great Argus | *Argusianus argus grayi* | Phasianidae |
| Great Slaty Woodpecker | *Mulleripicus pulverulentus* | Picidae |
| Greater Green Leafbird | *Chloropsis sonnerati zosterops* | Chloropseidae |
| Greater Racquet-tailed Drongo | *Dicrurus paradiseus* | Dicruridae |

| | | |
|---|---|---|
| Green Broadbill | *Calyptomena viridis gloriosa* | Calyptomenidae |
| Green Iora | *Aegithina viridissima* | Aegithinidae |
| Grey-bellied Bulbul | *Ixodia cyaniventris paroticalis* | Pycnonotidae |
| Grey-hooded Babbler | *Cyanoderma bicolor bicolor* | Timaliidae |
| Grey-rumped Treeswift | *Hemiprocne longipennis harterti* | Hemiprocnidae |
| Hairy-backed Bulbul | *Tricholestes criniger* | Pycnonotidae |
| Helmeted Hornbill | *Rhinoplex vigil* | Bucerotidae |
| Horsfield's Babbler | *Malacocincla sepiaria* | Pellorneidae |
| Indian Cuckoo | *Cuculus micropterus concretus* | Cuculidae |
| Javan Myna | *Acridotheres javanicus* | Sturnidae |
| Lesser Green Leafbird | *Chloropsis cyanopogon cyanopogon* | Chloropseidae |
| Little Green Pigeon | *Treron olax* | Columbidae |
| Little Spiderhunter | *Arachnothera longirostra buettikoferi* | Nectariniidae |
| Long-billed Spiderhunter | *Arachnothera robusta robusta* | Nectariniidae |
| Long-tailed Parakeet | *Psittacula longicauda longicauda* | Psittaculidae |
| Malaysian Blue Flycatcher | *Cyornis turcosus* | Muscicapidae |
| Malaysian Pied Fantail | *Rhipidura javanica longicauda* | Rhipiduridae |
| Orange-backed Woodpecker | *Reinwardtipicus validus xanthopygius* | Picidae |
| Orange-bellied Flowerpecker | *Dicaeum trigonostigma dayakanum* | Dicaeidae |
| Oriental Dollarbird | *Eurystomus orientalis orientalis* | Coraciidae |
| Oriental Dwarf Kingfisher | *Ceyx erithaca motleyi* | Alcedinidae |
| Pacific Swallow | *Hirundo tahitica* | Hirundinidae |
| Plaintive Cuckoo | *Cacomantis merulinus threnodes* | Cuculidae |
| Plume-toed Swiftlet | *Collocalia affinis cyanoptila* | Apodidae |
| Purple-naped Spiderhunter | *Kurochkinegramma hypogrammicum* | Nectariniidae |
| Raffles's Malkoha | *Rhinortha chlorophaea* | Cuculidae |
| Red-bearded Bee-eater | *Nyctyornis amictus* | Meropidae |
| Red-billed Malkoha | *Zanclostomus javanicus pallidus* | Cuculidae |
| Red-crowned Barbet | *Psilopogon rafflesii* | Megalaimidae |
| Red-naped Trogon | *Harpactes kasumba* | Trogonidae |
| Red-throated Barbet | *Psilopogon mystacophanos* | Megalaimidae |
| Rhinoceros Hornbill | *Buceros rhinoceros* | Bucerotidae |
| Rufous Piculet | *Sasia abnormis* | Picidae |
| Rufous-collared Kingfisher | *Actenoides concretus borneanus* | Alcedinidae |
| Rufous-crowned Babbler | *Malacopteron magnum saba* | Pellorneidae |
| Rufous-fronted Babbler | *Cyanoderma rufifrons* | Timaliidae |
| Rufous-tailed Shama | *Copsychus pyrropygus* | Muscicapidae |
| Rufous-tailed Tailorbird | *Orthotomus sericeus sericeus* | Cisticolidae |
| Rufous-winged Philentoma | *Philentoma pyrhoptera pyrhoptera* | Vangidae |
| Scarlet Minivet | *Pericrocotus speciosus insulanus* | Campephagidae |
| Scarlet-breasted Flowerpecker | *Prionochilus thoracicus* | Dicaeidae |
| Scarlet-rumped Trogon | *Harpactes duvaucelii* | Trogonidae |
| Short-tailed Babbler | *Pellorneum malaccense poliogene* | Pellorneidae |
| Silver-rumped Spinetail | *Rhaphidura leucopygialis* | Apodidae |
| Slender-billed Crow | *Corvus enca compilator* | Corvidae |
| Sooty-capped Babbler | *Malacopteron affine phoeniceum* | Pellorneidae |
| Spectacled Bulbul | *Ixodia erythropthalmos* | Pycnonotidae |
| Spectacled Spiderhunter | *Arachnothera flavigaster* | Nectariniidae |
| Spotted Dove | *Spilopelia chinensis tigrina* | Columbidae |
| Square-tailed Drongo-cuckoo | *Surniculus lugubris brachyurus* | Cuculidae |
| Stork-billed Kingfisher | *Pelargopsis capensis inominata* | Alcedinidae |
| Streaked Bulbul | *Ixos malaccensis* | Pycnonotidae |
| Sunda Scimitar-Babbler | *Pomatorhinus bornensis* | Timaliidae |
| Sunda Scops Owl | *Otus lempiji lempiji* | Strigidae |
| Temminck's Sunbird | *Aethopyga temminckii* | Nectariniidae |

