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Molecular identification of yeasts from Turkish traditional cheeses: Extracellular enzyme activities and physiological properties important for dairy industry

MELİH GÜNAY[1], TÜLAY TURGUT GENÇ[2,♥]

[1]Graduate School of Natural and Applied Sciences, Çanakkale Onsekiz Mart University. 17020, Çanakkale, Turkey
[2]Department of Biology, Faculty of Sciences, Çanakkale Onsekiz Mart University. 17020, Çanakkale, Turkey. Tel. +90-2862180018,
♥email: tturgutgenc@comu.edu.tr

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Abstract. *Gunay M, Genc TT. 2023. Molecular identification of yeasts from Turkish traditional cheeses: Extracellular enzyme activities and physiological properties important for dairy industry. Nusantara Bioscience 15: 1-11.* The determination of yeast microbiota in cheeses and the physiological properties of yeasts are very important for the dairy industry. In addition, the physiological features, proteolytic and lipolytic activities, and stress tolerance of yeasts have a significant role in the selection of starter yeast species for cheese ripening. This study aimed to determine industrially important yeasts isolated from cheese samples. Molecular techniques identified the isolated yeast strains. The yeast strains' extracellular enzyme activities, fermentation capacities, and thermotolerance and osmotolerance properties were also evaluated. A total of 81 yeast strains were isolated and characterized from three types of cheese samples. PCR-RFLP determined the isolated yeast strains and sequence analysis of ITS1-5.8S-ITS2 and 26S rDNA regions. A maximum parsimony tree was constructed by MEGA X software to evaluate the phylogenetic relationship of identified yeast strains. *Candida intermedia*, *Candida parapsilosis*, *Clavispora lusitaniae*, *Debaryomyces hansenii*, *Kluyveromyces marxianus*, *Pichia kudriavzevii*, and *Wickerhamomyces anomalus* yeast species were identified on cheese samples. The distribution of identified yeast species on cheese samples was determined as 48.1% for *W. anomalus*, 17.3% for *K. marxianus*, 14.8% for *C. parapsilosis*, 8.6% for *D. hansenii*, 4.9% for *Cl. lusitaniae*, 3.7% for *C. intermedia* and 2.5% for *P. kudriavzevii*. The *W. anomalus* yeast species was common in three cheese types. All strains of *W. anomalus* and *P. kudriavzevii* yeast species, three *C. parapsilosis*, and two *Cl. lusitaniae* yeast strains have important physiological properties for industrial applications. These yeast strains have the potential to be used in combination as starter cultures to improve cheese maturation in the future. This comprehensive study identifies yeast species by ITS1-5.8S-ITS2 and 26S rDNA regions and determines industrially important yeast species using multiple criteria (extracellular enzyme activity, stress tolerance, and fermentation capacity).

Keywords: Cheese-related yeast, fermentation, lipase, protease, stress tolerance

INTRODUCTION

Yeasts have been used in industrial and biotechnological applications. They also possess significant roles in various fields such as food, pharmacy, agriculture, and the fermentation process. *Saccharomyces cerevisiae* and other non-*Saccharomyces* yeast species are used in baking, wine making, dairy production, biomedical research, and drug discovery. They are also used as a biocontrol agent, as in *Metschnikowia pulcherrima* and *Aureobasidium pullulans* (Parafati et al. 2015; Settier-Ramírez et al. 2021). Conventional and non-conventional yeast strains are also frequently used in the production of bioethanol, heterologous proteins and industrial enzymes (Steensels and Verstrepen 2014; Settier-Ramírez et al. 2021; Vincent et al. 2021). Stress-resistant yeasts with high osmo-, thermo- and ethanol tolerance are preferred especially in industrial areas involving fermentation processes such as wine and bioethanol production (Balakumar and Arasaratnam 2012). Yeasts are used in the fermentation process for different purposes such as increasing the flavor of the product, controlling microbial spoilage, and adjusting the alcohol and nutrient levels (Steensels and Verstrepen 2014). The main producers of industrially important microbial enzymes are bacteria, filamentous fungi and a limited number of yeast species. *Candida boidinii*, *Candida pseudotropicalis*, *Candida rugosa*, *Cryptococcus laurentii*, *Geotrichum candidum*, *Kluyveromyces marxianus*, *Komagataella pastoris*, *Ogataea polymorpha*, *Pseudozyma antarctica*, *Rhodotorula* spp., *S. cerevisiae*, *Sporobolomyces salmonicolor*, *Trichosporon fermentum*, *Yarrowia lipolytica*, and *Zygosaccharomyces rouxii* yeast species are used for the production of industrial enzymes such as phenylalanine ammonia lyase (PAL), L-gutaminase, α-galactosidase, phytase, chymosin, lactase, inulinase, invertase, lipase and protease (Fonseca et al. 2008; Johnson and Echavarri-Erasun 2011; Johnson 2013a,b). Yeast proteases and lipases are important as they contribute to cheese flavor during the ripening process, even if they cause adverse taste, appearance and odor. However, the identification of new yeast strains with high protease and lipase activity is important for the dairy industry to increase product efficiency. It is known that the strains of *Clavispora*

lusitaniae and *Candida parapsilosis* yeast species have proteolytic activity while the strains of *K. marxianus* yeast species have both proteolytic and lipolytic activity (Binetti et al. 2013). The extracellular enzyme properties of yeast species may differ between strains of the same species. They can also be different depending on growth conditions and environmental factors (de Araújo et al. 2010; Molnárová et al. 2014; Delgado-Ospina et al. 2020).

The maturation and production of cheese is a very sophisticated process that involved different biochemical reactions. The cheese microbiota, which includes bacteria, molds and yeasts, contributes to the characteristic features of cheese and the ripening process, allowing different cheese varieties to be obtained (Montel et al. 2014; Banjara et al. 2015). Yeasts associated with cheese can have beneficial and non-beneficial effects on cheeses. Some yeasts may improve cheese quality by affecting its texture, ripening, and flavor, while others may cause spoilage, discoloration, and unpleasant odor or taste (Binetti et al. 2013; Hatoum et al. 2013). For example, *Debaryomyces hansenii* and *Candida krusei* yeast species promote the ripening of German Harzer and Quark cheeses, whereas *K. lactis* and *K. marxianus* cause spoilage of white-brined cheeses (Fröhlich-Wyder et al. 2019; Geronikou et al. 2020). Although the positive or negative contributions of yeasts vary at both species and strain levels, overgrowth of yeasts negatively affects cheese quality. Therefore, determining the microbial diversity of cheeses is important for the manufacturers in terms of increasing the quality and shelf life of cheese. In previous studies, different yeast strains have been isolated and identified from many commercial and local dairy products. The most commonly identified yeast species belong to *Candida*, *Cryptococcus*, *Debaryomyces*, *Geotrichum*, *Kluyveromyces*, *Trichosporon*, *Rhodotorula*, *Torulaspora*, *Saccharomyces*, and *Yarrowia* genera (Vasdinyei and Deak 2003). Yeast species isolated and identified from some local cheeses (white, tulum, Mihalic cheeses, etc.) belong to the genera of *Debaryomyces*, *Pichia*, *Geotrichum*, *Trichosporon*, *Kluyveromyces* and *Saccharomyces* (Hayaloğlu et al. 2002; Kavas et al. 2006; Çorbaci et al. 2012; Karasu-Yalcin et al. 2017). Generally, *D. hansenii*, *K. marxianus*, *K. lactis*, and *Y. lipolytica* are the predominant yeast species in the different cheese types (Togay et al. 2020; Çorbaci et al. 2012).

The diversity and density of yeasts vary according to temperature, ability to use lactose and other carbon sources, extracellular enzyme activities, stress tolerance, cheese-type, ripening process and also geographical locations (Merchan et al. 2021). Therefore, the main purpose of this study is to identify new yeast strains with potential for industrial applications. For this purpose, the isolated yeast strains from the traditional cheese samples (white, goat and cheddar) were identified by physiological (API-ID32C aux system) and molecular (PCR-RFLP and sequence analysis of both ITS1-5.8S-ITS2 and 26S rDNA regions) methods. Next, extracellular enzyme activities (for protease and lipase), stress tolerance and fermentation abilities of the identified yeast strains were determined to evaluate whether the yeast strains are industrially important. Seven yeast species were identified from a total of 81 yeast strains isolated from cheese samples. *Wickerhamomyces anomalus* and *Pichia kudriavzevii* were determined as potential yeast species for industrial applications. This is the first comparative study to identify yeast strains using PCR-RFLP and sequence analysis of both ITS1-5.8S-ITS2 and 26S rDNA regions at the same time. It is also a comprehensive study that determines yeast species of industrial importance by using more than one criterion (extracellular enzyme activity, stress tolerance and fermentation capacity).

MATERIALS AND METHODS

Sampling and yeast isolation

Three different cheese samples (white, cheddar and goat cheeses) were collected from 6 different dairy producers in Bolu, Turkey. Five grams from each cheese sample were homogenized in a 2% sodium citrate solution and spread onto yeast extract glucose chloramphenicol agar medium (40 g/L, YGC) including 0.1% sodium propionate. The colony-forming units (CFU) were determined after 3 days of incubation at 30°C. Yeast strains having different colony morphology (colour, shape, size, or texture) were selected randomly and streaked on the YP medium (10 g/L yeast extract, 20 g/L bacto-peptone, 20 g/L agar) supplemented with 2% Dextrose. Yeast isolates were maintained at 4°C for the biochemical tests and molecular identification, and stored at -80°C in 20% glycerol for further studies.

Physiological characterization of isolated yeasts

The biochemical profile of isolated yeast strains was performed by the API-ID32C aux system (Bio-Mérieux, France) following the manufacturer's instructions. The strip includes 32 wells to perform 29 assimilation tests for carbohydrates, organic acids, and amino acids. Exponentially grown yeast cells (OD_{600}=0.8-1.0) were resuspended in API C medium and 135 µL of the suspension was dispensed into each cupule of the strip. The results were recorded by direct reading after 24 hr or 48 hr of incubation at 30°C.

Molecular identification of isolated yeasts using rDNA sequence

Genomic DNA isolation was conducted by using a previously improved procedure (Lõoke et al. 2011). The one yeast colony was suspended in 100 µL 200 mM LiOAc and 1% SDS solution. The cell suspension was incubated at 70°C for 5 min, and then 300 µL of absolute ethanol was added. The cell suspension was centrifuged for 3 min at 15000 rpm and the supernatant was discarded. The pellet was washed with 1 mL 70% ethanol and centrifuged for 3 min at 15000 rpm again. The pellet was dissolved in 100 µLTE buffer (pH 8) and stored at -20ºC.

PCR-RFLP analysis

ITS1-5.8S-ITS2 rDNA region was amplified by using ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') primers (White et al.

1990) for PCR-RFLP (Restriction Fragment Length Polymorphism) analysis. D1/D2 rDNA region was amplified by using NL1 (5'-GCATATCAATAAGCGGAGGAAAAG-3') and NL4 (5'-GGTCCGTGTTTCAAGACGG-3') primers for PCR-RFLP analysis (Kurtzman and Robnett 1998). PCR amplification was studied with BIO-RAD thermal Cycler in 25 µL final volume, including 10X reaction buffer, 3mM $MgCl_2$, 10mM of dNTP, 10 pmol/µL of related primers, 1.25U Taq polymerase, and 50-100 ng DNA as a template. PCR conditions were determined as initial denaturation at 95°C for 5 min; 30 cycles of denaturing at 94°C for 3 min; annealing at 60°C (for ITS1-ITS4 primers) and 52°C (for NL1-NL4 primers) for 1 min, and extension at 72°C for 1 min; and a final extension step of 10 min at 72°C. PCR amplicons of ITS1-5.8S-ITS2 and D1/D2 rDNA region were digested with *Hae*III, *Hinf*I, *Hha*I (*Cfo*I), *Msp*I, and *Alu*I restriction endonucleases according to the supplier's instructions. PCR products and the restriction fragments were electrophoresed in 1.5% and 3% agarose gels, respectively, and photographed. The length of PCR amplicons and restriction fragments were calculated by using Gel-Pro Analyzer v4.0 software. The yeast strains were grouped according to restriction patterns.

Sequencing and phylogenetic analysis

Yeast strains having different restriction profiles were selected randomly and PCR products of ITS1-5.8S-ITS2 and D1/D2 rDNA were sequenced with the Applied Biotechnologies 3500xl Genetic Analyzer. The obtained sequences were analyzed with the Basic Local Alignment Search Tool (BLAST) on the National Center for Biotechnology Information (NCBI) web server (Altschul et al. 1990). ITS1-5.8S-ITS2 and D1/D2 rDNA region sequences of the selected yeast strains were submitted to the GenBank database to get the accession number. Phylogenetic analysis of sequenced yeast strains was determined with MEGA-X (Molecular Evolutionary Genetics Analysis) software (Kumar et al. 2018). ITS1-5.8S-ITS2 and D1/D2 rDNA sequences of yeast strains were aligned with ClustalW (v1.6) parameters. The maximum parsimony tree of these regions was constructed by utilizing Subtree-Pruning-Regrafting (SPR) parameters and the bootstrap method. 1000 bootstrap replicates were used to determine branch support and bootstrap values below 50% were not shown.

Proteolytic activity

Extracellular protease activity of the isolated yeast strains was determined with the skimmed milk agar (SMA) plate test (Abdelmoteleb et al. 2017). Skimmed milk powder stock solution (10%) and agar solution were autoclaved separately and mixed where the final concentration of skimmed milk was 1%. Yeast strains were grown in YPD broth up to exponential phase and inoculated as 5 µL droplets into SMA plates. Plates were incubated for up to 10 days at 30°C. The clear zone around the yeast colonies was considered protease activity. All yeast strains were grouped according to the zone diameter as having high, middle, and low protease activity.

Lipolytic activity

Lipolytic activities of yeast strains were detected with Tween 20/80 precipitation test (Kumar et al. 2012). Tween 20 and Tween 80 contain esters of low fatty acid chains and oleic acid, respectively. Therefore, Tween 20 is used for the detection of esterase activity and Tween 80 is used for lipase activity. Tween plates (10 g/L peptone, 5 g/L $NaCl_2$, 0.1 g/L $CaCl_2 \cdot 2H_2O$, 20 g/L agar) supplemented with 10 mL Tween 20 or Tween 80 were prepared and poured into plates. Yeast cells growing exponentially in YPD broth were inoculated onto Tween plates as 5 µl droplets and incubated at 30°C for 3-7 days. The appearance of white visible precipitate around the boundary of the colonies because of the deposition of calcium crystal salts was an indicator of lipolytic activity. All yeast strains were grouped according to the zone diameter as having high, middle and low lipase activity.

Temperature and glucose tolerance test

The temperature tolerance tests of yeast strains were performed on a YPD medium. The plates were incubated at 25, 30, 37, and 45°C for 2-3 days. The glucose tolerance tests of yeast strains were performed on a YP medium supplemented with 50% dextrose. The plates were incubated at 30°C for 2-3 days. The growth of yeast strains was signed positive or negative according to the growth situations.

Fermentation test

The carbohydrate utilization test was performed using a YP medium supplemented with 1.6% bromothymol blue as pH indicator and fermentable carbon sources (2% each of dextrose, galactose, sucrose, lactose and maltose). The Durham tubes were also placed into the media to detect gas production (Karki et al. 2017). The yeast strains were inoculated to cultures and incubated at 30°C for up to 15 days. The color change in the growth medium from blue-green to yellow indicated acid production, and the presence of bubbles in the inverted Durham tube indicated gas production. The yeast strains showing both acid and gas production were recorded as fermentation positive. All experiments were carried out under anaerobic conditions.

Statistical analysis

All the biochemical tests were assayed in at least triplicate. The results were analyzed using the SPSS software (version 10.0) to obtain means and standard deviations (SD). P values of <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Yeast identification and diversity

The white, cheddar and goat cheese samples were collected from six different dairy farms in Bolu, Turkey. A total of 81 yeast strains were isolated from cheese samples according to different colony morphology. The fungal load of cheese samples was determined as 5.34 log cfu/g for white cheese, 4.44 log cfu/g for cheddar cheese and 4.74

log cfu/g for goat cheese. Yeast counts in cheeses can vary between 2 and 9 log cfu/g depending on the ripening period and cheese types (Karasu-Yalcin et al. 2017; Bintsis 2021). In the present study, the yeast loads were found to be similar to the yeast counts in previous studies (Bintsis 2021). All isolated yeast strains were characterized using the API ID32C system and the results were analyzed with API-WEB v1.2.1 fungal database. The identified yeast strains were belonging to *Candida kefyr, C. parapsilosis, Candida pelliculosa, Candida famata, Candida intermedia, C. krusei* and *Candida lusitaniae* yeast species. It was observed that the assimilation profiles of the identified yeast strains were different. The yeast strains belonging to *C. kefyr* and *C. parapsilosis* showed seven different assimilation profiles (P1-P7). Similarly, six and three assimilation profiles were determined for *C. pelliculosa* and *C. famata* yeast strains, respectively. The other yeast strains belonging to *C. intermedia, C. krusei* and *C. lusitaniae* yeast species showed 2 assimilation profiles (Table 1).

Traditional identification methods are complicated processes and time-consuming and could give incorrect results (Pincus et al. 2007). Therefore, different commercial kit systems based on biochemical and assimilation tests, and DNA-based identification techniques (PCR-RFLP analysis, ITS1-5.8S-ITS2 and 26S rDNA sequences) have been used to identify yeast strains (Garnier et al. 2017; Karasu-Yalçın 2017; Benito et al. 2018; Moubasher et al. 2018; Haastrup et al. 2018; Genç and Günay 2020; Togay et al. 2020). Therefore, the isolated yeast strains were identified by PCR-RFLP and rDNA region sequence analysis. The genomic DNA extraction of all isolated yeast strains was carried out, and ITS1-5.8S-ITS2 and 26S rDNA regions were amplified. ITS1-5.8S-ITS2 and 26S rDNA regions of yeast strains were digested with five restriction endonucleases, and yeast strains were grouped for the restriction profiles (Table 2 and Table 3). Yeast strains showed 7 and 10 restriction profiles according to the PCR-RFLP results of ITS1-5.8S-ITS2 and 26S regions, respectively. It was determined that yeast strains grouped according to assimilation results showed a similar distribution according to ITS1-5.8S-ITS2 restriction profiles. However, there were differences between the groups of assimilations and the restriction profiles of the 26S region.

Table 1. Assimilation profile of yeast strains and identified species

Yeast species	Strain codes	Number of yeast strains
Candida kefyr	P1: W-13, W-14, W-18, W-19, W-20, W-22, W-24 P2: W-4, W-5 P3: W-9 P4: W-11 P5: C-28 P6: C-29 P7: C-20	14
Candida parapsilosis	P1: W-15, W-21 P2: W-6, W-7, W-12 P3: W-17 P4: W-3, W-8, W-25 P5: W-10 P6: W-16 P7: W-23	12
Candida pelliculosa	P1: W-2, C-2, C-3, C-7, C-8, C-10, C-11, C-16 P2: G-1, G-13, G-27 P3: C-1, C-5, C-6 P4: G-2, G-16 P5: C-4, C-9, C-12, C-13, C-14, C-15, C-21 P6: G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-14, G-15, G-19, G-21, G-23, G-24, G-25, G-26	39
Candida famata	P1: C-18, C-22, C-23 P2: C-24, C-25, C-26 P3: C-19	7
Candida intermedia	P1: C-17, C-27 P2: W-1	3
Candida crusei	P1: G-3 P2: G-4	2
Candida lusitaniae	P1: G-17, G-18 P2: G-20, G-22	4

Note: "P" indicates assimilation profile; "W" indicates white cheese; "C" indicates cheddar cheese; "G" indicates goat cheese

Table 2. PCR-RFLP results of ITS-5.8S rDNA region

Strain code	*Hae*III	*Hinf*I	*Hha*I	*Alu*I	*Msp*I
W-4, W-5, W-9, W-11, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	650-90	280-190-110-90-90	290-175-150-90	375-175-170	-
W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	-	315-310	575-60	500-105-50	-
W-3, W-6, W-7, W-8, W-10, W-12, W-15, W-16, W-17, W-21, W-23, W-25	450-125	300-300	320-240	540-50	500-90
C-18, C-19, C-22, C-23, C-24, C-25, C-26	425-140-85	315-310	290-290-60	-	-
G-17, G-18, G-20, G-22	-	200-200	200-190-90	-	275-110
W-1, C-17, C-27	-	200-200	190-180	-	300-100
G-3, G-4	390-95	260-210	200-190-90	390-95	250-215

Table 3. PCR-RFLP results of 26S rDNA region

Strain code	*Hae*III	*Hinf*I	*Hha*I	*Alu*I	*Msp*I
W-4, W-5, W-9, W-11, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	475-150	400-200	-	240-150-120-90	-
W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	475-150	250-180-175	-	250-190-110-80	-
G-5	290-200-125	390-175-80	-	250-190-110-80	-
W-3, W-6, W-7, W-8, W-10, W-12, W-15, W-16, W-17, W-21, W-23, W-25	475-150	400-200	-	250-190-110-80	490-150
C-18, C-19, C-22, C-23, C-24, C-25, C-26	475-150	400-200	-	225-200-100-90	-
G-17, G-18, G-20, G-22	290-110-100-85	300-115-100-80	290-125-120-75	225-200-100-90	400-175-60
W-1, C-17	390-95-90	380-225	340-150-100	250-190-110-80	290-180-140
C-27	475-150	400-200	340-150-100	225-200-100-90	290-180-140
G-3	290-200-125	300-300	250-280-150	430-190	420-200
G-4	225-220-90-90	400-120-75	250-280-150	430-190	420-200

Note: *All the yeast strains have the same PCR length for 26S rDNA region of about 600-650bp. Thus, data not shown in the table

Table 4. Identification of selected yeasts isolated from Turkish traditional cheeses

Yeast Strains	Identified yeast strains (Ref. Acc. Number)	GenBank Acc. No	Identified yeast strains (Ref. Acc. Number)	GenBank Acc. No
W-1	*C. intermedia* (DQ657830.1)	MT321268	*C. intermedia* (KX981200.1)	MT334438
W-3	*C. parapsilosis* (MK998693.1)	MT321173	*C. parapsilosis* (MK940816.1)	MT334455
W-16	*C. parapsilosis* (MH445556.1)	MT321276	*C. parapsilosis* (FJ432673.1)	MT334446
W-22	*K. marxianus* (KY103821.1)	MT321174	*K. marxianus* (KJ641888.1)	MT334456
W-24	*K. marxianus* (MH595342.1)	MT321278	*K. marxianus* (KJ641888.1)	MT334448
C-4	*W. anomalus* (KY105866.1)	MT321170	*W. anomalus* (MG773348.1)	MT334452
C-17	*C. intermedia* (DQ657830.1)	MT321171	*C. intermedia* (KX981200.1)	MT334453
C-21	*W. anomalus* (KY105868.1)	MT321272	*W. anomalus* (MG773348.1)	MT334442
C-22	*D. hansenii* (KP835570.1)	MT321273	*D. hansenii* (KY107525.1)	MT334443
C-25	*D. hansenii* (KY103209.1)	MT321172	*D. hansenii (*KY107525.1)	MT334454
C-27	*C. intermedia* (DQ657830.1)	MT321275	*C. intermedia* (KX981200.1)	MT334445
G-3	*P. kudriavzevii* (MH545928.1)	MT321265	*P. kudriavzevii* (MK881743.1)	MT334435
G-4	*P. kudriavzevii* (MH545928.1)	MT321167	*P. kudriavzevii* (MF377363.1)	MT334449
G-5	*W. anomalus* (KY105880.1)	MT321168	*W. anomalus* (KF612003.1)	MT334450
G-7	*W. anomalus* (KY105894.1)	MT321266	*W. anomalus* (MG773348.1)	MT334436
G-18	*Cl. lusitaniae* (KY102565.1)	MT321169	*Cl. lusitaniae* (MH892862.1)	MT334451

When assimilation groups were compared with the restriction groups of the 26S region, one of 39 yeast strains (G-5) defined as *C. pelliculosa* was separated from the other 38 yeast strains with *Hae*III and *Hinf*I restrictions. Similarly, C-27 yeast strain was separated from other two *C. intermedia* strains (W-1 and C-17) with the *Hae*III restriction. In addition, the strains of *C. krusei* yeast species, G-3 and G-4, showed different profiles with the *Hae*III and *Alu*I restrictions. It was observed that *C. kefyr*, *C. famata*, *C. parapsilosis* and *C. lusitaniae* yeast strains distributed in similar groups according to the assimilation test and restriction profile of the 26S region.

The different restriction profiles obtained from PCR-RFLP analysis can represent the different yeast species (Gibson et al. 2011). Although seven yeast species have been identified based on assimilation tests, ten different yeast species may have been isolated depending on the ten restriction profiles of the 26S region. Therefore, at least one yeast strain was randomly selected from each restriction profile of ITS-5.8S and 26S regions, and the amplification product of both ITS-5.8S and 26S regions was sequenced. The sequences of sixteen yeast strains (W-1, W-3, W-16, W-22, W-24, C-4, C-17, C-21, C-22, C-25, C-27, G-3, G-4, G-5, G-7, G-18) were analyzed by BLAST online tool from NCBI web server. The nucleotide sequences were submitted to the GenBank database and accession numbers were obtained for all sequences (Table 4). According to BLAST results, all yeast strains showed 97-99% similarity with reference strains for ITS-5.8S and 26S regions. G-3 and G-4 yeast strains were identified as *P. kudriavzevii*, G-5, G-7, C-4 and C-21 yeast strains identified as *W. anomalus*, G-18 yeast strain identified as *Cl. lusitaniae*, C-22 and C-25 yeast strains identified as *D. hansenii*, W-22 and W-24 yeast strains identified as *K. marxianus*, W-1, C-17 and C-27 yeast strains identified as *C. intermedia* and W-3 and W-16 yeast strains identified as *C. parapsilosis*. According to the APIWEB current database and previous studies, *C. krusei*, *C. pelliculosa*, *C. lusitaniae*, *C. famata*, and *C. kefyr* yeast species are synonymous with *P. kudriavzevii*, *W. anomalus*, *Cl.*

lusitaniae, *D. hansenii*, and *K. marxianus* yeast species, respectively (Kurtzman et al. 2011). Therefore, the yeast species identified by assimilation tests were identical to yeast species identified by sequence analysis. It has been previously reported that the percent confidence of API-ID32C results is less than that of molecular identification methods (Pincus et al. 2007). However, in recent studies, it has been reported that the reliability between the ID32C API System and 26S rDNA sequencing methods revealed a high correlation (Ceugniez et al. 2015). In this study, the results obtained with the API-ID32C identification kit system were found to be fully compatible with the results obtained by molecular identification methods. In addition, although the API-ID32C assimilation profile varies between strains of the same species, it was observed that the identification of yeasts was quite accurate. This may be because updates and revisions made in the API-ID32C kit and database allow for correct identification of yeasts.

The percent distribution of identified yeast species was determined as 48.1% for *W. anomalus*, 17.3% for *K. marxianus*, 14.8% for *C. parapsilosis*, 8.6% for *D. hansenii*, 4.9% for *Cl. lusitaniae*, 3.7% for *C. intermedia* and 2.5% for *P. kudriavzevii*. The diversity of yeast species on cheese types was given in Figure 1. The *W. anomalus* was abundant in goat cheese (21 of 27 strains) and cheddar cheese (17 of 29 strains). The *K. marxianus* and *C. parapsilosis* yeast species were distributed predominantly in white cheese samples. The other yeast species *Cl. lusitaniae* and *P. kudriavzevii* were distributed only in goat cheese samples, and *D. hansenii* was distributed only in cheddar cheese samples.

So far, *C. parapsilosis*, *C. albicans*, *C. diddensiae*, *C. haemulonii*, *C. membranifaciens*, *C. sake*, *C. tropicalis*, *C. versatilis*, *C. zeylanoides*, *Cl. lusitaniae*, *D. hansenii*, *Galactomyces geotrichum*, *K. lactis*, *K. marxianus*, *Meyerozyma guilliermondii* (formerly *P. guilliermondii*), *P. anomala*, *Rhodotorula mucilaginosa*, *S. cerevisiae*, *Torulaspora delbrueckii*, *Williopsis californica* and *Y. lipolytica* yeast species have been identified from different traditional Turkish cheeses (tulum, Kashkaval, Mihalic, örgü, white, sepet, and goat) (Yalçın and Uçar 2009; Çorbacı et al. 2012; Togay et al. 2020; Esen and Çetin 2021).

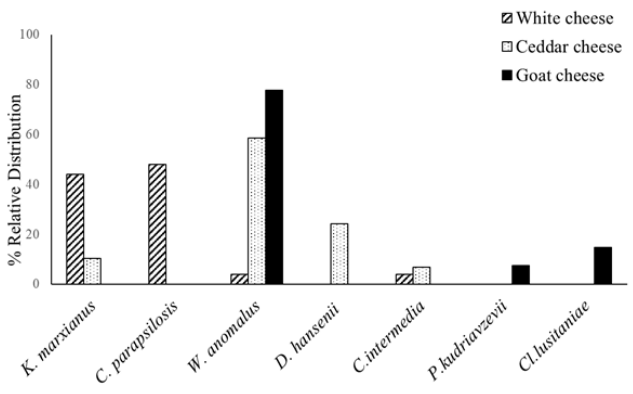

Figure 1. The relative distribution of identified yeast species

In addition to these species, many yeast species have been isolated and identified from local cheeses of different countries in the last decade. The *C. boidinii*, *C. butyric*, *C. mogii*, *C. sphaerica*, *Cr. albidus*, *K. blattae*, *K. thermotolerance*, *P. farinose*, *P. membranifaciens*, *R. glutinis* and *Z. rouxii* yeast species were recorded from Cyprus, Denmark, Macedonia, France and South Africa (Bintsis 2021). The *C. inconspicua*, *C. xylopsoci*, *G. candidus* and *P. kudriavzevii* yeast species were identified from Bryndza cheese of Slovakia (Pangallo et al. 2014). Similarly, *C. catenulate*, *C. etchellsii*, *C. glaebosa*, *G. candidum*, *Kazachstania unispora*, *Kodomaea ohmeri*, *Saturnispora mendoncae* and *T. ovoides* yeast species were identified from local cheeses of Brazil, England, France, Italy, Mexico and Spain (Gkatzionis et al. 2014; Tofalo et al. 2014; Padilla et al. 2014; Cardoso et al. 2015; Ceugniez et al. 2015; Chombo-Morales et al. 2016; Dugat-Bony et al. 2016). These studies showed that *D. hansenii*, *K. lactis*, *K. marxianus* and *Y. lipolytica* are the most common yeast species in many cheese types. In this study, *K. marxianus* and *W. anomalus* yeast species were found at high prevalence in cheese samples. Also, *P. kudriavzevii* yeast strain was reported for the first time in this study as a yeast species isolated from Turkish cheeses.

Phylogenetic analysis of identified yeast species was carried out by MEGA-X software. The obtained sequence of ITS-5.8S and 26S regions were aligned using ClustalW v1.6 tool, and the maximum parsimony tree was generated by utilizing MEGA-X (Kumar et al. 2018). *S. cerevisiae* was selected as an outgroup. Yeast species were divided into three main clades according to the maximum parsimony tree result of the ITS-5.8S region (Figure 2). The first clade including 6 yeast species was separated into 2 sub-clades. The *C. intermedia* and *Cl. lusitaniae* species are localized in the same sub-clade while the other sub-clade includes *P. kudriavzevii* yeast species. The second clade consists of eight yeast species and is divided into 3 sub-clades, the first sub-clade contains four *W. anomalus* yeast species, the second sub-clade includes *D. hansenii*, and the third sub-clade includes *C. parapsilosis* yeast species. The last clade includes only one yeast species, *K. marxianus*. According to the maximum parsimony tree of the 26S rDNA region, yeast strains were divided into 2 main clades (Figure 3). The first clade consists of 3 sub-clades including *C. intermedia*, *C. parapsilosis*, *Cl. lusitaniae*, *D. hansenii*, *P. kudriavzevii* and W. *anomalus* yeast species and the second clade contains only one yeast, *K. marxianus*.

Screening of protease and lipase activities

Extracellular proteases and lipases have been used in various industrial areas, including the pharmaceutical, detergent, leather, waste management, drug designs, cosmetic, biodiesel production, food and dairy industry (Escribano et al. 2017; Liu et al. 2020; Naveed et al. 2021). Several studies revealed that anti-inflammatory drugs, pesticides, anti-Alzheimer drugs, and analgesics could be produced by using yeast lipases. In addition, the importance of biodiesel production is increasing rapidly due to the worldwide depletion of fossil fuels (Adlercreutz

2013; Sharma and Kanvar 2014; Gupta et al. 2015). So, the isolation and identification of non-*Saccharomyces* yeast species with high protease and lipase activity are important for industrial applications. Therefore, protease and lipase activities of all isolated yeast strains were determined (Table 5). All strains of *P. kudriavzevii* and *W. anomalus* yeast species showed a high protease activity. In addition, all strains of *C. intermedia*, three yeast strains of *D. hansenii* (C-24, C-25, C-26) and three yeast strains of *K. marxianus* (W-4, W-9, W-11) yeast species displayed a protease activity in a moderate level. The strains of *C. parapsilosis* and *Cl. lusitaniae* yeast species did not have protease activity. Interestingly, lipase activity was not detected in all isolated yeast strains except for three strains of *C. parapsilosis* (W-7, W-17, W-21), which exhibited low lipase activity.

When the protease and lipase activities of 81 yeast strains were evaluated, it was determined that 61.7% of the yeast strains showed protease activity, while only 2.5% showed lipase activity. In previous studies, it was determined that *C. parapsilosis*, *Cl. lusitaniae*, *K. marxianus*, *M. pulcherrima* and *W. anomalus* yeast species have industrially important protease activity (Binetti et al. 2013; Escribano et al. 2017). And, *R. mucilaginosa*, *C. parapsilosis*, *K. marxianus* and *W. anomalus* yeast species have industrially important lipase activity (Binetti et al. 2013; Yalçın et al. 2014; Gupta et al. 2015).

Table 5. Extracellular enzyme profiles of cheese-related yeast strains

Yeast species	Yeast strains	Protease	Lipase
K. marxianus	W-4, W-9, W-11	++	-
	W-5, W-13, W-14, W-18, W-19, W-20, W-22, W-24, C-20, C-28, C-29	-	-
W. anomalus	W-2, C-1, C-2, C-3, C-4, C-5, C-6, C-7, C-8, C-9, C-10, C-11, C-12, C-13, C-14, C-15, C-16, C-21, G-1, G-2, G-5, G-6, G-7, G-8, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-16, G-19, G-21, G-23, G-24, G-25, G-26, G-27	++++	-
C. parapsilosis	W-3, W-6, W-8, W-10, W-12, W-15, W-16, W-23, W-25	-	-
	W-7, W-17, W-21	-	++
D. hansenii	C-18, C-19, C-22, C-23	-	-
	C-24, C-25, C-26	++	-
Cl. lusitaniae	G-17, G-18, G-20, G-22	-	-
C. intermedia	W-1, C-17, C-27	++	-
P. kudriavzevii	G-3, G-4	++++	-

Note: Negative sign indicates the absence of a clear zone around the colony; Zone diameter (mm): ++++ (>15); +++ (6–15); ++ (3–6); + (1–3)

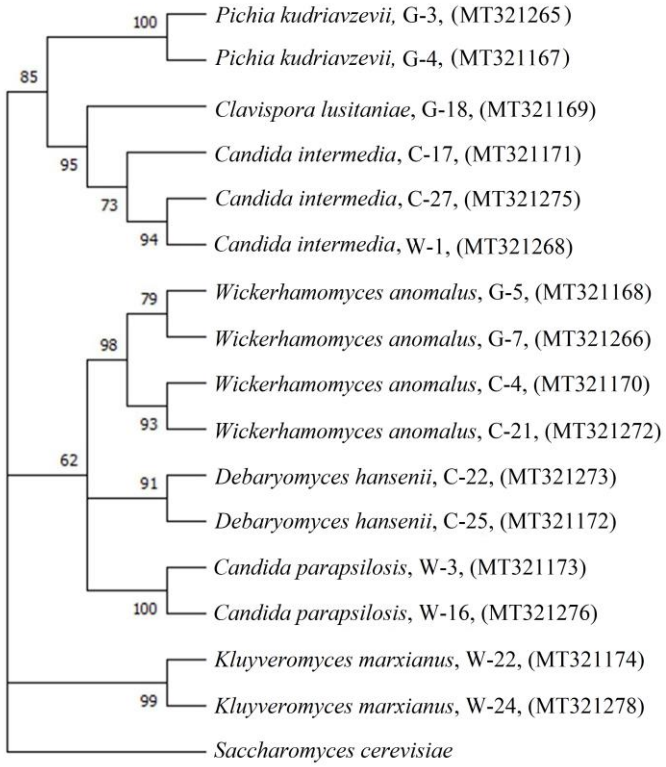

Figure 2. Phylogenetic placement of yeast species based on the sequence of ITS1-5.8S-ITS2 rDNA region. Reference sequences retrieved from the GenBank database are included. The tree was constructed with the maximum parsimony method and the Subtree-Pruning Regrafting algorithm. Numbers on branches represent the bootstrap values (>50%) from 1000 random replicates. The consistency index is (0.689320), the retention index is (0.777003), and the composite index is 0.545709 (0.535604) for all sites and parsimony-informative sites (in parentheses)

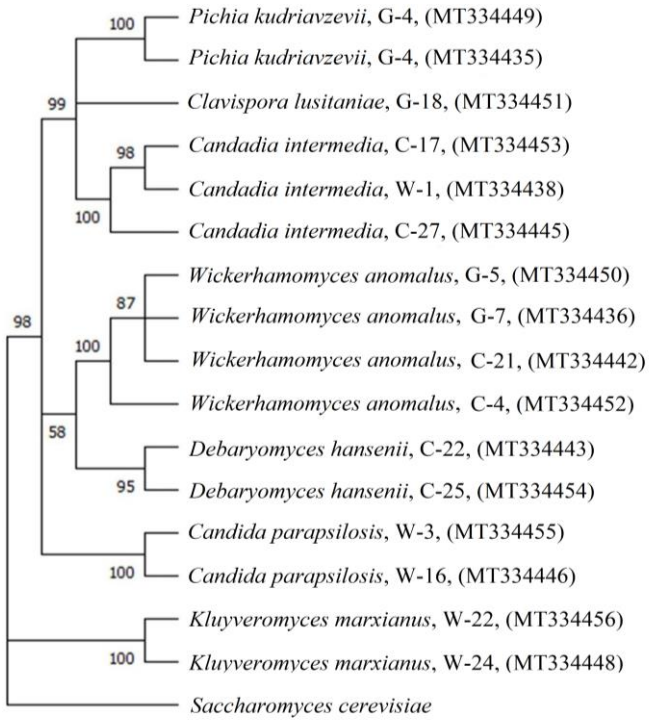

Figure 3. Phylogenetic placement of yeast species based on the sequence of D1/D2 rDNA region. Reference sequences retrieved from the GenBank database are included. The tree was constructed with the maximum parsimony method and using the Subtree-Pruning Regrafting algorithm. Numbers on branches represent the bootstrap values (>50%) from 1000 random replicates. The consistency index is (0,695906), the retention index is (0,828383), and the composite index is 0,610828 (0,576477) for all sites and parsimony-informative sites (in parentheses)

However, in this research, the strains of *C. parapsilosis*, *Cl. lusitaniae* and *K. marxianus* (except three strains) yeast species did not show proteolytic activity. Similarly, all isolated yeast strains of *C. parapsilosis* (except three strains), *K. marxianus* and *W. anomalus* yeast species did not show lipolytic activity. The extracellular enzyme activities of yeast species can show variations between the strains of the same species. For example, the production of proteases is affected by growth conditions (such as components of medium, inoculum size and dissolved oxygen) and environmental factors (such as pH, temperature and incubation time) (de Araújo et al. 2010; Molnárová et al. 2014). In this study, it was observed that the proteolytic and lipolytic activities were strain-specific as previously stated. In addition, all strains of *P. kudriavzevii* and *W. anomalus* yeast species have great potential as candidate yeasts for the dairy industry due to their high proteolytic activity.

Screening thermotolerant and osmotolerant properties

Since cheese is a fermentation product, the composition of cheese is indirectly regulated by microbial biota. The presence of thermotolerant and osmotolerant yeasts in this microbial biota is important. Thermotolerant and osmotolerant yeasts are capable of surviving and growing at high temperatures and osmotic environments (such as high sugar or salt concentrations), respectively. So, thermotolerance and osmotolerance properties of yeast strains were also investigated in this work. Thermotolerance properties of yeast strains were determined by incubation in a rich medium at 25, 30, 37 and 45°C (Table 6). All yeast strains showed a well growth at 25 and 30°C (data not shown). *D. hansenii* and *C. intermedia* yeast strains did not grow at both 37 and 45°C. The strains of *W. anomalus* yeast species grew at 37°C but not at 45°C. *K. marxianus*, *C. parapsilosis*, *Cl. lusitaniae* and *P. kudriavzevii* yeast strains showed a well growth at both 37 and 45°C. The osmotolerant yeast strains were determined by incubating yeast strains in the rich medium supplemented with 50% glucose at 30°C (Table 6). The results showed that all the yeast strains (except *K. marxianus*) showed growth in an osmotic environment. It has been previously reported that some strains of *C. famata, C. parapsilosis, D. hansenii, P. kudriavzevii* and *W. anomalus* possess osmophilic properties (Breuer and Harms 2006). Furthermore, *D. hansenii*, *K. marxianus* and *P. kudriavzevii* yeast species have both thermophilic and osmophilic properties (Breuer and Harms 2006; Yamamoto et al. 2015; Choi et al. 2017). In this research, *C. parapsilosis*, *Cl. lusitaniae* and *P. kudriavzevii* yeast strains were determined as both thermotolerant and osmotolerant yeast species. In addition, no variation was observed between the thermotolerant and osmotolerant profiles of yeast strains belonging to the same species.

Table 7. Fermentation capacity of yeast strains in different carbon sources

Yeast species	Yeast strains	Dex	Gal	Suc	Lac	Mal
K. marxianus	W-4, W-11, W-13, W-14, W-20, W-5, W-9, W-19, W-22, W-24, C-20, C-29	+	+	+	+	+
	W-18, C-28	+	+	+	+	-
W. anomalus	W-2, C-2, C-4, C-5, C-6, C-7, C-9, C-10, C-11, C-13, C-14, C-16, G-5, G-6, G-7, G-9, G-10, G-11, G-12, G-13, G-14, G-15, G-21, G-23, G-26, G-27	+	+	+	+	+
	C-1, C-3, C-8, C-12, C-15, C-21, G-1, G-2, G-8, G-16, G-19, G-24, G-25	+	+	+	-	+
C. parapsilosis	W-3, W-6, W-7, W-12, W-17, W-23, W-8, W-15, W-16	+	+	+	-	+
	W-10, W-21, W-25	+	+	+	+	+
D. hansenii	C-23, C-24, C-25, C-26	+	+	+	+	+
	C-18, C-19, C-22	+	+	+	+	-
Cl. lusitaniae	G-17, G-18	+	+	+	+	+
	G-20, G-22	+	+	+	-	+
C. intermedia	W-1, C-17, C-27	+	+	+	+	+
P. kudriavzevii	G-3	+	+	+	-	+
	G-4	+	+	-	-	-

Note: Negative sign indicates the absence of growth; Positive sign indicates the presence of growth. Dex: Dextrose, Gal: Galactose, Suc: Sucrose, Lac: Lactose, Mal: Maltose

Table 6. Thermotolerant and osmotolerant yeast strains

Yeast species	37°C	45°C	50% Dex
K. marxianus	+	+	-
W. anomalus	+	-	+
C. parapsilosis	+	+	+
D. hansenii	-	-	+
Cl. lusitaniae	+	+	+
C. intermedia	-	-	+
P. kudriavzevii	+	+	+

Note: Negative sign indicates the absence of growth; Positive sign indicates the presence of growth

Screening fermentation ability

The fermentation ability of the yeast strains was analyzed by using five different fermentable carbon sources: dextrose, galactose, sucrose, lactose, and maltose. Fermentation tests of all yeast strains yielded positive results on dextrose, galactose, and sucrose carbon sources, except G-4 yeast strain of *P. kudriavzevii* which gave a negative result on sucrose (Table 7). The strains of *C. intermedia*, *D. hansenii* and *K. marxianus* yeast species showed positive results on lactose. However, thirteen yeast strains of *W. anomalus*, nine yeast strains of *C. parapsilosis*, two yeast strains of *Cl. lusitaniae* and all strains of *P. kudriavzevii* yeast species gave negative results for the lactose. *C. intermedia*, *C. parapsilosis*, *Cl. lusitaniae* and *W. anomalus* yeast strains showed positive results in the maltose fermentation test, while two yeast strains of *K. marxianus*, three yeast strains of *D. hansenii* and one strain of *P. kudriavzevii* showed negative results in the maltose test (Table 7). As a result, 61.8% of isolated yeast strains gave positive results in all fermentable carbon sources used.