| | | |
|---|---|---|
| Thick-billed Spiderhunter | *Arachnothera crassirostris* | Nectariniidae |
| Van Hasselt's Sunbird | *Leptocoma brasiliana brasiliana* | Nectariniidae |
| Ventriloquial Oriole | *Oriolus consobrinus* | Oriolidae |
| Verditer Flycatcher | *Eumyias thalassina* | Muscicapidae |
| Whiskered Treeswift | *Hemiprocne comata comata* | Hemiprocnidae |
| White-bellied Woodpecker | *Dryocopus javensis javensis* | Picidae |
| White-chested Babbler | *Pellorneum rostratum macropterum* | Pellorneidae |
| **White-crowned Shama** | *Copsychus stricklandi* | Muscicapidae |
| Yellow-bellied Prinia | *Prinia flaviventris latrunculus* | Cisticolidae |
| Yellow-crowned Barbet | *Psilopogon henricii brachyrhynchus* | Megalaimidae |

## APPENDIX II

### Bird families recorded in May 2024 at the Sungai Talibu Forest Reserve (Class I), Telupid, Sabah, Malaysia.

The list is sorted according to the highest number of species.

| Family | No. of species | No. of individuals |
|---|---|---|
| Pycnonotidae | 10 | 45 |
| Nectariniidae | 8 | 41 |
| Cuculidae | 8 | 22 |
| Pellorneidae | 7 | 15 |
| Megalaimidae | 5 | 16 |
| Picidae | 5 | 8 |
| Timaliidae | 4 | 22 |
| Cisticolidae | 4 | 14 |
| Muscicapidae | 4 | 9 |
| Alcedinidae | 4 | 5 |
| Apodidae | 3 | 10 |
| Eurylaimidae | 3 | 10 |
| Trogonidae | 3 | 4 |
| Strigidae | 3 | 3 |
| Psittaculidae | 2 | 19 |
| Chloropseidae | 2 | 18 |
| Dicaeidae | 2 | 17 |
| Monarchidae | 2 | 15 |
| Hemiprocnidae | 2 | 8 |
| Bucerotidae | 2 | 7 |
| Sturnidae | 2 | 7 |
| Accipitridae | 2 | 4 |
| Hirundinidae | 2 | 4 |
| Columbidae | 2 | 2 |
| Aegithinidae | 1 | 13 |
| Phasianidae | 1 | 9 |
| Estrildidae | 1 | 8 |
| Zosteropidae | 1 | 7 |
| Pityriasidae | 1 | 5 |
| Oriolidae | 1 | 4 |
| Alcippeidae | 1 | 3 |
| Calyptomenidae | 1 | 2 |
| Campephagidae | 1 | 2 |
| Coraciidae | 1 | 2 |
| Corvidae | 1 | 2 |
| Dicruridae | 1 | 2 |
| Meropidae | 1 | 2 |
| Rhipiduridae | 1 | 2 |
| Vangidae | 1 | 2 |
| Irenidae | 1 | 1 |

# APPENDIX III

## Bird diversity metrics from 22 forest reserves and sites in Sabah, Malaysia.[1]

| Forest Reserve/Site | No. of individuals | H' | $H_{max}$ | $E_H$ | No. of species, S | $Num_{eff}$ | Chao1 est. | No. of families | Habitat type | Notes |
|---|---|---|---|---|---|---|---|---|---|---|
| Balingkadus | 388 | 3.890 | 4.45 | 0.87 | 86 | 48.91 | 114.00 | 37 | Disturbed upland & montane ultramafic forest | Petol et al., 2022a |
| Bukit Gemok | 273 | 3.870 | 4.23 | 0.91 | 69 | 47.94 | 91.13 | 34 | Isolated, disturbed lowland MDF | Surveyed 2019, unpubl. data |
| Bukit Hampuan | 408 | 3.440 | 4.26 | 0.81 | 71 | 31.19 | 90.50 | 33 | Isolated, heavily disturbed upland & lower montane ultramafic forest | Petol et al., 2021a |
| Bukit Mentapok & Bukit Mongkobo | 422 | 4.016 | 4.49 | 0.89 | 89 | 55.47 | 114.25 | 37 | Lowland to upland MDF & montane | Surveyed 2024 at northern uplands, unpubl. data |
| Gn. Tingkar | 654 | 4.270 | 4.74 | 0.90 | 114 | 71.52 | 135.63 | 38 | Old-growth logged lowland MDF | Surveyed 2020, unpubl. data |
| Kabili-Sepilok* | 1306 | 4.460 | 5.07 | 0.88 | 159 | 86.47 | 195.24 | 53 | Undisturbed lowland MDF | Surveyed 2019, unpubl. Data |
| Kawang | 225 | 3.390 | 3.85 | 0.88 | 47 | 29.67 | 60.02 | 29 | Disturbed lowland to upland MDF | In print |
| Meliau Range | 161 | 4.325 | 4.50 | 0.96 | 90 | 75.57 | 156.18 | 39 | Logged-over lowland to montane ultramafic forest | Surveyed 2025 at northern lowland ultramafic forest, unpubl. Data |
| Menghilan | 486 | 3.860 | 4.32 | 0.89 | 75 | 47.47 | 91.00 | 33 | Logged-over upland MDF & lower montane kerangas forest | Petol et al., 2021b. Surveyed at upland MDF. |
| Mensalong | 566 | 4.180 | 4.62 | 0.91 | 101 | 65.37 | 118.00 | 37 | Logged-over lowland & upland MDF | Joeman et al., 2020b |
| Mt. Mandalom | 242 | 4.250 | 4.52 | 0.94 | 92 | 70.11 | 133.31 | 38 | Logged-over lowland & upland MDF | Joeman et al., 2024 |
| Nuluhon-Trusmadi (cumulative) | 946 | 3.968 | 4.83 | 0.82 | 124 | 52.88 | 163.00 | 45 | Old-growth logged-over montane forest | Surveyed 2023 & 2024, unpubl. data |
| Rainforest Discovery Centre, Sepilok‡ | 2296 | 4.372 | 5.04 | 0.87 | 154 | 79.20 | 169.83 | 45 | Old-growth logged lowland MDF | Surveyed 2020, unpubl. data |
| Sepagaya | 624 | 4.170 | 4.74 | 0.88 | 114 | 64.72 | 146.56 | 43 | Lowland ultramafic & MDF forest | Joeman et al., 2020a |
| Sg. Talibu | 391 | 3.880 | 4.17 | 0.87 | 107 | 37.34 | 138.00 | 40 | Logged-over ultramafic forest | Current publication |
| Sg. Tindikon & Sg. Tikolod | 328 | 3.670 | 4.79 | 0.91 | 79 | 79.04 | 109.26 | 36 | Disturbed upland MDF | Petol et al., 2022b. |
| Sg. Tongod | 342 | 4.070 | 4.48 | 0.92 | 85 | 62.18 | 144.44 | 38 | Logged-over lowland & upland MDF | Joeman et al., 2023 |
| Sg. Pin Conservation Area | 333 | 3.620 | 4.67 | 0.83 | 65 | 48.42 | 83.78 | 33 | Disturbed lowland riverine forest | Surveyed 2025, unpubl. data |
| Sg. Rawog CA (cummulative) | 1072 | 4.490 | 4.94 | 0.91 | 140 | 89.12 | 162.78 | 44 | Logged-over MDF | Petol & Rudolf (2019), Petol et al. (2024), cumulative data unpubl. |
| Tawai | 158 | 3.940 | 4.44 | 0.92 | 68 | 58.56 | 100.43 | 29 | Lowland to montane ultramafic forest | Surveyed 2024, unpubl. data, surveyed at northern lowlands |
| Timbah & Tangkulap | 639 | 3.950 | 4.22 | 0.93 | 92 | 51.42 | 108.24 | 38 | Logged-over lowland & upland MDF | Surveyed 2025, unpubl. data |
| Ulu Kalang | 261 | 4.020 | 4.52 | 0.87 | 85 | 51.94 | 115.18 | 36 | Disturbed upland MDF | Joeman et al., 2019. |
| **Mean, μ** | 569 | 4.005 | 4.54 | 0.89 | 95.73 | 59.29 | 124.58 | 37.95 | | |
| **Std dev** | 486 | 0.301 | 0.30 | 0.04 | 28.90 | 16.67 | 33.03 | 5.58 | | |
| **CV** | 85.42 | 7.524 | 6.61 | 4.24 | 30.19 | 28.12 | 26.51 | 14.69 | | |

‡30-day survey.
* 4-day survey, 2 teams.
[1]For sites without corresponding publications, all species data and diversity metrics are derived from the authors' original field surveys (2018–2025) and remain unpublished.