Due to their high fermentation capacity in different carbon sources and thermotolerant/osmotolerant properties, twenty-six strains of *W. anomalus*, three strains of *C. parapsilosis* (W-10, W-21 and W-25) and two strains of *Cl. lusitaniae* (G-17 and G-18) have been identified as yeast species with industrial potential. On the other hand, fermentative yeasts are often responsible for the deterioration of cheeses. Although *P. kudriavzevii* yeast strain (G-4) can grow in sucrose, lactose and maltose carbon sources, it cannot ferment them. For this reason, it may not cause deterioration even if it is used as a starter culture in cheese production. In this respect, G-4 strain of *P. kudriavzevii* is industrially important.

In conclusion, yeasts are an important part of the cheese microbiota. While yeasts have positive effects on improving the sensory properties of cheeses, they sometimes cause the cheese to deteriorate. Therefore, the determination of yeast diversity in cheese biota is important for the development of new protective precautions and new starter cultures. Biochemical and molecular identification methods are of great importance for the identification of yeast samples in cheese biota. In this study, seven different yeast species were identified from traditional cheese samples, and *C. parapsilosis*, *Cl. lusitaniae*, *P. kudriavzevii* and *W. anomalus* yeast species were determined to be industrially important. The strains of these yeast species have the potential for industrial applications according to their extracellular enzyme activities, fermentation capacity and thermotolerant-osmotolerant properties.

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In silico comparative analysis of the complete chloroplast genome sequences in different jewel orchid species

MINH PHUONG NGUYEN[1], THI HUONG TRINH[1], THI KIM ANH NGO[1], SASANTI WIDIARSIH[2], VIET THE HO[1,♥]

[1]Faculty of Biology and Environment, Ho Chi Minh City University of Food Industry. 140 Le Trong Tan Street, Ho Chi Minh City 72009, Vietnam. Tel./Fax. +84-933-025-55-23, ♥email: thehv@hufi.edu.vn.

[2]Research Center for Radiation Process Technology, Research Organization of Nuclear Energy, National Research and Innovation Agency. Jl. Lebakbulus Raya No. 49, Jakarta 12440, Indonesia

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Abstract. *Nguyen MP, Trinh TH, Ngo TKA, Widiarsih S, Ho VT. 2023. In silico comparative analysis of the complete chloroplast genome sequences in different jewel orchid species. Nusantara Bioscience 15: 12-21.* Jewel orchid is the common name of several orchid species which can be alike in morphological characteristics but variable in medicinal properties. As these plants are utilized to treat several diseases, their natural existence in the wild habitat is rapidly diminished. Therefore, a better understanding of the genetic information of this plant for better genetic conservation and development of these plants is necessary. In this study, a total of 18 published chloroplast genomes of 18 jewel orchid species determined by the next-generation sequencing method were retrieved from NCBI GenBank and targeted for genomic characterization and phylogenetic analyses. Different bioinformatics tools were utilized to characterize these genomes' genomic structure, repetitive sequences, interspecific variation, divergence, and phylogenetic relationships. The obtained data revealed that the chloroplast genomes of different jewel orchid species varied in length between 151,414 (*Anoectochilus formosanus* MN880624.1) and 154,375 (*Goodyera biflora* OM314910.1). Each species contains 34-87 SSR loci which could be useful as molecular markers for further genetic diversity study of this plant. Structural variations in the expansion and contraction of inverted repeat regions were also considered. Phylogenetic analysis identified a close relationship among species belonging to the *Goodyera* genus, and this genus is distinctive from other genera such as *Anoectochilus*, *Cystorchis*, *Dossinia*, *Ludisia*, and *Macodes*. The obtained results show a high potential of deeper characterizing the chloroplast genome of jewel orchids for species classification, identification, molecular breeding, and evolutionary exploration of these important herbal plants.

Keywords: Chloroplast genome, jewel orchid, next-generation sequencing, phylogenetic relationship, SSR

INTRODUCTION

Jewel orchid is a general name of several plant species in the Orchidaceae family. This name is used for plants that have smooth brocade leaves with beautiful veins. These plant species have been used for numerous purposes, such as ornamental plants for their beautiful foliage and health care purposes since they contain several valuable medicinal properties such as antioxidant, antitumor, and immunomodulatory agents (Winarto and Samijan 2018). Therefore, these herbs are used as treatments for several diseases as well as cancer prevention. However, because of their treasured medicinal values, they are exhaustedly exploited in the wild. Thus, the study for a better understanding of the genetic composition of this plant type for proper identification, conservation, and development of these plants is necessary. Since several visual characteristics of jewel orchid species are nearly similar, properly conserving species with high economic and pharmaceutical values is challenging. The current jewel orchid identification is mainly based on the morphological characteristics of leaves, flowers, and stems. Nevertheless, these methods are easily compromised by several factors such as different plant developmental stages or environmental conditions. Therefore, seeking a new marker for a more accurate classification of this plant group for better development and conservation is urgently needed.

Several phylogenetic studies have provided huge information about these herbal plants' relationships and evolutionary processes. However, these studies are mostly based on fragmentation analysis, such as RAPD and ISSR (David et al. 2020; Tran et al. 2022) or DNA barcodes (Ho et al. 2021; Raskoti and Ale 2021). Due to the methods' nature based on fragment length analysis, variations in internal DNA sequence are easily overlooked. On the other hand, DNA barcoding identification is based on the sequence of only a limited number of genome regions which does not provide sufficient discriminating power due to the similarity of sequences between species (Galimberti et al. 2014). Currently, the highest discriminating ability of DNA barcodes is only 70%, and this may be reduced in plants with complex genomes (Besse et al. 2021). Consequently, the relationship among jewel orchid species still lacks convincing evidence and needs further investigation.

The variation in chloroplast genomes in plants has been widely applied in studies on population genetics, evolutionary relationships, and genetic relationships to serve the conservation of plants under threatened extinction or developed molecular markers to accelerate the breeding process of plants with higher efficiency. Recently, next-

generation sequencing (NSG), a method simultaneously sequences several DNA or RNA molecules in a short time, with low cost and high accuracy, are widely used to replace the Sanger method for DNA sequencing in most applications that require a sequence of several target DNA or RNA molecules at the same time or identify the entire genomes. NGS enables a rapid increase in the completion of chloroplast genomes and has shifted the study of phylogenetics to phylogenomics (Behura 2015). In addition, many studies show that NGS can solve the remaining problems of DNA barcode technology, especially in determining plant origin, checking the mixing of poor-quality ingredients into products as well as traceability of plant-derived materials (Galimberti et al. 2014). At present, with the development of many new generation sequencing platforms, the sequencing of whole organism genomes, in general, and chloroplast genomes, in particular, are done easily and quickly. Consequently, several chloroplast genome sequences of different jewel orchid species have been published. A deeper understanding of the information on several chloroplast sequences simultaneously from availably published chloroplast genomes is an important basis for developing conservation and development programs for these plants.

In this study, the complete chloroplast genome sequences from 18 jewel orchid species were obtained from public databases and used for analysis. Based on the sequence comparison results, variable DNA regions between species found in this study would be used to design specialized primer pairs to help distinguish species to serve the conservation, breeding, and development of orchid species.

MATERIALS AND METHODS

Sequence annotation and comparison of chloroplast genomes

Eighteen complete chloroplast genome sequences of different jewel orchid species were retrieved from NCBI GenBank (MW589500.1 *Anoectochilus chapaensis*; LC057212.1 *Anoectochilus emeiensis*; MN880624.1 *Anoectochilus formosanus*; MW589501.1 *Anoectochilus hainanensis*; MN880626.1 *Anoectochilus roxburghii*; MW173020.1 *Anoectochilus zhejiangensis*; MW589507.1 *Cystorchis variegata*; MW589508.1 *Dossinia marmorata*; OM314910.1 *Goodyera biflora*; OM314911.1 *Goodyera henryi*; KT886429.1 *Goodyera procera*; OM314912.1 *Goodyera pubescens*; OM314914.1 *Goodyera schlechtendaliana*; OM314915.1 *Goodyera striata*; OM314916.1 *Goodyera velutina*; MN317571.1 *Ludisia discolor*; MW589527.1 *Macodes petola*; and MW589528.1 *Macodes sanderiana*). For species with several sequences available, only one sequence was randomly selected for further analysis. The Geseq program (https://chlorobox.mpimp-golm.mpg.de/geseq.html) was used to annotate and locate genes in the chloroplast genomes (Tillich et al. 2017). Chloroplot software (https://irscope.shinyapps.io/Chloroplot/) was used to identify the number of protein-coding genes, rRNA genes, tRNA genes, and GC content in each chloroplast genome (Zheng et al. 2020).

Repeat element analysis

The whole chloroplast genome sequences of 18 jewel orchid species were aligned by the MAFFT program (https://mafft.cbrc.jp/alignment/server/) with the following parameters: BLOSUM62 for scoring matrix for amino acid sequences and 200PAM/k=2 for the scoring matrix to find sequence variation (Katoh et al. 2019). The alignment result was then used to determine the DNA polymorphism by the DnaSP software to analyze nucleotide diversity (Pi) and the total number of mutations (Eta). Evolutionary divergence for each data set and pattern of nucleotide substitution was performed by MEGA X (https://www.megasoftware.net/) with default parameters (Kumar et al. 2018). The evolutionary distance between sequences will be calculated based on the p-value (p-distance) through the Kimura two-parameter algorithm of the MEGA 7.0 software to determine the genetic differences among chloroplast genomes. Chloroplast genomes were compared using the VISTA program (https://genome.lbl.gov/vista/index.shtml) in Shuffle-LAGAN mode (Brudno et al. 2003). The comparison of the LSC/IRB/SSC/IRA junctions among these related species was visualized by IRscope (https://irscope.shinyapps.io/irapp/) based on the annotations of their available chloroplast genomes in GenBank (Amiryousefi et al. 2018). Simple Sequence Repeat (SSRs) motifs were detected by MISA (http://pgrc.ipk-gatersleben.de/misa/misa.html) using parameters of the minimum repeats of ten for mononucleotides, six for dinucleotides, five for trinucleotides, four for tetra-nucleotides, and three each for penta- and hexa-nucleotides (Beier et al. 2017). Long repeat regions were defined using REPuter software (https://bibiserv.cebitec.uni-bielefeld.de/reputer) using default parameters such as repeat size of ≥ 30 bp and 90% minimum identity to find four types of repeats, namely forward (F), reverse (R), complementary (C), and palindromic (P) (Kurtz et al. 2001).

Phylogenetic analysis

The MAFFT alignment results were then used to determine the phylogenetic relationship among genomes. Phylogenetic trees of 18 chloroplast genomes were constructed based on Neighbor Joining (NJ), which represents distance methods (Kang et al. 2017) using 1000 bootstrap replicates with chloroplast genome of *Oryza sativa* (MK348618.1) and *Zea mays* (KP966116.1) belonging to Poaceae family as outgroups. Kimura 2-parameter nucleotide substitution model was applied for phylogenetic trees as this is one of the most widely used models for estimating genetic differences due to nucleotide substitution (Nishimaki and Sato 2019). To evaluate the classification resolution of given chloroplast genomes, a genus was considered as clear resolution if all its species are grouped into one monophyletic branch of dendrogram with strong bootstrap support and if species in a specific genus are separated in different branches that genus was considered as unresolved (Sikdar et al. 2018).

RESULTS AND DISCUSSION

Sequence annotation and comparison of chloroplast genomes

Orchidaceae, one of the largest and species-richest families in flowering plants, comprises approximately 880 genera, with 26,000 species distributed worldwide (Fay and Chase 2009). In this study, 21 chloroplast genomes of jewel orchids were obtained from GenBank from 18 species. There are 2 chloroplast sequences in 3 species: *Anoectochilus roxburghii*, *Goodyera schlechtendaliana*, and *Ludisia discolor*. For the remaining 15 species, only one chloroplast sequence is available for each species. Therefore, for equality, each chloroplast sequence from each species was kept for further analysis (Table 1). By using the Geseq program, the structural characteristics and gene contents of 18 chloroplast genomes were obtained (Figure 1). Similar to other chloroplast genomes, all chloroplast genomes in this study have a four-part structure consisting of Large Single Copy (LSC) region, Small Single Copy (SSC) region, and two Inverted Repeat (IRs) regions.

The genome size of 18 jewel orchids ranged from 151,414 bp in *A. formosanus* to 154,375 bp in *G. biflora*, which is slightly smaller than the chloroplast genome of other species in Orchidaceae in *Paphiopedilum* genus such as *P. barbigerum* (155,965 bp), *P. bellatulum* (156,567 bp), *P. henryanum* (155,886 bp), *P. hirsutissimum* (156,571 bp), and the hybrid cultivar *P. 'GZSLKY' Youyou* (160,503 bp) (Liu et al., 2022) or *Cypripedium* genus such as *C. palangshanense* (207,142 bp), *C. debile* (162,773 bp), *C. subtropicum* (212,668 bp), *C. tibeticum* (197,815 bp), *C. japonicum* (174,417 bp), *C. formosanum* (178,131 bp) and *C. calceolus* (175,122 bp) (Zhang et al., 2022). The protein coding gene numbers vary from 81 (*G. procera)* to 93 (*A. emeiensis*). Eight rRNA genes were detected in all chloroplast genomes, similar to rRNA gene numbers in different genera in the Orchidaceae family, such as *Paphiopedilum* (Liu et al. 2022); *Cypripedium* (Zhang et al. 2022). Similarly, the number of genes encoding for tRNA was negligibly different among chloroplast genomes ranging from 37 to 39 genes, except *A. emeiensis*, with up to 46 genes. The average GC content of the chloroplast genomes in the 18 species was comparable and ranged from 37% to 38%. GC content is an important parameter in the DNA sequence that directly alters protein amino acid composition in plants to cope with specific environments. The genome with high GC content will be more conserved, making it more stable and harder to transcribe. Thus, the differences in GC content could be due to the different pressure of natural selection among species. The LSC lengths ranged from 81,879 bp (*A. formosanus*) to 83,596 bp (*G. henryi*)), the SSC lengths ranged from 17,026 bp (*C. variegata*) to 18,406 bp (*G. procera*) and the length of the IR region was enlarged to 26,069 bp (*A. hainanensis)* to 26,572 bp (*G. striata)*. However, the tRNA number is conserved in all chloroplast genomes of 18 species with 35 tRNA for each chloroplast genome.

Using the Dnasp program, 10,788 polymorphic sites were detected and the nucleotide diversity value is at 0,01712 lower than similar values from four other orchid species, namely *D. densiflorum*, *G. densiflorum*, *C. aloifolium* and *R. retusa* (Roy et al. 2016). However, the nucleotide diversity value obtained from our study is almost two times higher than reported in the *Paphiopedilum* orchid with an average of 0.00962 (Liu et al. 2022) and even higher than other plants in distant taxonomy such as *Pennisetum* (0.00638) (Xu et al. 2021). Generally, plants' low level of nucleotide diversity is due to the selection pressure of humans with economical plants. On the other hand, the low nucleotide diversity of wild plants could result from collecting samples in a narrow area. The divergence of 18 chloroplast genomes ranged from 0.001 to 0.028 (Table 2).

Table 1. Size comparison of plastome features of 18 jewel orchid species

Accession code	Scientific name	Genome size (bp)	LSC size (bp)	SSC size (bp)	IR size (bp)	Coding genes	rRNA	tRNA	GC content (%)
MW589500	*A. chapaensis*	152,395	82,630	17,125	26,320	90	8	38	37
LC057212.1	*A. emeiensis*	152,650	82,670	17,342	26,319	93	8	46	37
MN880624	*A. formosanus*	151,414	81,879	17,342	26,313	90	8	37	37
MW589501	*A. hainanensis*	152,645	82,881	17,626	26,069	90	8	38	37
MN880626.1	*A. roxburghii*	152,821	82,683	17,478	26,324	91	8	37	37
MW173020.1	*A. zhejiangensis*	152,509	82,247	17,026	26,498	90	8	38	37
MW589507	*C. variegata*	152,269	82,336	17,443	26,551	90	8	38	37
MW589508	*D. marmorata*	152,881	83,466	17,893	26,508	90	8	38	37
OM314910	*G. biflora*	154,375	83,596	17,720	26,488	89	8	38	37
OM314911	*G. henryi*	154,292	82,496	18,406	26,169	89	8	38	37
KT886429.1	*G. procera*	153,240	82,101	17,876	26,220	81	8	39	38
OM314912.1	*G. pubescens*	152,417	82,674	17,999	26,535	89	8	38	37
OM314914	*G.schlechtendaliana*	153,743	82,081	17,871	26,395	89	8	38	37
OM314915.	*G. striata*	152,742	82,922	17,258	26,572	89	8	38	37
OM314916	*G. velutina*	153,997	82,659	17,513	26,438	89	8	38	37
MN317571.1	*L. discolor*	153,324	82,777	17,413	26,463	87	8	38	37
MW589527	*M. petola*	153,048	82,670	17,342	26,319	90	8	38	37
MW589528	*M. sanderiana*	153,116	81,879	17,342	26,313	90	8	38	37

Table 2. Estimates of evolutionary divergence among chloroplast genome sequences of 18 jewel orchid species

No.	Accession code	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	*A. chapaensis*																	
2	*A. emeiensis*	0.004																
3	*A. formosanus*	0.005	0.004															
4	*A. hainanensis*	0.005	0.004	0.001														
5	*A. roxburghii*	0.005	0.005	0.001	0.001													
6	*A. zhejiangensis*	0.005	0.005	0.002	0.002	0.002												
7	*C. variegata*	0.017	0.017	0.017	0.017	0.017	0.017											
8	*D. marmorata*	0.016	0.015	0.016	0.015	0.016	0.016	0.012										
9	*G. biflora*	0.016	0.016	0.016	0.016	0.016	0.016	0.012	0.006									
10	*G. henryi*	0.017	0.017	0.017	0.017	0.017	0.017	0.016	0.015	0.015								
11	*G. procera*	0.025	0.025	0.025	0.025	0.025	0.025	0.026	0.025	0.025	0.026							
12	*G. pubescens*	0.022	0.022	0.022	0.022	0.022	0.022	0.023	0.022	0.022	0.023	0.011						
13	*G. schlechtendaliana*	0.023	0.023	0.023	0.023	0.023	0.023	0.023	0.022	0.022	0.024	0.012	0.006					
14	*G. striata*	0.024	0.024	0.025	0.025	0.025	0.025	0.025	0.024	0.024	0.025	0.017	0.013	0.013				
15	*G. velutina*	0.026	0.026	0.026	0.026	0.027	0.027	0.027	0.026	0.026	0.027	0.019	0.015	0.016	0.015			
16	*L. discolor*	0.027	0.027	0.028	0.028	0.028	0.028	0.028	0.027	0.027	0.028	0.020	0.017	0.017	0.018	0.020		
17	*M. petola*	0.021	0.021	0.021	0.021	0.022	0.021	0.022	0.021	0.020	0.022	0.026	0.022	0.023	0.025	0.027	0.028	
18	*M. sanderiana*	0.026	0.026	0.026	0.026	0.026	0.026	0.027	0.026	0.026	0.027	0.020	0.017	0.018	0.020	0.022	0.022	0.027

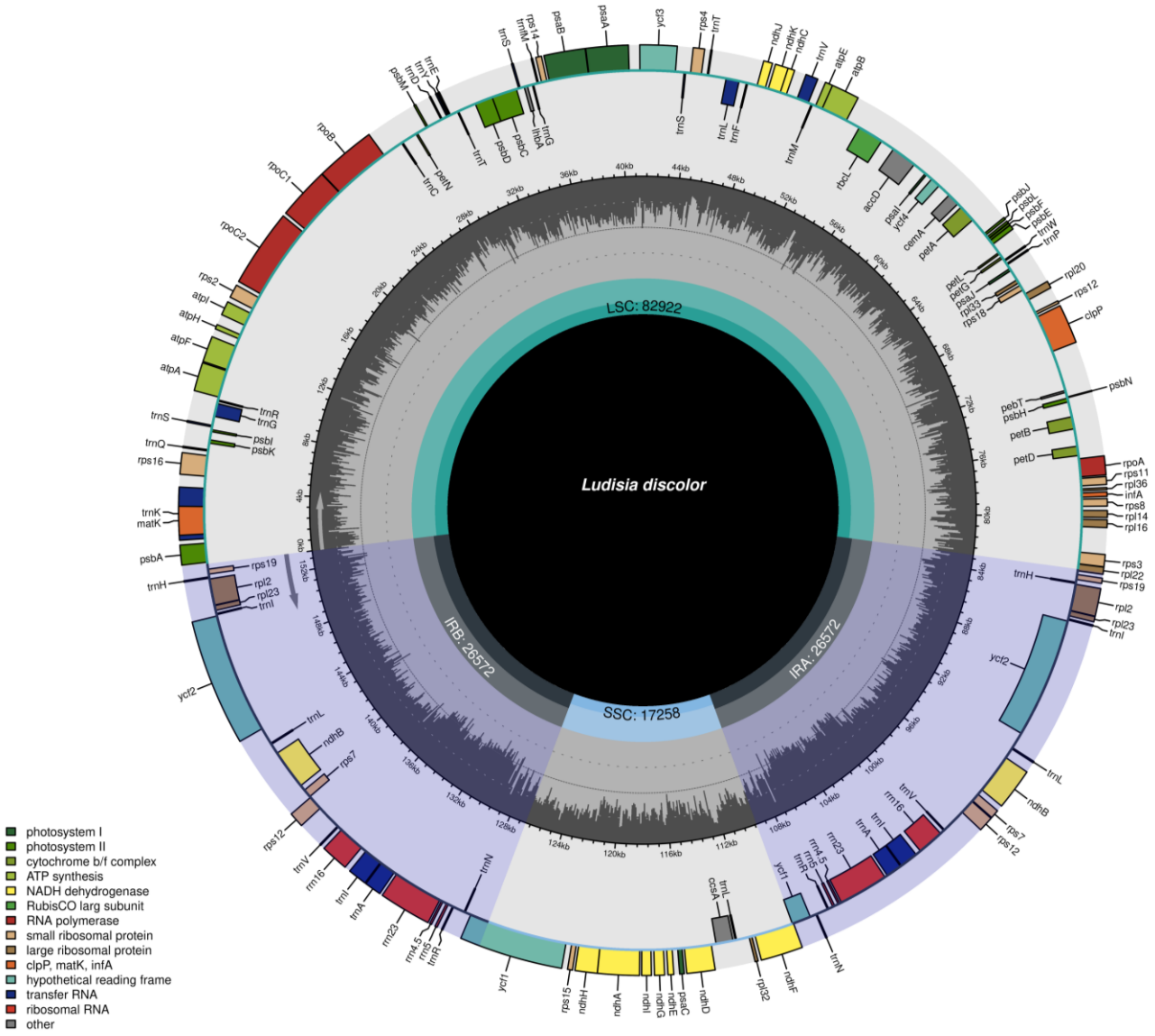

Figure 1. Typical map of jewel orchid chloroplast genome using MN317571.1 of *Ludisia discolor* as example (the genes drawn outside and inside of the circle are transcribed in clockwise and counterclockwise directions, respectively. The main parts of chloroplast genome are written as LSC, SSR, IRA, and IRB. The dark gray color and the light gray color of the inner circle shows the GC content and AT content, respectively)

Table 3. The pattern of nucleotide substitution among chloroplast genome sequences of 18 jewel orchid species (in percentage)

Nitrogenous bases	A	T	C	G
A	-	6.69	4.02	***10.81***
T	6.52	-	***10.81***	3.87
C	6.52	***18.0***	-	3.87
G	***18.19***	6.69	4.02	-

(Patterns and rates of substitutions were estimated under the Tamura-Nei model. Rates of different transitional substitutions and those of transversionsal substitutions are shown in bold and italics, respectively)

The chloroplast genome of *L. discolor* shows the largest difference with the remaining chloroplast genomes. The substitution of different nucleotides in whole genomes was evaluated on the entire codon position and shown in Table 3. Theoretically, there are 4 types of transitions; the substitution of a purine for a purine (A or G) nucleotide or a pyrimidine for a pyrimidine (C and T) nucleotide), also 8 types of transversions (the substitution of a purine (A or G) nucleotide for a pyrimidine nucleotide or vice versa). The expected ratio between transition and transversion is 0.5. In this study, the transitional substitution (57,8%) was significantly higher than the transversional substitution (43,2%). The high frequency of transitional substitution was also found among different species in the *Dracunculus* clade (Abdullah et al. 2021) or *Catalpa* genus (Li et al. 2022). Substitution is the most common mutation that causes variation and diversity among individuals and functions as a force for species evolution. It is also vital for phylogenetic construction since the transition bias in nucleotide transitions provides important information for clustering analysis.

Repeat element analysis

With the default parameters of MISA program of tandem repeat sequences consisting of 1-6 nucleotide repeat units, the relative abundance of SSR is detected. Microsatellites or SSRs are commonly used to identify the variable in the genomes of species. In total, 1,078 SSRs were detected among 18 jewel orchid species from 34 SSRs (*G. schlechtendaliana*) to 87 SSR (*D. marmorata*) with an average of approximately 60 SSRs per chloroplast genomes (Table 4). Seven SSR motifs were detected, namely A, T, C, G, AT, TA, and TTC. Two mononucleotide types consisting of T and A are the most dominant, with a frequency of 676 (62.7%) and 339 (31.4%), respectively. In contrast, C and G mononucleotide types are rarely detected, with only 3 and 1. Another dinucleotide (AT and TA) and trinucleotide (TTC) motifs were also identified with a low percentage. The A and T motifs seem common among plant chloroplast genomes. Similarly, Liu and colleagues reported the appearance of A and T motifs up to 66.39% in six oak species (*Quercus* L.) (Liu et al. 2021). The repeat motif type of chloroplast genomes in this study is less than those from other plants, such as two species in the *Morus* genus, which possess up to 18 motif types (Li et al. 2016) or up to 27 motif types in oak (Liu et al. 2021).

In addition, the 18 chloroplast sequences were analyzed with the REPuter program to determine the abundance of four oligonucleotide repeat types, namely forward (F), palindromic (P), reverse (R), and complementary (C). The number and type of repeat elements are largely variable among 18 jewel orchid species (Figure 2), ranging from 37 (*G. schlechtendaliana*) to 50 units (*A. chapaensis, A. emeiensis, A. formosanus, A. hainanensis*, *C. variegate*, *G. biflora*, *G. procera*, and *G. striata*). A total of 852 repeat elements were identified, palindromic repeats are the most commonly found, accounted up to 355 (41.67%) of the number of repeat elements. The second position was 274 (32.16%) forward, followed by 176 (20.66%) reverse and 47 (5.52%) complement repeat elements. These SSRs have a high potential to be used as candidate genetic markers. They are distributed widely in chloroplast genomes and serve as molecular markers for phylogenetic relationship inference. Moreover, SSRs are also associated with different types of genome rearrangement, recombination, and large inversions, which are useful for further phylogenetic studies.

Although the structure of chloroplast genomes is highly conserved among terrestrial plants, significant variation in the expansion and contraction of IR regions affects different genome sizes among plants. The LSC/IRb/SSC/IRa/LSC borders and adjacent genes were characterized to find similarities and differences among 18 jewel orchid species (Figure 3). Although the genomic structure and size were highly conserved in the 18 chloroplast genomes, the IR/SC boundary regions still showed considerable differences. The four regions are varied in length, of which *rps*3, *rpl*22, *rpl*19, *ycf*1, and *ndh*F genes were present at the junctions of the LSC/IR and SSC/IR borders. Notable variations were observed in the expansion and contraction of the IR regions. For the LSC/IR borders, *rpl*22 genes of 17/18 species are extended 12-95 bp into the IRb regions, whereas only this gene of *G. procera* was localized completely in LSC region. It indicates that this border has moved toward the LSC region compared to *G. procera* (Huang et al. 2020). On the contrary, only *ycf*1 gene in *L. discolor* stays extended from IRb to SSC regions. Interestingly, they are missing in *ndh*F (chloroplast NADH dehydrogenase F) genes in IRb/SSC regions of *D. marmorata*, *A. hainanensis* and *M. petola*, suggesting that the loss of this gene should have occurred independently among jewel orchids species. A previous study also reported that this gene is present in *Viburnum dilatatum* but not in at least six other species in the *Viburnum* genus (Park et al. 2020). This gene is often commonly pseudogenized or lost in different species in the *Paphiopedilum* genus (Liu et al. 2022).

Table 4. The different repeat types in the chloroplast genomes of 18 jewel orchid species

Scientific name	Repeat motifs							Total SSRs
	A	T	C	G	AT	TA	TTC	
A. chapaensis	21	43	0	0	2	3	0	69
A. emeiensis	21	49	1	0	2	3	1	77
A. formosanus	19	45	0	0	3	2	1	70
A. hainanensis	19	48	0	0	2	2	0	71
A. roxburghii	17	48	1	0	2	3	1	72
A. zhejiangensis	21	49	0	0	2	3	1	76
C. variegata	13	24	0	0	2	4	0	43
D. marmorata	32	54	0	0	0	1	0	87
G. biflora	18	24	0	0	0	1	0	43
G. henryi	11	25	0	0	1	1	0	38
G. procera	13	27	0	0	2	0	0	42
G. pubescens	15	34	0	0	0	1	0	50
G.schlechtendaliana	10	20	0	1	0	3	0	34
G. striata	17	31	0	0	0	1	0	49
G. velutina	14	32	1	0	0	4	0	51
L. discolor	22	38	0	0	1	1	0	62
M. petola	29	49	0	0	0	1	0	79
M. sanderiana	27	36	0	0	1	1	0	65
Total	676	339	3	1	20	35	5	1,078

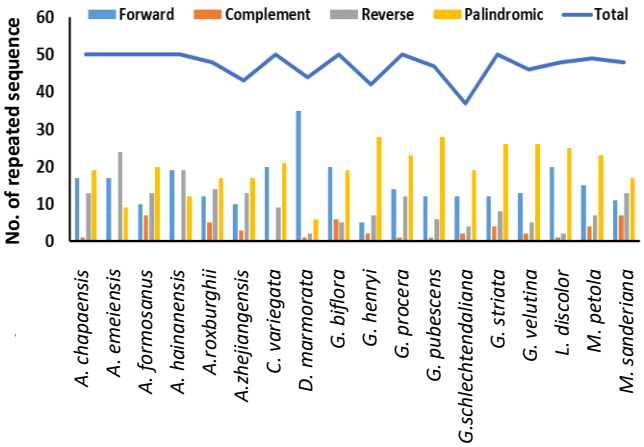

Figure 2. Number of repeated sequences in 18 jewel orchid chloroplast genomes

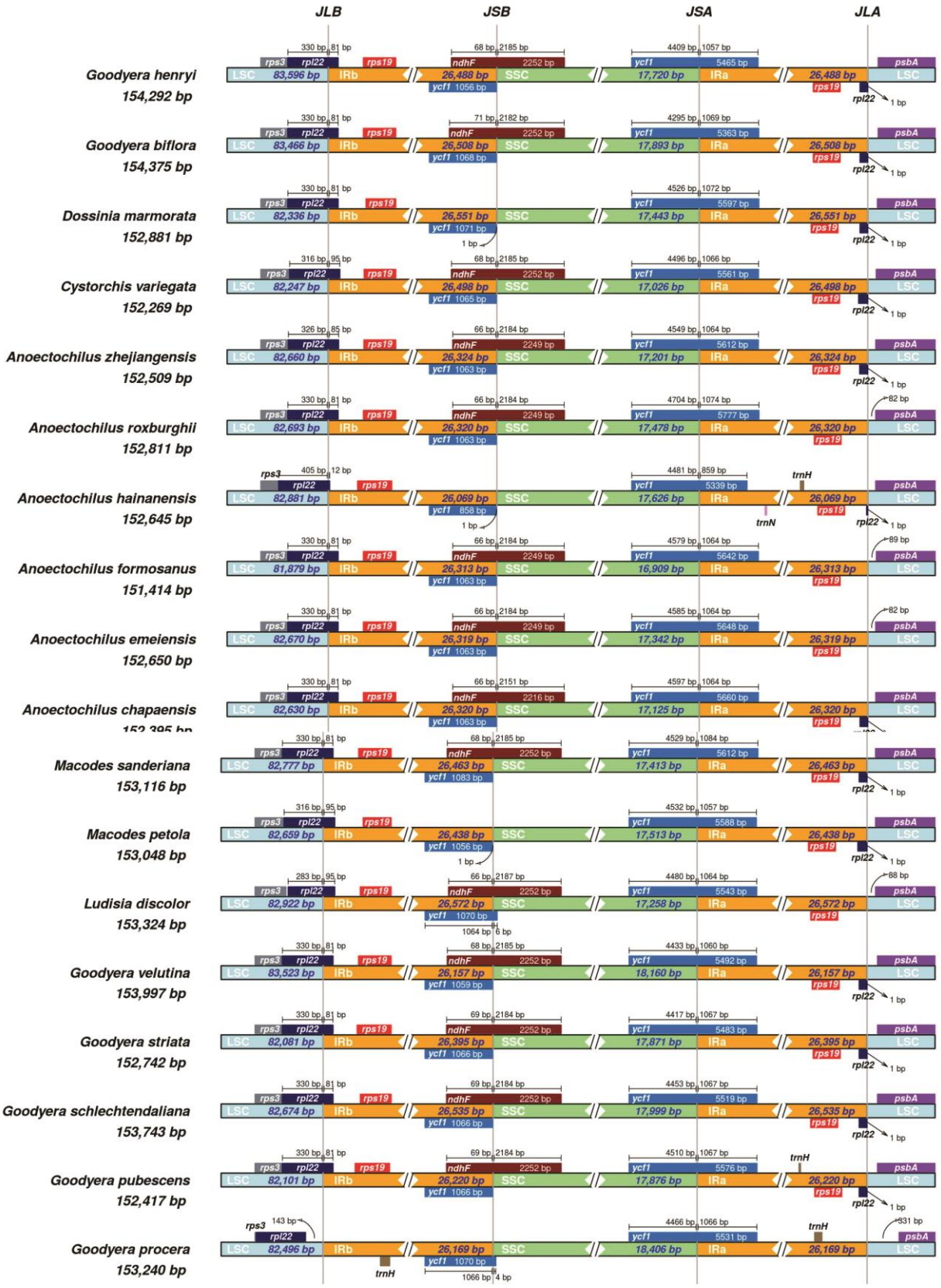

Figure 3. The comparison of the Large Single Copy (LSC), inverted repeat (IR) and Small Single Copy (SSC) border regions among 18 jewel orchid chloroplast genomes. Boxes above or below the main lines represent the genes at the IR/SC borders whereas the numbers above the gene indicate the distance from the gene terminal to the boundary region

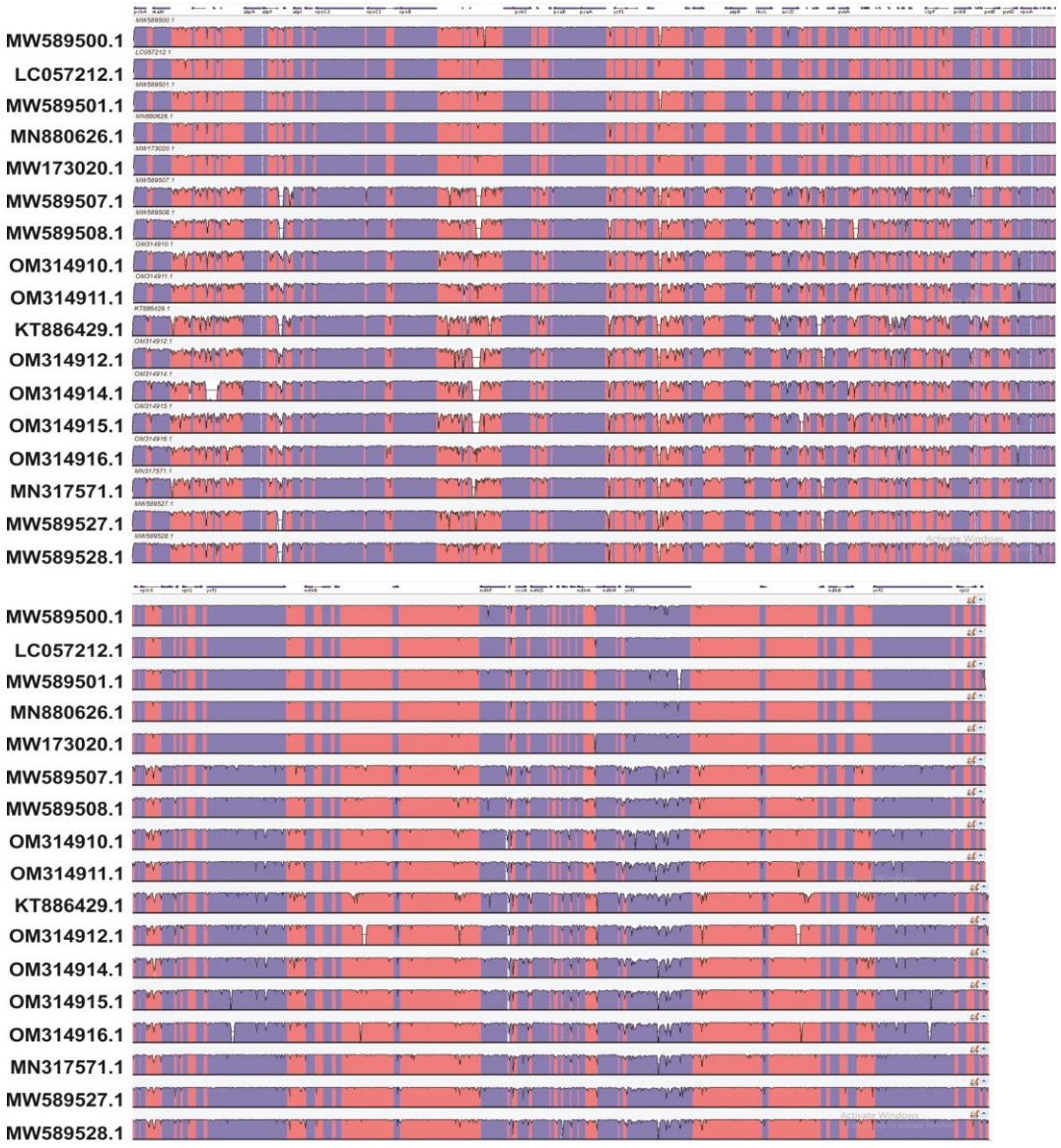

Figure 4. Comparison of chloroplast genomes of 18 jewel orchid species with *Anoectochilus formosanus* (MN880624.1) as the reference using mVISTA program. Coding regions are in blue and non-coding regions are in orange.

In contrast, the structure of SSC/IRa boundary regions is relatively stable. The gene *ycf*1 in the SSC region exhibited an interesting astride at the border of SSC/IRa with the extension from 859 bp (*A. roxburghii*) to 1,084 bp (*M. sanderiana*) into the IRa regions. The related expansions and contractions at SSR and LSC junctions with IRs suggest that the relationships among jewel orchid species may play evolutionary signals. Furthermore, the contractions and expansions at these positions may contribute to the variations in the chloroplast genomes and the IR expansions or contractions are likely to result from the gene conversion during plant speciation (Huang et al. 2020).

The annotated MN880624.1 chloroplast genome was used as a reference in mMISTA for alignment of the chloroplast genome among 18 jewel orchid species (Figure 4). Generally, the size and gene order of 18 analyzed chloroplast genomes are conserved. Nevertheless, some identified divergent regions are *acc*D, *ccs*A, *ycf*1, and *ycf*2.

Phylogenetic analyses

The results of phylogenetic analysis among 18 chloroplast genomes show a significant relationship among jewel orchid species with high bootstrap values (Figure 5). The *Goodyera* and *Anoectochillus* genera were found to be the best conserved clustered in two monophyletic groups. However, this result is opposite to Zhou and colleague's report, which is based on the phylogenetic dendrogram of the chloroplast genome, *G. velutina*, in the same cluster with *A. emeiensis* and *L. discolor* and separated from other species in the *Goodyera* genus such as *G. schlechtendaliana*, *G. goliosa*, *G. fumata* and *G. procera* (Zhou et al. 2019).

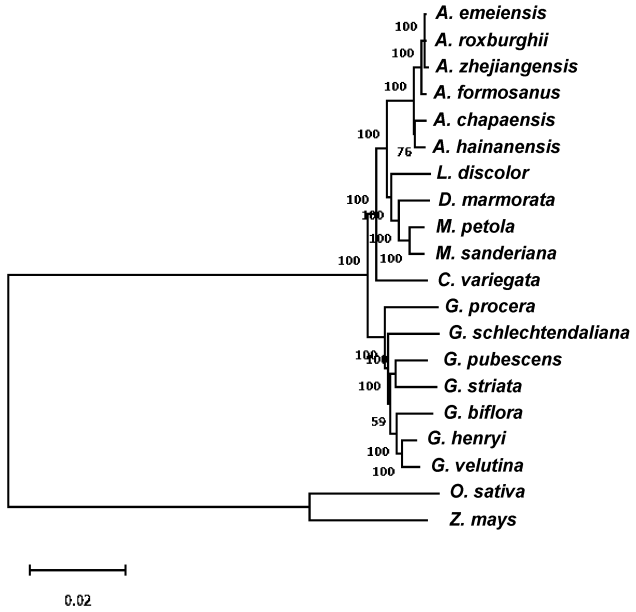

Figure 5. Phylogenetic tree of 18 chloroplast genomes of jewel orchid (The chloroplast sequences of *O. sativa* (rice) and *Z. mays* (maize) are used as outgroups. Numbers near branches are bootstrap values)

In conclusion, in silico analysis of many chloroplast genomes may play crucial roles in studying phylogeny, gene flow, and population genetics among different jewel orchid species. This study reveals the typical structure and content of the chloroplast genomes among the 18 jewel orchid species, an economically important herbal plant. This information regarding similarities and divergence among chloroplast genomes would enrich our understanding of jewel orchid genetic structure. Moreover, information about highly polymorphic regions from *acc*D, *ccs*A, *ycf*1, and *ycf*2 genes would also contribute to molecular markers and highly divergent regions, which might be useful for further studies of the taxonomy and phylogeographic of jewel orchid species.

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Effect of dolomite and pig manure on growth and production of carrots (*Daucus carota*)

NOVITA DEWI SAMBI, YUSUF LIMBONGAN♥, ADEWIDAR M. PATA'DUNGAN
Department of Agrotechnology, Faculty of Agriculture, Universitas Kristen Indonesia Toraja. Jl. Jenderal Sudirman No. 9, Bombongan, Tana Toraja 91811, South Sulawesi, Indonesia. Tel. +62-423-22468, ♥email: yusuflimbongan67@gmail.com

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Abstract. *Sambi ND, Limbongan Y, Pata'dungan AM. 2023. Effect of dolomite and pig manure on growth and production of carrots (*Daucus carota*). Nusantara Bioscience 14: 22-29.* This study aims to see how dolomite and pig manure affect carrot (*Daucus carota* L.) plant development and taproot production. The research was conducted from May to August 2021 in the Padangiring Village, Rantetayo Sub-district, Tana Toraja District, South Sulawesi, Indonesia. Three levels of dolomite in combination with four levels of pig manure were used: control (no treatment), and dolomite at 3 tons/ha, 6 tons/ha, and pig manure at control (no treatment), and 20 tons/ha, 30 tons/ha, and 40 tons/ha. The optimal dolomite level of 3 tons/ha significantly influenced plant height, the number of leaves, taproot length, and taproot diameter but had no significant effect on individual taproot weight, plot taproot weight, or taproot weight per hectare. The optimal pig manure dose of 4 kg/plot (40 tons/ha) showed a substantial influence on all investigated variables. The interaction between dolomite and pig manure substantially influenced taproot length but had no effect on the other observable variables. The optimum treatment combination was 3 tons/ha of dolomite with 40 tons/ha of pig manure, yielding 38.67 tons/ha of carrot taproots. Thus, commercial carrot growth and production may be boosted by a combination of dolomite and pig manure.

Keywords: Carrot, dolomite, pig manure

INTRODUCTION

Carrot (*Daucus carota* L.) is an agricultural product in great demand by the people in Indonesia because it has beneficial vitamin and mineral contents (Stefl et al. 2017) and a delicious taste. Carrot plants are classified as seasonal plants and are bush-shaped and can grow in the rainy and dry seasons (Gadomska et al. 2018). The market demand for carrots is quite high. According to data from the Central Statistics Agency (2020), the production of carrot vegetable commodities reached 674,634 metric tons in 2019. In addition to meeting domestic needs, carrots are one of the vegetables in demand abroad, so the prospect of carrot exports is very promising. The prospect of developing carrot plants in Indonesia is very bright because market demand continues to increase along with the increasing rate of population growth, especially in Tana Toraja.

The success of carrot cultivation is highly dependent on several factors, one of which is the nutrient content in the soil. The lack of nutrients in the soil can be overcome by providing additional nutrients to the soil, namely by fertilization. One type of fertilizer that farmers often use is inorganic fertilizer because the response of plants to inorganic fertilizers is quite fast. However, the tendency of some farmers to use inorganic fertilizers continuously causes residues that can damage the environment and result in poor-quality production. The use of organic fertilizers can overcome the problem of inorganic fertilization. Organic fertilizers have benefits, including preparing organic material in the soil or media, providing essential micronutrients needed by plants, and having a significant role in improving soil properties, both physical, chemical, and biological properties of the soil (Hayanti et al. 2012; Mokaya et al. 2018; Valšíková-Frey 2021; Satriawan et al. 2022).

Organic fertilizer is one type of fertilizer whose ingredients come from agricultural waste. Applying organic matter to agricultural land increases land fertility, improving the soil's chemical, physical, biological, and environmental properties. Organic fertilizers applied to the land will undergo several phases of an overhaul by degrading bacteria to become humus or organic matter (Lee et al. 2012). Pig manure is one of the wastes that can be used as organic fertilizer. Based on data from the Livestock Service Office of North Toraja District, the total population of pigs in North Toraja is 298,895 heads. The production of feces in pigs is 7 kg per day (Rahim et al. 2019). So that, pig manure production reaches 2.092 million tons per day.

Soil acidity is one of the important factors besides fertility that needs to be considered, especially for carrot plants. The soil acidity level can affect the nutrient absorption from the soil by plants. Carrot plants can grow well at a neutral pH with an acidity degree of 5.5-6.5. One way to overcome low acidity is to use minerals that can contain calcium oxide and magnesium oxide such as dolomite (Schultz et al. 2014)

Dolomite is one of the minerals containing calcium oxide with high magnesium oxide levels, which can neutralize soil pH. Lack of calcium and magnesium nutrients in the soil will cause plants to produce less than optimal production. Therefore, the use of dolomite plays a

very important role in plant growth and production. Dolomite is a mineral that contains several nutrients, such as calcium (CaO) and magnesium (Mga0) which are high enough to neutralize pH that is too acidic. Soil that lacks calcium and magnesium nutrients can cause plants to not produce well (Noviana 2021).

Based on the need for information on soil amendments to improve carrot production, we conducted a study to determine the effects of different levels of pig manure and dolomite on the growth and production of carrot plants.

MATERIALS AND METHODS

The study was conducted in the Padangiring Village, Rantetayo Sub-district, Tana Toraja District, South Sulawesi, Indonesia, from May to August 2021. Altitude 900 m above sea level, humidity 85-97%, temperature 15-27^{o}C with type of soil Ultisol and pH=4,0.

Experimental design

The study was carried out in the form of a field experiment with a Two-Factoral Randomized Block Design (RBD). The treatments consisted of 3 levels of dolomite and 4 levels of pig manure. The treatment doses to be tested were: Factor I, the amount of dolomite which consists of treatment levels: D0 = Control, D1 = 300 g/plot (3 tons/ha), D2 = 600 g/plot (6 tons /Ha). Factor II, the amount of pig manure which consists of 4 levels of treatment: B0 = Control B1 = 2 kg/plot (20 tons/ha) B2 = 3 kg/plot (30 tons/ha), and B3 = 4 kg/plot (40 tons/ha). Therefore, there were 12 treatment combinations.

Land preparation

The land is plowed and processed twice so that the soil is completely loose, so that the physical, chemical and biological conditions of the soil become better. Furthermore, the beds are made with a height of 30 cm, a width of 100 cm and a bed length of 100 cm. Making beds is done so that air and water circulation is good. Between the beds made a trench with a width of 30 cm.

Liming

Liming was carried out 1 week before planting with the use of dolomite at a dose according to the experimental dose D0 without treatment D1 300 g/plot and D2 600 g/plot

Carrot planting

Before planting, the first thing to do is to make a planting hole with a depth of 3-5 cm with a distance of 10 x 10 cm then the seeds are mixed with sand to make it easier to plant because the size of the carrot seeds are very small and then sown on top of the array and covered with soil.

Fertilization

Fertilization is done by giving pig manure according to the treatment given to carrot plants one week before planting. Then follow-up fertilization was carried out at 14 and 28 days after fertilization according to the dose before fertilization was carried out.

Watering and weeding

Watering carrot plants is done in the morning and evening every day depending on the weather. Weeding was done by removing weeds that grew in the research plot. While embroidery is done after weeding by replacing plants that do not grow or die.

Carrot plant pest control

Control of plant-disturbing organisms is carried out manually by pulling weeds that grow and catching or making pest traps, while for diseases using natural pesticides or removing infected plants so they don't spread.

Harvest and postharvest

Harvesting is done when the carrot plant is 100 days after planting and already has the characteristics of being ready to harvest, including 75% of the upper leaves falling or drying out. Harvesting is done by pulling the plant carefully.

Observation variables

Plant height

Observation of plant height was carried out by measuring the height of the carrot plant from the base of the stem or soil surface to the tip of the highest leaf and carried out at the age of 2 weeks after planting and repeated until the plants were 6 weeks old with an interval of 2 weeks.

Number of leaves

The number of leaf branches was counted by counting the number of leaf branches in the plant and was carried out at the age of 2 weeks after planting and repeated until the plants were 6 weeks old with an interval of 2 weeks.

Taproot length

The length of the tubers was measured using a ruler and measured from the base to the tip and was carried out after harvesting. The tubers measured were plant taproots that were used as samples.

Taproot diameter

The tuber diameter was measured using a roll meter. The tubers measured were plant taproots that were used as samples.

The weight of the taproot

The weight of the plots was calculated by weighing the tubers from the plots that had been cleaned of dirt.

Weight of taproots per hectare

The weight per hectare is calculated by converting the weight of the plots to hectares with the equation of area per hectare / area of research plot x yield of plots.

Data analysis

The results of the observations were analyzed using analysis of variance (ANOVA), and if the treatment had a significant effect, a further test was carried out using the Tukey's Honestly Significant Difference Test (HSD). (Stigler 1986).

RESULTS AND DISCUSSION

Based on the results of the analysis using the variable variance test for carrot plant height at the age of 2, 4 and 6 weeks after sowing in Table 1 and Figure 1, it was shown that the dose of dolomite lime and pig manure had a very significant effect on plant height. However, the interaction of dolomite lime dose and pig manure had no significant effect on carrot plant height.

Table 1 shows the HSD test for plant height at a level of $p < 0.05$ of 2 weeks after planting. The treatment of the dolomite at 300 g/plot (D1) resulted in the highest average plant height (18.03 cm) after sowing which was significantly different from the dolomite level D2, and different from the lowest average plant height (12.83) cm) at level D0. Pig manure with a dose of 40 ton/ha (B3) gave the highest average carrot plant height (19.80 cm) at the age of 2 weeks after planting, which was not significantly different from the B2 level (15.81 cm) but was very significantly different from the B1 and B0 levels. The lowest plant height was found in treatment B0 with an average plant height of 12.21 cm.

Dolomite treatment of 3 ton/ha (D1) resulted in the highest average carrot plant height (32.63 cm) at the age of 4 weeks after sowing which was not significantly different from the dolomite level D2 but was very significantly different from the D0 level with average plant height 12.83 cm (lowest). Pig manure at a dose of 4 kg/plot (B3) gave the highest average carrot plant height (34.41 cm) at the age of 4 weeks after planting which was not significantly different from the B2 level (30.41cm) but very significantly different from the B1 and B0 levels, while The lowest plant height was found in treatment B0 with an average plant height of 26.81 cm.

Dolomite treatment of 3 ton/ha (D1) resulted in the highest average carrot plant height (47.15 cm) at the age of 6 weeks after sowing which was not significantly different from the dolomite level D2 but was very significantly different from the D0 level with average plant height 41.99 cm (lowest). Pig manure at a dose of 4 kg/plot (B3) gave the highest average carrot plant height (48.90 cm) at the age of 6 weeks which was significantly different from other levels of manure treatment, while the lowest plant height was in treatment B0 with an average plant height 41.51 cm.

The combination of treatments with the best plant height growth was achieved at a dose of 3 tons/ha (D1) of dolomite and 40 tons/ha (B3) of pig manure at 2 weeks, 4 weeks and 6 weeks after sowing.

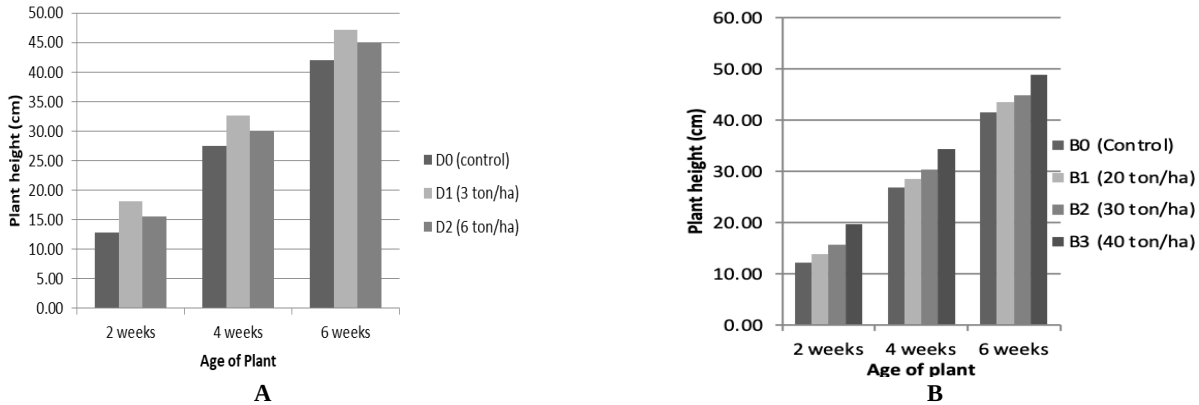
Number of leaves

Based on the results of the analysis using the test of variance of the variable number of carrot leaves at the age of 2, 4 and 6 weeks after sowing in Table 2 and Figure 2, it was shown that the dose of dolomite and pig manure had a very significant effect on the number of carrot leaves. Meanwhile, the interaction of dolomite lime and pig manure did not significantly affect the number of carrot leaves.

Table 1. Average of plant height 2, 4, and 6 weeks after planting

Treatments	Plant height					
	2 weeks		4 weeks		6 weeks	
Dolomite						
D0 (control)	12.83	v	27.43	v	41.99	v
D1 (3 ton/ha)	18.03	w	32.63	w	47.15	x
D2 (6 ton/ha)	15.48	v	30.08	w	45.03	wx
Pig manure						
B0 (control)	12.21	p	26.81	p	41.51	p
B1 (20 ton/ha)	13.96	p	28.56	p	43.55	pq
B2 (30 ton/ha)	15.81	pq	30.41	pq	44.93	q
B3 (40 ton/ha)	19.80	q	34.40	q	48.90	r

Note: The average value followed by the different letter in the column for averages across dolomite treatments (v, w, x) and for averages across pig manure treatments (p, q, r, s), is significantly different at level of HSD $p<0.05$



A B

Figure 1. The effect of dolomite (A) and pig manure (B) on plant height

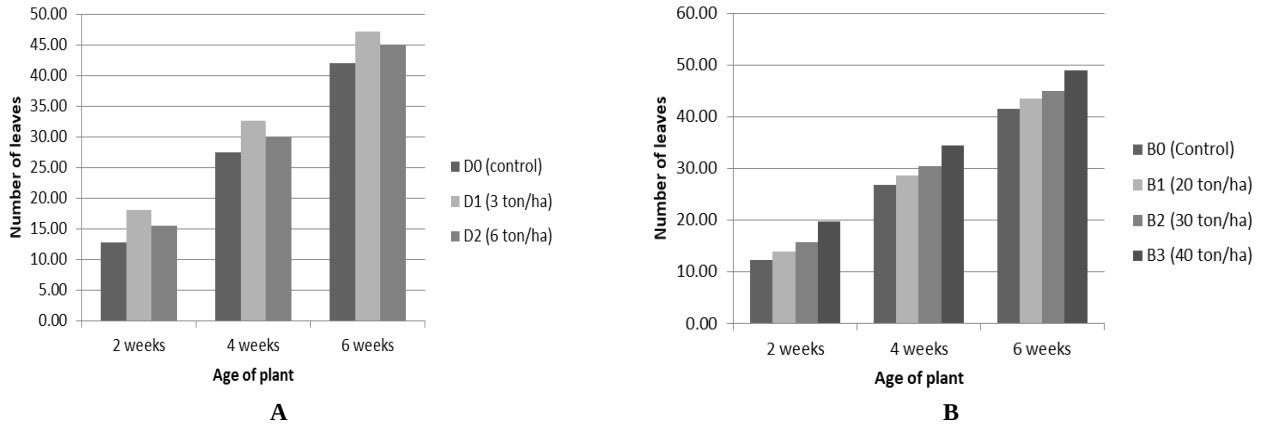


Figure 2. The effect of dolomite (A) and pig manure (B) on number of leaves

Table 2. Average number of leaves 2, 4 and 6 weeks after planting

Treatments	Plant height					
	2 weeks		4 weeks		6 weeks	
Dolomite						
D0 (control)	3.40	v	4.70	v	3.40	v
D1 (3 ton/ha)	5.04	w	6.34	w	5.04	x
D2 (6 ton/ha)	4.04	v	5.34	vw	4.04	vx
Pig manure						
B0 (Control)	2.77	p	4.07	p	2.77	p
B1 (20 ton/ha)	3.14	p	4.44	p	3.14	pq
B2 (30 ton/ha)	4.59	q	5.89	q	4.59	q
B3 (40 ton/ha)	6.13	r	7.43	r	6.13	r

Note: The average value followed by the different letter in the column for averages across dolomite treatments (v, w, x) and for averages across pig manure treatments (p, q, r, s), is significantly different at level of HSD p>0.05

Dolomite treatment of 300 g/plot (D1) resulted in the highest average number of carrot leaves (5.04 strands) at the age of 2 weeks after sowing, which was significantly different from the other dolomite levels, while the lowest average number of leaves was found in DO level (3.40 pieces). Pig manure with a dose of 4 kg/plot (B3) gave the highest average number of carrot leaves (6.3 pieces) at the age of 2 weeks which was significantly different from other levels of manure treatment, while the lowest number of leaves was in treatment B0 with an average the number of leaves 2.77 pieces. Treatment B2 is also significantly different from B1 and B0.

Dolomite treatment of 3 ton/ha (D1) resulted in the highest average number of carrot leaves (6.43 pieces) at the age of 4 weeks after sowing, which was not significantly different from the dolomite level D2 but was very significantly different from the dolomite level B0, with an average of 4.70 leaves (lowest). Pig manure with a dose of 40 ton/ha (B3) gave the highest average number of carrot leaves (7.43 strands) at the age of 4 weeks which was significantly different from other levels of pig manure treatment, while the lowest number of leaves was found in treatment B0 with an average the average number of leaves 4.19 pieces. Treatment B2 is also significantly different from B1.

Dolomite treatment of 3 ton/ha (D1) resulted in the highest average number of carrot leaves (8.64 pieces) at the age of 6 weeks after planting, which was not significantly different from the dolomite level D2 but very significantly different from the dolomite level B0, with an average number of leaves 7.00 pieces (lowest). Pig manure with a dose of 40 ton/ha (B3) gave the highest average number of carrot leaves (9.72 strands) at the age of 6 weeks which was significantly different from other levels of pig manure treatment, while the lowest number of leaves was found in treatment B0 with an average of the average number of leaves 6.37 leaves. Treatment 30 ton/ha pig manure B2 is also significantly different from 20 ton/ha (B1). The combination of treatments with the best number of leaf growth was achieved at a dose of 3 tons/ha (D1) of dolomite and 40 tons/ha (B3) of pig manure at 2 weeks, 4 weeks and 6 weeks after sowing.

Taproot length

Based on the results of the analysis using the variable variance test of carrot length in Table 3, it was shown that the dose of dolomite lime, pig manure, and the interaction of the two treatments had a very significant effect on carrot taproot length.

The dolomite treatment of 3 ton/ha (D1) resulted in the highest average carrot taproot length (16.81cm), which was not significantly different from the dolomite level D2 (15.18 cm) but very significantly different from the dolomite level B0, with an average taproot length of 14.49 cm (lowest). Pig manure at a dose of 40 tons/ha (B3) gave the highest average length of carrot s taproots (17.58 cm) which was significantly different from other levels of pig manure treatment, while the lowest taproot length was found at the level of pig manure B0 with an average taproot length 14.04 cm. Mention B2 also different from B1 and B0. The interaction of the D1B3 treatment gave the highest taproot length (20.60 cm), which was not significantly

different from the D1B2 (17.43 cm) treatment but was very significantly different from other treatment combinations. While the lowest taproot length was found in the interaction combination treatment D0B0 (13.93 cm).

Taproot diameter

Based on the results of the analysis using the variance test Based on the results of the analysis using the variance test for the variable diameter of the carrot taproot in Table 3, it was shown that the dose of dolomite lime and pig manure had a very significant effect on the diameter of the carrot taproots. Meanwhile, the interaction of dolomite lime and pig manure did not significantly affect the diameter of the carrot taproot.

Dolomite treatment of 3 tons/ha (D1) resulted in the highest average diameter of carrot taproots (3.29 cm), significantly different from other levels of dolomite. In contrast, the lowest average taproot diameter was at the D0 dolomite level (2.44 cm). Pig manure with a dose of 40 tons/ha (B3) gave the highest average carrot taproot diameter (3.69 cm), which was not significantly different from the B2 level (2.82 cm) but was very significantly different from the B1 and B0 levels. In contrast, the lowest taproot diameter was found in treatment B0 with an average taproot diameter of 2.21 cm. Treatment B2 is also significantly different from B1 and B0.

Taproot weight

Based on the analysis results using the test of variance of the variable weight of taproot weights (Table 3), it was shown that the treatment of pig manure had a very significant effect on the weight of carrot taproots. Meanwhile, the dose of dolomite lime and the interaction of dolomite lime and pig manure did not significantly affect the taproot weight of carrot planting.

Treatment of pig manure at a dose of 40 tons/ha (B3) gave the highest average carrot taproot weight (137.10 g) which was significantly different from the other levels of manure treatment, while the lowest taproot weight was found at the level of pig manure B0 with an average taproot weight of 71.03 g. Treatment B2 is also significantly different from B1 and B0.

Taproot weight per hectare

Based on the results for taproot weight per plot of the analysis using the test of variance of the taproot weight variable per hectare of carrots, it shows that the treatment of pig manure has a very significant effect on the weight of taproots per hectare of carrot plants. Meanwhile, the dose of dolomite lime and the interaction of the dose of dolomite lime and pig manure did not significantly affect the weight of taproots per hectare of carrot plants.

There was no difference among dolomite treatments for taproot weight per hectare. However, the treatment of pig manure at a dose of 40 ton/ha (B3) gave the highest average taproot weight per hectare of carrot plants (38.39 tons) which was significantly different from other levels of pig manure treatment, while taproot weight per hectare. The lowest level was found at the level of pig manure B0, with an average taproot weight of 19.89 tons per hectare. Treatment B2 is also significantly different from B1 and B0.

Table 3. Average taproot length and taproot diameter (cm)

Treatments	Variable							
	Length of taproot		Diameter of taproot		Weight of taproot per plant		Weight of taproot per ha	
Dolomite								
D0 (control)	14.49	v	2.44	v	87.20	v	24.42	v
D1 (3 ton/ha)	16.81	w	3.29	x	106.10	v	29.71	v
D2 (6 ton/ha)	15.18	vw	2.65	v	99.85	v	27.90	v
Pig manure								
B0 (Control)	14.04	p	2.21	p	71.03	p	19.89	v
B1 (20 ton/ha)	14.56	p	2.45	p	79.96	p	22.39	v
B2 (30 ton/ha)	15.79	q	2.82	pq	102.78	q	28.70	x
B3 (40 ton/ha)	17.58	r	3.69	q	137.10	r	38.39	wx
Interaction								
D0B0	13.93	A	1.79		63.10		17.67	
D0B1	14.27	A	2.12		73.21		20.50	
D0B2	14.60	A	2.57		86.31		24.17	
D0B3	15.15	AB	3.27		126.19		35.33	
D1B0	14.13	A	2.80		80.36		22.50	
D1B1	15.07	AB	3.00		83.93		23.50	
D1B2	17.43	BC	3.30		113.10		31.67	
D1B3	20.60	C	4.06		147.02		41.17	
D2B0	14.07	A	2.03		69.64		19.50	
D2B1	14.33	A	2.23		82.74		23.17	
D2B2	15.33	AB	2.60		108.93		30.27	
D2B3	17.00	B	3.73		138.10		38.67	

Discussion

The results of the analysis of all observational variables showed that the dolomite treatment had a significant and beneficial effect on carrot plants, namely the variables of plant height, number of leaves, taproot length, and taproot diameter, but had no significant effect on plant taproot weight either on an individual taproot or hectare basis. In contrast, pig manure treatment significantly affected all observed variables. However, the dolomite and pig manure interaction was only significant for the taproot length.

Pig manure contains essential nutrients needed by plants in the vegetative phase, especially elements of N, P, and K, which stimulate the photosynthesis process to produce photosynthate, which is translocated to all parts of the carrot plant for cell formation and cell enlargements such as leaves, stems, and roots so that it has an impact to increased production (Duan et al. 2012).

Dolomite had few effects on taproot weights, likely due to environmental conditions, namely high rainfall at the beginning of the study, so most of the dolomite given was washed away by rainwater. In addition, rainwater also has a fairly wet pH, so the absorption of nutrients in the soil by carrot plants is not optimal (Gadomska et al. 2018) so the impact on the interaction that occurs between pig manure and dolomite is not optimal in affecting the growth and production of carrot plants.

Dolomite lime influence

Based on the results of the variance analysis, it was shown that the dolomite treatment had a significant effect on the variables of plant height, number of leaves, taproot length, and taproot diameter but had no significant effect on plant taproot weight, plot taproot weight and taproot weight per hectare of carrot plants (Figure 4). Meanwhile, the HSD test at 0.05 level showed that the best dose of dolomite was 300 g/plot (3 tons/ha).

Using dolomite at a dose of 300 g/plot (3 tons/ha) increased plant height, number of leaves, and taproot length and diameter. Furthermore, the results of observations of soil pH before and after dolomite administration showed an increase from an average pH of 5.4 to 6.0 to provide both macro and micro nutrients needed by carrot plants. This result is to the opinion of Ilham et al. (2019), who said that dolomite can provide nutrients in the soil and contains other micronutrients to support plant growth and development. Amri et al. (2016) and Krismawati et al. (2021) further stated that the application of dolomite fertilizer can significantly influence plant height, stem circle, number of leaves, and leaf chlorophyll content.

Giving lime CaMg(CO3)2 can provide nutrients, Ca and Mg will stimulate cell turgor and the formation of chlorophyll so that the photosynthesis process increases and the product of photosynthesis also increase (Sirait et al. 2018). Magnesium plays a very important role in forming leaf green matter (chlorophyll) and helps plant metabolic processes such as photosynthesis, cell formation, protein formation, starch formation and carbohydrate distribution throughout plant tissues (Thana and Haryati 2021).

Giving dolomite can also provide P nutrients needed in plants in cell development that can increase plant growth, such as the number of leaves and plant height. Syahputra et al. (2015) stated that the application of dolomite lime in a certain range increased the soil's available P. Furthermore, La Habi et al. (2018) state that the availability of element P is strongly influenced by soil acidity. P cannot be dissolved in acidic soil conditions, so plants cannot absorb and use it (Sirait et al. 2018). Dolomite at a dose of 3 tons/ha improved the pH of acidic soil (pH 4.0) to neutral (pH 6.0) so that P nutrients were available for carrot plants used for growth.

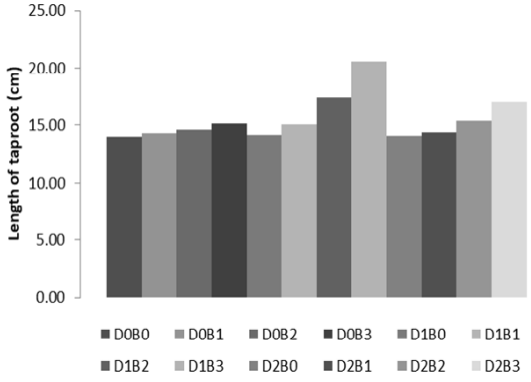
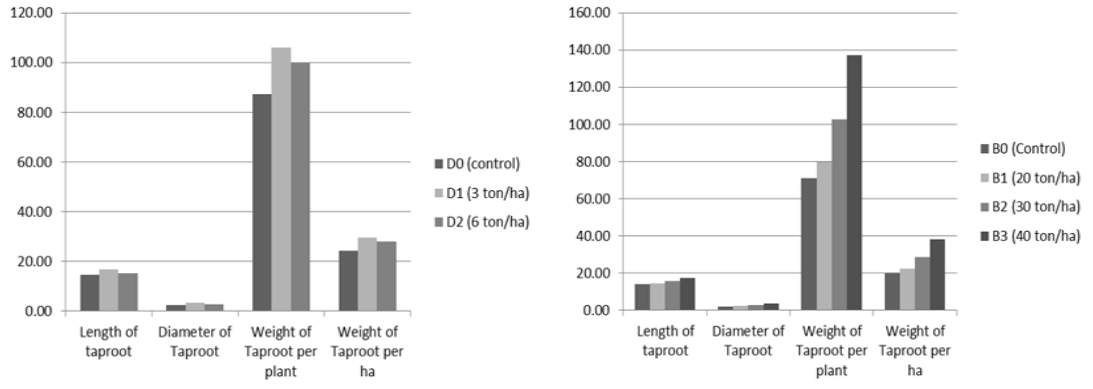


Figure 3. Interaction of dolomite and pig manure on length of taproot



A **B**

Figure 4. The effect of dolomite (A) and pig manure (B) on length of taproot, diameter of taproot, weight of taproot and weight of taproot per ha

Giving dolomite lime can increase the nutrients available to plants, increase the decomposition process by microorganisms in the soil and increase the availability of nutrients in the soil in the form of ions that plants can take up (Widodo et al. 2017; Rosalyne 2020). Sirait et al. (2018) stated that besides providing elements of Ca and Mgdolomite, it could affect the availability of nitrogen and other nutrients bound by heavy metal content such as Al, Fe, and Mn. So that gives a dose of 3 tons/ ha of nutrients that can be absorbed by plants that will be used to increase growth and production.

A dolomite dose of 3 tons/ha gave the highest taproot weight, but it was not significantly different from the control treatment (without dolomite). This was thought to be caused by washing some of the dolomite from rainwater because the study was carried out in the rainy season.

Effect of pig manure

The results of the variance test of pig manure showed a significant effect on all observed variables. Based on the results of the HSD test at 0.05 level, the best level was treatment with pig manure at 4 kg/plot (40 tons/ha).

The highest dose of pig manure, 4 kg/plot, resulted in the greatest response for all observed variables. It is suspected that the high nutrient content influenced this result in pig manure (Pujiastuti et al. 2021; Vamvuka and Raftogianni 2021) stated that the nutrient content of pig manure includes 3.75% nitrogen, 3.13% phosphorus, and 2.50% potassium with 68% humidity. The element N is the highest nutrient element in pig manure which functions as a form of plant vegetative organs such as plant height and several leaves. Plants containing sufficient N elements will form wider leaves and higher chlorophyll content, if this happens, it will affect the process of forming assimilate/ carbohydrates that are sufficient for plants to use in forming vegetative organs (Duan et al. 2012; Bhato 2015; Marlina et al. 2015). In line with that, Nokas et al. (2015) and Thana (2019) stated that nitrogen plays a role in increasing plant growth, such as the formation of green leaves, increasing protein levels, and the proliferation of microorganisms in the soil.

The level of pig manure also plays a role in increasing the activity of microorganisms, especially in the process of decomposition of organic matter to produce nutrients that are available and can be absorbed by plants. Galla and Naman (2021) and Chinyaeva et al. (2022) suggested that adding manure to the soil will stimulate microorganisms to increase their activity. This is because the number of sources of energy needed by microorganisms for activities increases, impacting plants' absorption of nitrogen and phosphorus.

The high macronutrient content in manure likely had a beneficial effect on the growth of carrot plants, such as several leaves increasing, while their chlorophyll content also increased, resulting in a high photosynthetic rate. Limbongan (2019a) and Yasa et al. (2019) stated that a high number of leaves indicates a higher photosynthetic efficiency of plants so that photosynthesis assimilation for growth and development of taproots is relatively fast. Therefore, giving high doses of pig manure can provide high carrot production so that the right dose of pig manure is used to get maximum carrot production above 40 tons/ha (Figure 4).

Interaction of dolomite and pig manure

Based on the interaction variance test results between dolomite and pig manure, the best treatment combination was 3 tons/ha of dolomite with 40 tons/ha of pig manure resulting in 38.67 tons/ha of carrot taproot production. The interaction of dolomite and pig manure is linear where at a pig manure dose of 30 tons/ha, it shows an increase in taproot length with an increase in dolomite dose (Figure 3).

The interaction of dolomite and pig manure with a combination of 3 tons/ha of dolomite and 40 tons/ha of pig manure gave the highest yield on length variables. This is because in the initial phase of taproots, dolomite and pig manure together supply the nutrient needs of carrot plants in the growth phase, especially in taproot formation. In addition, the high content of P and K elements in pig manure is able to produce high photosynthate which is then translocated to all parts of the plant and the rest is channeled to the s. In line with that, Limbongan (2019b) stated that P and K elements greatly affect fruit weight because they form storage networks. The formation of better fruit flesh is strongly supported by the presence of microelements, especially Fe.

The combination of treatments that produced the highest taproot weight was 3 tons/ha of dolomite and 40 tons/ha of pig manure with a taproot weight of 38.67 tons per hectare. However, this result has not yet reached optimal production. This is because the dose of manure given has not been maximum, so growth and production with these interactions are not significantly different. In addition, dolomite and pig manure have not been able to act together to increase carrot plants' growth and production. In line with that, Steel and Torrie in Safei et al. (2014) state that there is no significant difference in an interaction caused by each treatment factor acting independently or the influence is independent.

In addition, it is suspected that part of the dolomite treatment given was washed away by rainwater because of the sloping experimental land and high rainfall, so the nutrient content was also partially washed off by rainwater which plant roots had not absorbed. So that the highest dose combination 3 tons/ha of dolomite and 40 tons/ha of pig manure has not been able to show a significant difference with the low dose combination.

In conclusion, dolomite lime influenced carrot plant height, the number of leaves, and taproot length and taproot diameter but had no significant effect on taproot weight, taproot weight per plot, and taproot weight per hectare. The optimal dose of dolomite was 300 g/plot (3 tons/ha) for a significant influence on the growth and production of carrots Pig manure influenced all investigated variables. The optimal dose of pig manure was 4 kg/plot (40 tons/ha), which substantially influenced all investigated variables. The interaction between dolomite and pig manure substantially influenced taproot length but did not affect the other observable variables; the optimum treatment combination was 3 tons/ha of dolomite with 40 tons/ha pig

manure, yielding 38.67 tons/ha of carrot taproots. To get maximum results, it is advisable to cultivate carrots on rainfed land with a combination was 3 tons/ha of dolomite with 40 tons/ha of pig manure.

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Physico-chemical, heavy metal analysis and physical composition of household solid waste, Shone Town, Ethiopia

GIZACHEW BALILO, ABEBE ASCHALEW, REJILA MANIKANDAN♥, ARBO FEYISA
School of Natural Resources Management and Environmental Sciences, College of Agriculture and Environmental Sciences, Haramaya University. Haramaya, Dire Dawa, Ethiopia. Tel.: +251-927847321, ♥email: rejularaja@gmail.com

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Abstract. *Balilo G, Aschalew A, Manikandan R, Feyisa A. 2023. Physico-chemical, heavy metal analysis and physical composition of household solid waste, Shone Town, Ethiopia. Nusantara Bioscience 15: 32-37.* In Ethiopia, Shone is one of the country's fast-developing towns; inadequate solid waste management was observed. Therefore, the objective of the study was to determine the generation rate and selected physico-chemical characterization and heavy metal analysis of household solid waste. The study was conducted on randomly selected 120 households from two Kebele of the town. The physico-chemical analysis results revealed that moisture content, pH, electrical conductivity (dS/cm), nitrogen, organic carbon, (%), phosphorus and potassium content of household solid waste were 54.6, 8.25, 2.52 (dS/cm), 2.47, 10.25, 0.54, and 0.82%, respectively. In addition, in the decomposed household solid waste samples, heavy metals such as iron, manganese, copper, zinc, nickel, cobalt, chromium, lead, and cadmium were analyzed using atomic absorption spectrophotometer. Results showed the concentration of these heavy metals in the decomposed solid waste samples was in the order of iron>manganese>zinc>copper>nickel>chromium>cobalt>lead>cadmium. Daily solid waste from those households was separated into its components, and each component was measured. That was conducted for 7 consecutive days to quantify and characterize household solid waste. The result revealed that the household solid waste generation rate in the study area was 0.206 kilogram/capita/day. The physical composition of the solid waste in Shone Town is mainly organic, constituting 92.8%. The organic waste of the town has a high content of biodegradable waste such as food waste (49.56%), ash and dust (29.74%), yard waste (4.95%), textile (4.006%), old shoes and bone (2.5%), paper and cardboard (2.04%) and recyclable material such as plastic (3.28%), glass and metal (3.88%) all account for 92.8% (biodegradable) and 7.17% (non-biodegradable), respectively.

Keywords: Generation rate, heavy metals, household, physico-chemical, Shone, solid waste

INTRODUCTION

Household solid waste management is one of the critical concerns facing developing countries because of the social, economic, and environmental implications once not properly managed. Household solid waste, normally termed garbage or trash, is an inevitable byproduct of human activity. Municipal solid waste is usually generated from human settlements, small industries, and commercial activities. Solid waste disposals (open dumps, landfills, sanitary landfills, or incinerators) represent a significant source of metals released into the environment. Household solid waste management is one of the critical concerns facing developing countries because of the social, economic, and environmental implications once not properly managed. Studies showed that household solid waste, directly and indirectly, affects the environment and human welfare. Improper household solid waste management causes serious health effects in daily life. Poor waste management, inadequate collection, and improper disposal of the waste facility could lead to various diseases, infections, and infestation. These include Malaria, Typhoid, Diarrhea, Cholera, Helminthiasis, and Dysentery (Yasmin and Rahman 2017; Ochieng et al. 2019). Moreover, 30-50% of the waste generated in developing countries is collected and managed properly. Still, the remaining is either burned or left to decompose in open space or dumped in unregulated landfills (Abebe et al. 2021).

Solid waste is a global environmental problem in today's world in both developed and developing countries due to rapid population growth, economic activities, and the rise of community demand, accelerated solid waste generation in the world. Activities in society generate large quantities of waste, posing a problem for their disposal. Therefore, improper disposal leads to unhygienic conditions besides spoiling the aesthetics (Gezahegn et al. 2018). Solid waste management practices differ between developed and developing countries, urban and rural areas, and residential and industrial producers. Solid waste management in most African countries has two problems: a lack of accurate data on waste generation and characterization and a lack of information about waste collection, processing, and disposal. The changing economic trends and rapid urbanization complicate solid waste management in developing countries.

Consequently, solid waste is increasing in quantity and changing in composition from less organic to more paper, packing waste, and plastics, glass, and metal waste, among other types, a fact leading to rates. But most household solid waste compositions generated in most parts of Africa are biodegradable organic wastes (Gezahegn et al. 2018). Therefore, solid waste management is a major public health and environmental concern in cities of many African

countries. The expansion of the urban population is one of the reasons many African countries continuously increase the amount of solid waste. However, because of the development of cities and the increase in the population, the country is doing the opposite boost in waste disposal (Emmanuel and Jiquan 2019).

Typically, components of most household solid waste are food waste, paper, cardboard, plastics, textiles, leather, yard waste, wood, glass, tin cans, aluminum, other metal, ashes, street leaves, special waste (including bulky items, consumer electronics, white goods, yard waste collected separately, batteries, oil, and tires), and household hazardous wastes (Tesfahun et al. 2022). The percentage of household components varies with location, season, economic condition, and many other factors. The composition of Solid Wastes plays a major role in determining the compaction, decomposition, and incineration process. Furthermore, the waste generated in low and middle-income countries has high moisture content and density (Syeda 2014).

Shone Town Administration is characterized by rapid population growth and urbanization. This rapid increase in population, coupled with the town development, has produced an increasing solid waste generation rate. The household solid waste generated in the study area is dumped in open areas. Roadsides and gully communities are unaware of recycling, reducing, and using the massive amount of waste generated in the town. As a result, household solid waste management in Shone Town Administration has not been carried out sufficiently and well. As a result, the aesthetic and sanitary conditions of the town have become more serious from time to time, and people are suffering health problems in such conditions. In addition, the generation rate, composition, and characteristics of household solid waste in the Shone Town Administration are unknown. Therefore, this study was designed to determine the generation rate and selected Physico-chemical characteristics of household solid waste in Shone Town Administration, Hadiya Zone, Southern Ethiopia.

MATERIALS AND METHODS

Description of the study area

The Shone Town is the administrative center of Shone Town Administration (Figure 1). It is located at a distance of 338 km from Addis Ababa, 121 km from Hawasa Town, and 98 km from Hosanna Town. Shone is a town located in the southern part of Ethiopia at 37° 56'30" to 37° 58'0" East and 7° 7'0" to 7° 10'0" North. According to the total population of Shone, the town administration was 47,420. Among them, 23,236 are male, and 24,184 are female. The district's mean annual maximum and minimum temperatures are 28°C and 8°C, respectively, with an altitude range between 1,000 to 2,300 masl and annual rainfall estimated at 531.1 mm to 1261 mm (STAMRMAO 2020).

Sample size determination and sampling technique

Shone Town Administration has seven administrative *kebele*. For this study, the two *kebele* named Arancha and Licha were selected purposively based on the households' settlement and the number. A total of 120 households were selected from two *kebele* (Arancha 50 and Licha 70 household) by using the following formula (Cochran 1977).

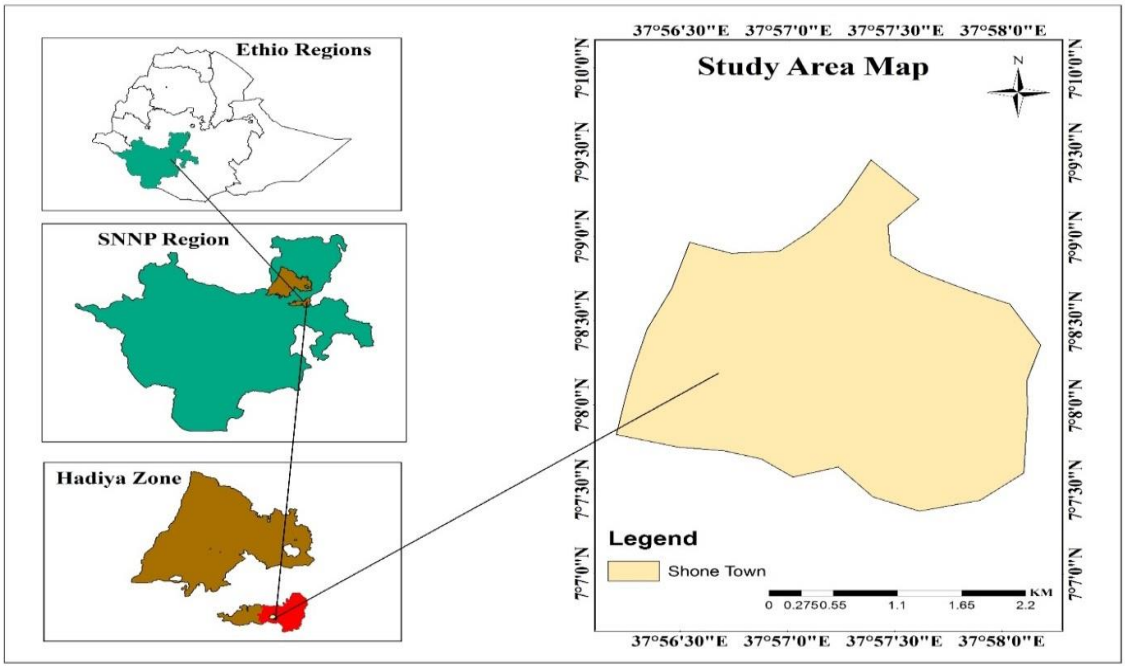

Figure 1. Map of the study area in Shone Town, Ethiopia

$$n = \frac{NZ^2PQ}{d^2(N-1)+Z^2(PQ)}$$

Where: n = total sample size, N = Total number of households housing units of the selected kebele, Z = standard normal deviation at the required confidence level that corresponds to a 95% confidence interval equal to 1.96, d = the level of statistical significance (0.05), P = housing unit variable (the proportion in the targeted population estimated to have characteristics being measured), i.e91% p=0.91, Q = 1-p i.e., 1-0.91= 0.09. According to data from Shone Town Administration Municipality Office (STAMO), the socio-economic profile is about 2,486 housing units N in the two selected kebele, Arancha, and Licha, in the Shone Town.

Hence $n = \frac{NZ^2PQ}{d^2(N-1)+Z^2(PQ)}$

$n = \frac{2486(1.96)^2 \times 0.91 \times 0.09}{(0.05)^2(2486-1)+(1.96)^2(0.91\times0.09)}$ n =120

Sample collection

Determination of the generation rate and composition of solid waste generated from selected 120 households should be done carefully. Accordingly, the collection and sorting of wastes from the areas of the household were conducted for 8 consecutive days. The first-day training was given to the enumerators, who distributed the plastic bags for each selected household one day before collection. Two plastic bags labeled with their house number was given as one for dry (red plastic) and another for wet waste (black plastic) for the selected household. Another bag with the same label was given for the next day's collection, and these processes were continued for one week. Every morning, the solid wastes were collected from selected households using donkey carts, and after that, the solid waste was transported to a temporary sorting site. Sorting was the next step after collecting waste in the long process of determining the generation rate and composition of waste from household solid waste. Finally, sorted wastes from households were measured, and the value of each sample was recorded.

Determination of household solid waste generation rate

The household solid waste generation rate was determined by Fobil et al. (2008). Accordingly, the Per Capita Per Day Solid Waste Generation Rate (PCPDSWGR) was calculated using the following formula.

$$\text{PCPDSWGR} = \frac{\text{Total Solid Waste Generation within 7 Days}}{\text{7 Days} \times \text{ (Total family size of 120 households)}}$$

Based on the above equation, the daily, weekly, monthly, and annual generation rate of household solid waste was calculated for the town as the town's total population (47,420) times per capita per day of household solid waste generation rate.

Sample preparation for physico-chemical analysis

From selected *kebeles*, two samples of household solid waste were collected in two days, and subsequently, for one week, four samples from two *kebele*. After that, the collected samples were mixed to get a composite. The composite household solid waste was well mixed (homogenized) by minimizing the size of each sample component, chopped and grounded material like a knife, mortar, and pestle into powder forms. A sample of 500 gm was obtained for Physico-chemical analysis.

Physico-chemical analysis

The determination of moisture content, a sample from which were selected components of the solid waste immediately after measuring the weight, was taken to Laboratory for 5 consecutive days. Fifty (50) grams of fresh compost samples of food and yard waste were weighed before being dried at 105°C and weighed moisture. Then, the samples were put into an oven for 24 h at 105°C. Afterward, the sample was removed from the oven, cooled in a desiccator for 30 minutes, and weighed. The percentage of moisture was calculated as follows (Hogarh 2012).

$$\%MC = \left(\frac{W-D}{W}\right) \times 100$$

Where,

MC = moisture content

W = initial weight of sample in grams,

D = weight of the sample after drying at 105°C in grams

The pH value of the sample was determined by a pH meter with a glass electrode. Next, 10 grams of the sample was placed in a flask; 500 ml of distilled water was added and stirred for 3 to 5 minutes. The mixtures were allowed to settle for 5 minutes, and pH was measured using a pH meter with a glass electrode (Philippe and Culot 2009). Finally, the Electrical conductivity of a 1:1 aqueous extract was measured, and the reading was corrected to conductivity at 25°C sample saturation with the chemical method (Rhoades 1996).

Total nitrogen content was analyzed using the Kjeldahl digestion described by Hogarh (2012). The total organic carbon content in the samples was measured by Walkley and Black (1934). Total phosphorus analysis in compost samples requires a conversion of insoluble phosphates to soluble forms by digestion with a mixture of nitric acid and sulphuric acid. Phosphorus was measured using a spectrophotometer. Total potassium analysis in compost samples requires a conversion of insoluble potassium to soluble forms by digestion with a mixture of nitric acid and sulphuric acid. The potassium content in the solution was estimated with a flame photometer (Hogarh 2012).

Analysis of heavy metals from household biodegradable solid waste

The solid waste composite samples were digested with concentrated nitric (HNO_3) and perchloric ($HClO_4$) acids. First, 3 mL of concentrated HNO_3 were added to 0.5-1.0 g samples. Next, the acid sample mixture was heated to about 145°C for 1 hour. After 1 hour of heating, 4 mL concentrated $HClO_4$ was added, and the mixture was heated to 240°C for a further 1 hour. After complete digestion of all samples, the digests were allowed to cool to room

temperature. The content of the digests was filtered through Whatman No. 42 filter paper and diluted to 50 mL volume with deionized water. The diluted digests were taken for subsequent analysis of heavy metals, as described by Saudi et al. (2021). The selected heavy metal concentrations (Fe, Mn, Cu, Zn, Cd, Ni, Pb, Co, and Cr) were measured by Atomic Absorption Spectrophotometer model number 210VGP. The level of each heavy metal was measured at a specific wavelength; Chromium (357.9nm), Lead (217nm), Zinc (324.8nm), Iron (248.3nm), and Cadmium (228.8nm).

RESULTS AND DISCUSSION

Physical compositions and household solid waste generation rate

This study showed that (Table 1) almost half (49%) of total solid waste generated by households of Shone Town was food waste compared to other waste categories. The fraction of food waste obtained in this study is in the same order of magnitude as those obtained in previous studies for cities in other parts of Africa. For example, in developing and even most developed countries, food waste represented a large fraction of household solid waste in Nsukka town, and almost half (47%) of total solid waste generated by households was food waste in Nigeria (Endrias and Solomon 2017). The quantity of food waste in household solid waste is commonly taken as a hint of living standards, but it also represents the lifestyle pattern. In line with this, the study conducted in Fonko Town of Analemo District, Southern Ethiopia, also revealed that the composition of food waste takes a larger quantity than other households' solid waste composition (Abebe et al. 2021).

In this study, ash and dust waste was 29.74%, possibly due to the main energy source being firewood in the study area. Moreover, this study cooperates with the study conducted in Sawulatown in Ethiopia revealed the cooking energy source for most of the households were wood, charcoal, cow dung, and yard trimmings (Haile et al. 2019). In addition, yard waste is 4.95%, textile is 4.006%, old shoes and bones 2.5%, and paper and cardboard 2.04%.

Generally, the present study also revealed a large quantity (92.8%) of biodegradable household solid waste in Shone Town. That was comparable with other studies conducted in Ethiopia, especially the physical composition of the household solid waste in Sodo town was 93.7% (Max and Timothy 2014). In line with this, Meseret et al. (2019) reported that the large quantity of organic content (compostable organic matter) in the solid waste indicates the necessity of recycling the organic waste into valuable resources like compost (organic fertilizer).

In the study area, non-biodegradable solid waste was identified, such as plastic at 3.28%, and glass and metal accounting for 3.88%. The non-biodegradable solid waste found in the study areas compared to biodegradable solid waste is very small, i.e., plastic, glass, and metals constitute 7.17% of the total solid waste generated from households. A study conducted in Jigjiga Town Ethiopia revealed that glass and metal appear negligible because they are not discarded for disposal but are sold to recyclable material buyers (Amdiya et al. 2022).

Physico-chemical properties of household solid waste

The moisture content in the study area was 54.6% (Table 2). The moisture content in the study area was high compared to other findings due to the large portion of household solid waste being organic, and the season was not too cool and not too dry it was medium. That is in line with this study conducted in Fonko Town of Analemo District, Ethiopia, which accounts for moisture content at 46.44% (Abebe et al. 2021). The variation in the amount of moisture content depended on the source, composition, and season fluctuation.

The pH value of this study was 8.25 for composite samples, which were collected from household solid wastes, indicating that the composite sample's pH values were slightly alkaline. However, it showed within the standard limits of 5.5-8.5 and is suitable for composting. In addition, Hiranmai and Anteneh (2016) observed that pH tended to a neutral value towards the end of the composting process, indicating that the matured compost will have neutral pH. The above finding revealed that the pH of a household's solid waste at the beginning step might be acidic or basic until it reaches the neutral pH at the end of matured compost, which means that when pH is acidic, composting is very slow because microorganisms are destroyed. Therefore, the pH of this study was 8.25, and the basic then rate of decomposition is fast at the beginning step of the process to reach the neutral value to form compost.

Table 1. The physical composition of household solid waste in Shone Town Administration, Ethiopia

Categories of solid waste	Types of solid waste	Weight in kg/week	Kg/capita/day	%
Biodegradable	Food waste	501.942	0.102	49.56
	Ash and Dust	301.1652	0.0612	29.74
	Yard waste	50.16	0.0144	4.95
	Textile	40.575	0.00824	4.006
	Old shoes and bones	25.58	0.0052	2.5
	Paper and cardboard	20.7	0.004	2.04
Non-biodegradable	Plastic	33.24	0.00675	3.28
	Glass and metals	39.368	0.008	3.88

Table 2. Physico-chemical characteristics of organic solid waste from households in Shone Town, Ethiopia

Parameters	Units	Values	Recommend standards
Moisture contents	%	54.6	45-65
pH	-	8.25	6.9-8.3
EC	dS/m	2.52	2-6
Nitrogen	%	2.47	0.5 to 2.5
Organic carbon	%	10.25	<30
Phosphorus	%	0.54	0.4-1.1
Potassium	%	0.82	0.6-1.7

Table 3. Heavy metals concentrations in decomposed household solid waste

Types of heavy metals	Concentration (mg/kg)
Fe	912.7
Mn	172.6
Cu	34.42
Zn	68.92
Ni	6.5
Cu	0.75
Cr	7.74
Pb	2.22
Cd	0.4

In the present study, the electrical conductivity (EC) value was 2.52 dS/m, which determines the amount of salt in solid waste. The EC values also found in solid waste were within the recommended range. Moreover, Saudi et al. (2021) reported that EC is an important parameter in determining the quality of solid waste concerning the composting process. The results of this study revealed that the organic carbon of dry solid waste is 10.25%. The carbon-rich waste, such as dried leaves, tree bark, cardboard, etc., whereas the fruits and vegetable waste contains a high percentage of nitrogen such as banana, cabbage, carrot, potato, etc. (Cristina and Ana 2020). Similarly, the Nitrogen content of the organic matter in the study area was 2.47% which shows that food waste contains the vegetable and fruit (cabbage, potatoes, tomato, banana, mango, etc.), which was the main source of nitrogen (green waste)

The phosphorus value for biodegradable composite samples of household solid waste in the study area was 0.54%. This finding was comparable to the other study of the phosphorus contents of the compost in Ethiopia in Fonko Town of Analemo District, Ethiopia (Abebe et al. 2021). The value of the total potassium for composite biodegradable household solid waste in the study area was 0.82%. This finding was supported by a study conducted on chat waste in Aweday, that the average value of potassium (0.68-0.12%) and the result for the organic waste composite sample lie reasonably within the acceptable range for use in the preparation of compost.

Finally, the results of these studies revealed that the physico-chemical composition of selected biodegradable solid waste from household solid waste in the Shone Town Administration was suitable for composting. Due to the high percentage of biodegradable organic matter in the study area, composting the solid waste and using the compost as an organic fertilizer would be the best option for sustainable household solid waste management. That could be a good source of organic manure or fertilizer for farmers to reduce inorganic (chemical fertilizers) use. Composting system is technically simple, economically viable easily adaptable to anywhere at the farmers' level for composting locally available feedstock (Hiranmai and Anteneh 2016).

Heavy metals concentrations in decomposed household solid waste

The selected heavy metal, such as Fe, Mn, Cu, Zn, Ni, Co, Cr, Pb, and Cd, was identified from solid waste. The results indicated in Table 3 revealed that the highest proportion of heavy metals such as Fe (912.7 mg/kg) followed by Mn (172.7 mg/kg) and Zn (68.92mg/kg) was recorded in the study area (Table 3). On the other hand, the least heavy metal in solid waste is Cd (0.4 mg/kg).

The concentrations of heavy metals such as Fe, Mn, Zn, and Cu in decomposed solid waste (Table 3) were relatively higher than the results reported by Alemayehu et al. (2016), which might be due to more metallic waste in the study area. On the other hand, the presence of Cd (0.4 mg/kg) and Pb (2.22 mg/kg) in decomposed wastes was relatively lower than in other heavy metals. Similar findings were reported by Alemayehu et al. (2016) for Harari city waste dumping sites. In addition, heavy metal concentration variation also depends on summer and monsoon seasons. In line with this, Saudi et al. (2021) reported that a high concentration of heavy metals was observed during the summer season in their study. However, the concentrations of heavy metals such as Cu, Zn, Ni, Cr, Pb, and cadmium in decomposed solid waste did not exceed the limits compared with Indian and USEPA standards (Anjanapriya and Lalitha 2016).

Saudi et al. (2021) pointed out that Pb, Cd, Cu, and Ni are potentially toxic to plants and animals and have been shown to accumulate in the food chain. Zinc (Zn) is a necessary micronutrient for plants, but at a high level is phytotoxic and might reduce the fertility of the land. Therefore, continuous dumping of wastes can disturb natural soil's physical, chemical, and biological characteristics, pollute groundwater and causes a hazardous impact on human health. In addition, the food crops grown on soils contaminated with heavy metals absorb the metal ions depending on their metal uptake and storage capabilities.

In conclusion, the household solid waste generation rate of Shone Town Administration was 0.206 kg/capita/day, and based on the per capita/per/day solid waste generation rate, the daily, weekly, monthly, and yearly generation rate of Shone Town Administration was 9,768.52, 68,379.64, 293,055.6 and 3,565,509.8 Kg respectively. The physical

composition of household solid waste reveals the presence of food, yard waste, ash, dust, textile, old shoes, bones, paper, cardboard, plastic, glass, and metal waste. Among these wastes, biodegradable waste was found to be in a large quantity in the study area. Regarding the physico-chemical analysis, such moisture content pH values were within the standard limited range. Moreover, the household biodegradable solid waste identified a sufficient quantity of organic carbon, nitrogen, phosphorus, and potassium contents. Finally, results showed the concentration of heavy metals in the decomposed solid waste samples was in the order of Fe>Mn>Zn>Cu>Ni>Cr>Co>Pb>Cd. Therefore, in the study area households, solid waste is suitable for composting and can be used as organic manure.

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Diversity and role of woody Non-Timber Forest Products in Doba District, Eastern Ethiopia

AMARE FASSIL ABEBAW[1,♥], PATRICK VAN DAMME[2,3]

[1]Department of Biology, Injibara University. Injibara, Ethiopia. Tel./Fax.: +251933798608, ♥email: amarefassil@gmail.com
[2]Laboratories for Tropical and Subtropical Agriculture and Ethnobotany, Department of Plants and Crops, Faculty of Bio-Science Engineering, Ghent University. Coupure Links 653, 9000 Gent, Belgium
[3]Faculties of Tropical AgriSciences, Czech University of Life Sciences Prague. Kamýcká 129, 16500 Praha-Suchdol, Czech Republic

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Abstract. *Abebaw AF, Damme PV. 2023. Diversity and role of woody Non-Timber Forest Products in Doba District, Eastern Ethiopia. Nusantara Bioscience 15: 38-47.* Non-Timber Forest Products (NTFP) play a pivotal role as local sources of medicine, household paraphernalia, and fodder and offer income opportunities that can mitigate poverty. This study was conducted in Doba District, West Hararghe Zone, Eastern Ethiopia, to analyze the diversity and economic contribution of Non-Timber Forest Products. Both ethnobotanical and vegetation data were cross-sectionally collected from February 2020 up to June 2021. A total of 422 informants selected randomly from forest inhabitants were interviewed using semi-structured questionnaires to explore NTFP utilization and their economic contributions. Vegetation data were collected from 56 sample plots of each 20m x 20m (400m^2) along transects lines. Vegetation data collected were analyzed for the relative density of species, Shannon-Wiener index, species evenness, and relative frequency. From the household survey, 58 plant species categorized in 55 genera and 35 families were identified as NTFP-bearing species. Analysis of the socio-economic data shows that NTFP collection is a day-to-day activity of the local communities. These communities use NTFPs in different types of use categories, namely medicinal plants (32, 55.17%), melliferous species (20, 34.48%), wild food and condiment species (22, 37.93%), farm implements (7, 12.06%), wood and flavoring (6, 10.34%), source of energy (20, 35.71%), and household utensils and construction materials (30, 51.72%). The market survey analysis showed that NTFPs account for about 15.77% of the annual household income. The vegetation survey showed that Ades Forest has a good status with an average density of 1,450 plants ha^{-1}, high Shannon-Wiener index (H =3.299), and species evenness (E = 0.81). Ades Forest harbors a diverse number of NTFP-bearing species used for several categories. The study reveals the real experiences of the local communities in utilizing NTFPs for their livelihood. Forest dependency rates tend to be higher among poor households. However, further study on production potential and market chain analysis should be done together with awareness creation activities to get a sustainable product for the community and conserve the forest resource.

Keywords: Ades Forest, diversity, Non-Timber Forest Products

INTRODUCTION

Non-Timber Forest Products (NTFPs) cover biological resources of plant and animal origin harvested from natural forests, artificial plantations, wooded land, farmlands, and trees outside the forest. Both have been domesticated (Debela 2019). These NTFPs yield diverse sets of products: leaves and twigs that may be used in decorative arrangements; food such as fresh fruits plus juices; wood carved or woven into pieces of art or utilitarian objects; and roots, leaves, and bark processed into herbal remedies or medicines (Solomon and Tajebe 2014). Worldwide, between 3.5 and 5.8 billion people in or around natural forests depend on NTFPs for subsistence income and livelihood security (Shackleton and De Vos 2021), doubling the numbers from previous studies (World Bank 2004; Solomon and Tajebe 2014). About 80% of the populations of developing countries use NTFPs to meet at least some of their health and nutritional needs (Pandey et al. 2016; Talukdar et al. 2021). It is hard to imagine humans and animals living without plants as they often provide a complex array of goods and services. The world benefits from forests (in various stages), deriving resources such as food, medicine, timber, fuel wood, and livestock fodder (Hlaing et al. 2017; Gitz et al. 2021). Moreover, in general, African forests and sub-Saharan African countryside inhabitants considerably depend on NTFPs for their livelihoods (World Bank 2004; Melese 2016; Soe and Yeo-Chang 2019).

The diverse character and scope for exploitation of NTFPs become product and location-specific (Ros-Tonen and Wiersum 2005). This finding has been prompted by the fact that communities living adjacent to forest reserves rely greatly on the NTFPs for their livelihoods. Therefore any effort to conserve such resources should, as a prerequisite, understand how the host communities interact with them (Suleiman et al. 2017). Therefore, scientific documentation and information on diversity use patterns and the economic contribution of species can authenticate the conservation and sustainable use of such plant resources in any given area (Masoodi and Sundriyal 2020).

Ethiopia is a tropical country well-endowed with a diverse floral that includes about 6,000 species of higher plants with 10-12% endemism (IBC 2005; Melese 2016).

Ethiopia's forest and other vegetation resources offer diverse NTFPs that provide substantial inputs for the livelihoods of a very large number of people in the country, with an estimated annual turnover of more than $US 2.3 billion to the national economy (Worku 2015; Melese 2016). Some 8-10% of higher plants recorded in Ethiopia are assumed to be edible, whereas some 10% are used for medicinal purposes for human and livestock diseases (Abebe et al. 2003; Duguma 2020).

Substantial study records (Chilalo and Wiersum 2011; Melaku et al. 2014; Meles et al. 2016; Beyene et al. 2020; Reta et al. 2020) were done in different regions of Ethiopia on the contribution of NTFPs to the local or national economy. However, those studies are confined to only some parts of the country and with patchy patterns. Nevertheless, the vagaries of resident engagement in forest management and their utilization of NTFPs provide knowledge relevant to sustainable forest management practices (Thammanu et al. 2021).

This study addressed the following four principal questions. First, does the local community exercise NTFP harvest at current times? Second, to what extent do these NTFPs contribute to improving the community's livelihoods? Third, what are the major constraints in NTFP utilization? Finally, how diverse is Ades Forest to allow continuing to provide for the current NTFPs utilization needs of the community?

Doba District, Eastern Ethiopia, is among the districts of West Hararghe Zone where different types of NTFPs and their significance were not extensively studied or well-documented. The district is characterized by low agricultural output and land productivity below what it should be to fulfill the area's minimum food requirements (Gizaw 2021). This study, therefore, aimed at documenting the ethnobotany and diversity of NTFPs in the district. This research has great significance in exploring the socio-economic importance of NTFPs and quantifying them in the study area.

MATERIALS AND METHODS

The study site

The study was conducted at Ades Forest, Doba District West Hararghe Zone of Oromia Regional State, Ethiopia. Doba District is 382 km east of the capital city, Addis Ababa, and 45 km from Chiro, the Zonal capital town. Geographically, the district lies at 9°15′N and 41°00′E with altitudes ranging from 1149 to 2773 m.a.s.l. (Figure 1). Ades Forest is among Ethiopia's national priority forest resources. The forest covers some 618 ha. Some of the dominant species in this forest are *Gymnosporia obscura*, *Podocarpus falcatus*, *Croton macrostachyus*, and *Mytenus* species (Atomsa and Dibbisa 2019).

Data collection

Ethnobotanical and vegetation data were collected from February 2020 to June 2021. Forum Group Discussions (FGD) collected qualitative data from key informants. Quantitative ethnobotanical and vegetation data were collected through semi-structured questionnaires and field surveys following (Teka et al. 2020).

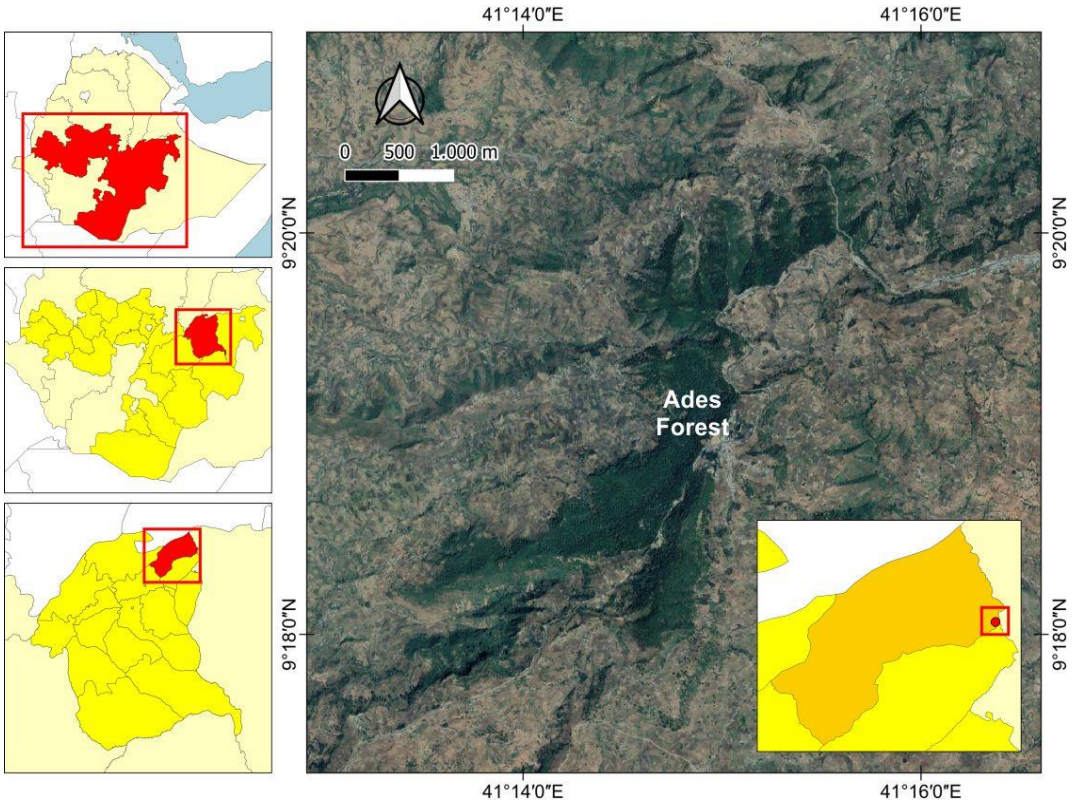

Figure 1. Map of the study area showing Doba District and Ades Forest, Ethiopia

Ethnobotanical data collection and documentation were done through key informant interviews and group discussions to gather information on NTFPs collection methods, use categories, their contribution to household income generation, and the factors that hamper the utilization mentioned above. The total sample size was determined following Cochran (1997) and using a 95% Confidence Level (CL), 0.05 margin of error, 50% proportion, and 10% non-respondent rate proportionally allocated to each of the five selected kebeles (the lowest administrative organization). The sample size was determined as follows.

$$n = \frac{(Z\alpha)2}{2} * \mathrm{P}\ (1\text{-}\mathrm{P})/\mathrm{W}^2$$

Where: n: sample size; W: Margin of error; P: population proportion, and hence

$$\mathrm{n} = \frac{(1.96)2(0.5)(0.5)}{(0.05)^2} = 384$$

Considering a 10% non-respondent rate (38), the final sample size was increased to 422 respondents.

Three Kebeles (The smallest administrative level in Ethiopia), namely Bekelcha Biftu, Ifa Aman, and Ifa Ramata, were selected for the study based on their proximity to the Ades Natural Forest and the local communities' dependency on forest products. Out of the 422 respondents, 36 key informants (i.e., 12 informants from each kebele) having confirmed herbal medicine extraction and local plant identification practices were chosen. The remaining 382 respondents were selected based on the population proportion criterion: Bekelcha Biftu (n = 103), Ifa Aman (n = 125), and Ifa Ramata (n = 154). Systematic sampling was used to select respondents from alphabetically listed households of the three kebeles and compute the skipping interval (K) by dividing the total number of households of each kebele with the total sample size for the kebele (i.e., K = 10). The first respondent of each kebele was determined by a lottery method; the other respondents were selected at every 'K' value from the mother list. A market survey was also done at the local Doba District market in October 2020 for four consecutive weeks for data consistency to assess the commercialization practices for NTFPs in the area.

A systematic sampling technique was used to collect vegetation data in Ades Natural Forest by laying three transect lines following Kent (2012). Sixty-nine sample plots (i.e., 2.76 ha.) of 400 m^2 each laid out along the transect lines at 100 m intervals were used for tree and shrub surveys. A total of 345 subplots of each 1 m^2 were used for evidencing saplings and seedlings. Those subplots are put at the four corners and one at the center of each larger quadrat. All woody plant species in each quadrat were recorded with their growth habits. In addition, older people in the community identified vernacular names (Oromipha language) of plants from the field. Scientific names were identified using Flora of Ethiopia and Eritrea (Volumes 1-8), compared with already identified specimens and using own knowledge, and validated using Plants of the World Online (POWO). Voucher specimens were collected, identified, and stored in Oda Bultum University Herbarium Room. Plant specimen collection permission was attained from Oda Bultum University, Chiro, Etiopia. Our field studies, including plant material collection, comply with the IUCN policy statement on research involving species at risk of extinction, the convention on the trade in endangered species of wild fauna and flora, and other relevant institutional and national guidelines and legislation.

Each plot measured the height and diameters of all woody plant species at breast height (DBH 1.3 meters above the ground) with height ≥ 2 m and DBH ≥ 2.5 cm. In addition, individuals having a height < 2 m and DBH < 2.5 cm were counted. Key informants (knowledgeable and older people in the area suggested by residents) were used to provide local names of all evidenced plants. Moreover, phytogeographic comparisons of Ades Natural Forest with other similar forests (data from literature) were made using of Shannon-Wiener's diversity (H'), Evenness (E), and richness diversity indices.

Data analysis

Ethnobotanical data were analyzed using descriptive statistics (frequencies and percentages) and presented with tables and figures. Associations among wealth groups against different agricultural activities were assessed using SPSS (Statistical Package for Social Sciences). Species utilized for NTFPs were sub-grouped based on their use category.

Microsoft Excel spreadsheets analyzed Vegetation data using diversity parameters (i.e., relative density, Shannon-Wiener's diversity index, species evenness, and relative frequency). Species density was computed as the number of species collected per area following Gotelli and Colwell (2001). Relative density is the proportion of the density of a given use category to the total density of the study area:

$$\text{Density} = \frac{\text{Total number of individual species}}{\text{Total sampled area}}$$

Species frequency

Species frequency was computed as the proportion of sample units that contain a given species following (Sewale and Mammo 2022).

Shannon-Wiener diversity index

The Shannon-Wiener diversity index was computed as follows:

$$H = -\sum^{s} pi \ln pi$$

Where:

H : Shannon-Wiener's diversity index

$\sum$: The sum of calculations

p : The proportion (n/N) of individuals of one particular species (n) divided by the total number of individuals found (N)

ln : Natural logarithm

s : Number of species

RESULTS AND DISCUSSION

Results

Analysis of the ethnobotanical survey revealed 58 woody plant species categorized in 55 genera and 35 families as NTFP-bearing species. The use of such a diverse number of plant species for NTFP use was also evident from similar studies (Fetene et al. 2010; Solomon and Tajebe 2014; Reshad et al. 2017) in the country. The analysis of the ethnobotanical data shows that NTFP collection is a daily activity of the local community in the study area. Local communities have multiple use categories for NTFP, which is in line with results from other studies in the country (Mullatu 2010; Chilalo and Wiersum 2011; Melaku et al. 2014; Reshad et al. 2017; Debela 2019). The ethnobotanical survey found seven use categories, Medicinal plants, wild food plants, melliferous plants, energy, farm implements, wood for smoking (and burning) and flavoring plants, household utensils, and construction. Studies by van Andel (2006) and Fetene et al. (2010) supported the NTFP types identified in this study. Concerning the type of use category, our study showed significant similarity with the studies of (Fetene et al. 2010; Solomon and Tajebe 2014; Reshad et al. 2017), which should imply the cultural exchange and intimacy between the communities.

The different use categories, medicinal plants with 32 species (55.17%), household utensils with 30 species (51.72%), and wild food plants with 22 species (37.93%), were ranked the top three use categories. Those top three use categories harnessing the largest number of plant species (Figure 2). Whereas plant species used for farm implements and hygiene, smoke wood, and flavoring plants were represented by 7 (11.86%) species each.

Ethnobotanical survey

Our study highlights the significant variation observed for the multi-purpose use of NTFPs in the seven use categories (Table 1). Twenty-four plant species are used for over 40% of the use categories. By the same token, *P. falcatus,* and *Olea europaea* subsp. *cuspidata* occur in five out of seven (71.42%) use categories. Whereas *Ficus sur*, *Mytenus obscura*, *Myrica salicifolia*, *Prunus africana*, and *Zehneria scabra* occur in four out of seven (57.14%) use categories. Using plant species for several use categories may threaten species diversity due to over-exploitation, as is also evident from another study (Corlett 2016), especially for threatened plant species like *P. falcatus* in our country.

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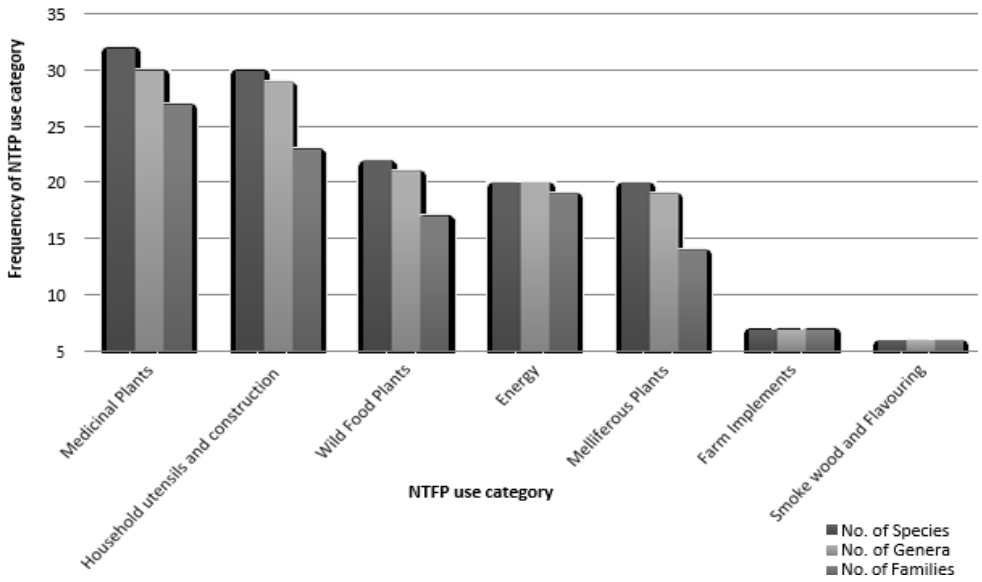

Figure 2. Proportions of NTFP categories in the study area

Table 1. NTFP-bearing species per different use categories

Species name	Family name	Vernacular name	Life-form	MP	MF	WFP	FI	SW	E	HUC
Agave sisalana Perrine	Agavaceae	*Alghee*	Shrub	-	-	-	-	-	-	+
Astropanax abyssinicus (Hochst. ex A.Rich.) Seem.	Araliaceae	*Gatama*	Tree	+	+	-	-	-	-	+
Bersama abyssinica Fresen.	Malianthaceae	*Lolcisa*	Shrub	+	-	-	-	-	-	-
Cadaba farinosa Forsk	Capparaceae	*Qalqalcha*	Shrub		-	-	-	-	+	-
Calpurnia aurea (Ait.) Benth.	Fabaceae	*Ceekaa*	Shrub	+	-	-	-	-	+	-
Canthium lactescens Hiern	Rubiaceae	*Galoo*	Shrub	-	-	+	-	-	-	-
Capparis cartilaginea Decne.	Capparaceae	*Goraa*	Shrub	+	+	+	-	-	-	-
Carissa spinarum L.	Apocynaceae	*Agamsa*	Shrub	+	-	+		+	+	-
Celtis africana Burm.f.	Cannabaceae	*Maxaqoma*	Tree	-	-	-	-	-	+	-
Cissampelos mucronata A.Rich	Menispermaceae	*Baltoke*	Climber	+	-	+	-	-	-	+
Croton macrostachyus Del.	Euphorbiaceae	*Bakanisaa*	Tree	+	+	-	-	-	-	+
Cucumis ficifolius A. Rich.	Solanaceae	*Haregogee*	Climber	+	-	+	-	-	-	-
Cupressus lusitanica Mill.	Cupressaceae	*Gatira faranjii*	Tree	-	-	-	-	-	+	+
Dombeya torrida (J. F. Gmel.) P. Bamps	Malvaceae	*Daannisa*	Tree	-	+	+	-	-	-	+
Ekebergia capensis Sparrm.	Meliaceae	*Sombo*	Tree	-	+	-	-	+	+	+
Embelia schimperi Vatke	Primulaceae	*Haanquu*	Shrub	+	+	+	-	-	-	+
Englerina woodfordioides Gilbert	Loranthaceae	*Digaluu*	Shrub	-	+	-	-	-	-	-
Eucalyptus globulus Labill.	Myrtaceae	*Citaa fura*	Tree	+	-	-	-	-	+	+
Ficus sur Forssk.	Moraceae	*Harbuu*	Tree	+	+	+	-	-	-	+
Gardenia ternifolia Schumach. & Thonn.	Rubiaceae	*Gambeela*	Tree	-	-	-	-	-	-	+
Grewia bicolor Juss.	Malvaceae	*Harooreessa*	Tree	-	-	+	+	-	-	+
Gymnanthemum amygdalinum (Delile) Sch.Bip.	Asteraceae	*Eebicha*	Shrub	+	+	-		+	-	-
Gymnanthemum auriculiferum (Hiern) Isawumi	Asteraceae	*Reejjii*	Tree	+	-	-	-	-	-	-
Hagenia abyssinica (Bruce) J.F. Gmel.	Rosaceae	*Heexo*	Tree	+	-	+	-	-	-	+
Helichrysum schimperi (Sch.Bip.ex A.Rich.) Moeser	Asteraceae	*Baalchii*	Shrub	-	+	-	-	-	-	-
Helinus mystacinus (Ait.) E. Mey.ex Steud.	Rhamnaceae	*Hidda xarii*	Climber	+	-	-	-	-	-	+
Jasminum floribundum L.sub sp. *Floribundum* (R.Br. ex. Freesen.)	Oleaceae	*Biluu*	Shrub	+	-	-	-	-	-	-
Juniperus procera Hochst. ex Endl.	Cupressaceae	*Gatira abesha*	Tree	+	-	-	-	-	-	+
Maesa lanceolata Forssk.	Primulaceae	*Abbayi*	Tree	-	+	-	-	-	+	+
Mytenus obscura (A.Rich.) Cuf.	Celestraceae	*Kombolcha*	Tree	-	+	+	-	-	+	+
Mytenus undata (Thumb.) blackelock	Celestraceae	*Wanta fulas*	Tree	-	-	-	+	-	-	-
Myrica salicifolia Hochst ex A. Rich.	Myricaceae	*Macheensoo*	Shrub	+	-	+	-	-	+	+
Myrsine Africana L.	Primulaceae	*Qachaamu*	Shrub	-	+	+	-	-	-	+
Myrsine melanophloeos (L.) R. Br.	Primulaceae	*Tuu1a*	Tree	-	+	+	-	-	-	+
Ocimum gratissimum subsp. *Gratissimum*	Lamiaceae	*Hanchabi*	Shrub	+	-	-	-	-	-	-
Ocimum lamiifolium Hochst. ex Benth.	Lamiaceae	*Daamma kaasee*	Shrub	+	-	-	-	-	-	+
Olea europaea L. subsp. *cuspidata* (Wall. Ex G. Don) cif.	Oleaceae	*Ejersa*	Tree	+	-	-	+	+	+	+
Olinia rochetiana A. Juss.	Oliniaceae	*Noolee*	Tree	-	+	-	-	-	-	+
Opuntia ficus-tndioa (L.) Miller	Cactaceae	*Tini*	Shrub	+	-	+	-	-	-	-
Osyris lanceolata Hochst. & Steud.	Santalaceae	*Waatoo*	Tree	-	-	+	-	-	-	+
Pentanema confertiflorum (A.Rich.)	Asteraceae	*Haxawwii*	Shrub		+	-	-	-	-	-
Periploca linearifolia Quart.Dill. & A.Rich.	Apocynaceae	*Hidda aannannoo*	Liana	+	-	-	-	+	-	-
Phyllanthus ovalifolius Forssk.	Euphorbiaceae	*Jilolafaa*	Tree	-	-	-	-	-	+	-
Phytolacca dodecandra L.'Hérit.	Phytolaccaceae	*Handodee*	Climber	+	-	-	-	+		+
Podocarpus falcatus (Thunb.) R.B.ex Mirb.	Podocarpaceae	*Birbirsa*	Tree	+	-	+	+	-	+	+
Premna schimperi Engl.	Lamiaceae	*Urgeessaa*	Tree	+	-	-	-	-	-	-
Protea gaguedi J. F. Gmel.	Proteaceae	*Daanisa/ gaarrii*	Shrub	+	-	-	-	-	+	-
Prunus africana (Hook.f.) Kalkm	Rosaceae	*Muka guracha*	Tree	-	+	-	+	-	+	+
Rhamnus prinoides L.Herit	Rhamnaceae	*Garabagush*	Shrub	+	-	-	-	-	-	+
Rosa abyssinica Lindely	Rosaceae	*Qajima/goraa*	Shrub	+	+	-	-	-	-	-
Rubus steudneri Schweing.	Rosaceae	*Enjorii*	Shrub	-	-	+	-	-	-	-
Rytigynia neglecta (Hiern) Robyns	Rubiaceae	*Mete-guree*	Shrub	-	-	+	+	-	+	-
Sageretia thea (Osbeck) M. C.Johnston	Rhamnaceae	*Asgori*	Shrub	-	-	-	-	-	+	+
Scolopia theifolia Gilg.	Flacourtiaceae	*Qilisa*	Tree	+	-	-	-	-	+	-
Searsia glutinosa (Hochst. ex A.Rich.) Moffett	Anacardiaceae	*Xaaxeessaa*	Tree	-	-	-	+	-	-	+
Vangueria madagascariensis Gmel	Rubiaceae	*Abba bunee*	Shrub	-	+	+	-	-	+	-
Verbascum sinaiticum Benth.	Scrophulariaceae	*Gurra harree*	Shrub	+	-	-	-	-	-	-
Zehneria scabra (L.f.) Sond.	Cucurbitaceae	*Shimbirqoolii*	Climber	+	+	+	-	-	+	-

Note: MP: Medicinal Plants, MF; Melliferous Plants, WFP: Wood Food Plats, FI: Farm implements, SW: Smoke Wood and Flavoring, E: Energy, HUC: Househpld Utensils and Construction

Medicinal plant species

Of the total 58 NTFPs recorded in the study area, 32 species (55.17%) distributed under 30 genera (54.54%), and 25 families (71.42%) were identified as medicinal plants used for one or several diseases. Medicinal plant species identified in our study showed about 28-35% overlap with previous studies (Reshad et al. 2017; Fassil and Gashaw 2019; Assefa et al. 2021). The high proportion of plant species used for traditional medicine can be attributed to the greater preference of the local community for traditional medication as their primary healthcare system (Pathy et al. 2020).

Three species represented Lamiaceae. Asteraceae, Apocynaceae, Oleaceae, Rhamnaceae, and Rosaceae were represented by two species, while the rest families represented one species each. The medical importance of the 32 species in the medicinal plant use category, the types of treatments, and preparation and administration methods are presented in Table 2.

Woody medicinal plants fall under four plant habits, i.e., shrubs, trees, climbers, and lianas. Of the 32 medicinal plant species identified, the majorities are shrubs (15, 46.88%) and trees (11, 34.38%), while the remaining are climbers (5, 15.62%) and lianas (1, 3.12%). Seven plant parts are used in treatments (Table 2); these are leaves (50%), roots (18.75), seeds (18.75), fruits (9.38%), bark (9.38%), shoot (9.38%), and stems (9.38%). This preference for using leaves, roots, and seeds in traditional medicine preparations was also seen in other studies (Duguma 2020; Gonfa et al. 2020; Kassa et al. 2020).

Using plant parts such as leaves and seeds for medical purposes can be mutual with forest conservation schemes. In contrast, harvesting roots, stems, barks, and shoot parts needs reconsideration or proper control as they result in resource exhaustion and sometimes even species extinction (Chen et al. 2016; Van Wyk and Prinsloo 2018).

A total of 24 diseases were evidenced to be treated by the medicinal plant species in the study area (Table 2). Ascariasis and Tenidiasis were treated with 3 (9.38%) medicinal plants each, whereas blood clotting, dermatitis, intestinal parasites, toothaches, and wounds were treated with 2 (6.25%) medicinal plant species each. The remaining 17 diseases only had a single (3.13%) species.

Melliferous species

Of the 58 species recorded in this study, 20 (34.48%) species in 19 (33.93%) genera and 14 (37.83%) families were identified as Melliferous plants (Table 1). Some families, like Primulaceae (4 species), Asteraceae (3 species), and Rosaceae (2 species), are represented by more than one species, whereas the remaining families are represented by a single species each (Table 1). More than 80% of identified Melliferous species were also cited in the Honey Bee Flora of Ethiopia book written by Fichtl and Adi (1994), which signposts the good knowledge of the local community on this category species. Such awareness of local communities on the importance of melliferous species greatly impacts sustainable conservation and quality-honey production (Coh-Martínez et al. 2019).

Wild food and condiment plant species

Of the 58 NTFP-bearing plants recorded in the study area, 21 (36.21%) species in 20 (36.36%) genera and 15 (42.85%) families were identified as wild foods for both humans and livestock (Table 1). Among the 20 wild food-bearing families, Primulaceae and Rubiaceae are represented by three (20%) species each, followed by Rosaceae with two (13.33%) species with the remaining 14 families represented by one species (6.67%) each. Of these wild food plants, *Carissa spinarum*, *Cucumis ficifolius*, *P. falcatus*, and *Cissampelos mucronata* are evidenced in their medicinal and dietary values by Lulekal et al. (2011), Fassil and Gashaw (2019), and Maroyi (2020).

Farm implements

We recorded 7 (12.72%) plant species in 7 (12.5%) genera and 7 (20%) families to produce farm implements (Table 1). These species were evidenced to have good wood hardness, strength, and elasticity properties. For example, *Grewia bicolor* and *O. europaea* have good wood hardness, strength, and specific weight, which make them well-placed for traditional plowing implements and hence ideal candidates for agricultural farming equipment (Govorčin et al. 2010; Ruffo et al. 2002).

Wood for burning and flavoring plants

Out of the 58 NTFP-bearing species, 6 (10.91%) species in 6 (10.71%) genera and 6 (17.14%) families were used as wood for burning (and smoking) and flavoring (Table 1). When preparing food and beverages, either they need to improve the taste that might have deteriorated due to the containers they use (such as removing the bitter taste of Cucurbitacins from storage jars of *Lagenaria siceraria*) or adding good flavors through fumigation and washing. The characteristic smoke flavor of plants is attributed to phenolic compounds found in them (Shahidi and Ambigaipalan 2015). Previous studies signpost the presence of Oleuropein in *O. europaea* (Nediani et al. 2019) and Cardiac glycosides in *C. spinarum* (Wangteeraprasert et al. 2012).

Plant species used for combustion

Twenty (34.48%) species in 19 (34.54%) genera and 19 (54.28) families recorded in this study are used as wood fuel (Table 1). Only Rubiaceae contribute two (10.52%) species, while the remaining 18 families only contribute one species each. Our study reveals that the local community prefers selected plant species for fuel wood purposes which is in line with the studies of Reshad et al. (2017) and Dadile et al. (2020). About 35% of recorded plant species to be used for fuel wood overlapped with another similar study in the country (Fetene et al. 2010).

Household utensils and construction materials

Thirty (51.72%) species in 29 (52.72%) genera and 23 (65.71%) families were identified as household paraphernalia (Table 1). From the 23 families identified, Primulaceae, Rhamnaceae, Cupressaceae, and Rosaceae families are represented by 4 (17.39%), 3 (13.04%), 2 (8.70%), and 2 (8.70) species, respectively. The remaining 19 families were represented by only one species each. Of the 30 plant species identified, 11 (36.67%) were also cited by previous studies (Fetene et al. 2010; Reshad et al. 2017).

Table 2. List of medicinal plants with their modes of preparation, diseases treated, way of administration, and plant parts used

Species name	Family name	Vernacular name	Life-form	Diseases treated	Plant parts	Modes of preparation and administration
Astropanax abyssinicus (Hochst. ex A.Rich.) Seem.	Araliaceae	*Gatama*	Tree	Ectoparasite	Leaf	Pounding the leaf and rubbing over the affected body part with ectoparasite
Bersama abyssinica Fresen.	Malianthaceae	*Lolcisa*	Shrub	Wound	Leaf	The leaf is pounded and tied on the affected body part
Calpurnia aurea (Ait.) Benth.	Fabaceae	*Ceekaa/cekata*	Shrub	Intestinal parasites	Seed	Pounding the seeds and drink them with water
Capparis cartilaginea Decne.	Capparaceae	*Goraa (ankusa)*	Shrub	Ascariasis	Sees	Chewing the seed and swallowing it
Carissa spinarum L.	Apocynaceae	*Agamsa*	Shrub	Blood clotting	Bark	Pounding the bark and tie on the affected body part
Cissampelos mucronata A.Rich	Menispermaceae	*Baltoke*	Climber	Stomach ache	Root	Chewing the root or pounding it and drink with water
Croton macrostachyus Del.	Euphorbiaceae	*Bakanisaa*	Tree	Ringworm	Shoot	Cut the shoot tip and rub over the affected body part (Skin)
Cucumis ficifolius A. Rich.	Solanaceae	*Haregogee*	Climber	Stomach ache, rabies	Root leaf	Chewing the root and taking the juice Pounding the leaves and allowing the dogs to drink it with milk
Embelia schimperi Vatke	Primulaceae	*Haanquu (enkoko)*	Shrub	Tapeworm	Seed	Pounding the dried seed and drink it with water
Eucalyptus globulus Labill.	Myrtaceae	*Citaa fura*	Tree	Common cold	Leaf	Boiling the leaf in water and fumigating it
Ficus sur Forssk.	Moraceae	*Harbuu*	Tree	Toothache	Fruit	Fire the fruit and chew with the teeth
Gymnanthemum amygdalinum (Delile) Sch.Bip.	Asteraceae	*Eebicha*	Shrub	Tonsillitis, diarrhea	Leaf leaf	Pounding the leaf and taking the leaf extract Pounding the leaf and drinking the extracts
Gymnanthemum auriculiferum (Hiern) Isawumi	Asteraceae	*Reejjii*	Tree	Wound	Leaf	The leaf is pounded and tied on the affected body part
Hagenia abyssinica (Bruce) J.F. Gmel.	Rosaceae	*Heexo*	Tree	Tapeworm	Seed	Pounding the seed and drinking with water
Helinus mystacinus (Ait.) E. Mey.ex Steud.	Rhamnaceae	*Hidda xarii*	Climber	Rheumatism	Root	Pounding the root and wash the body with it for seven days
Jasminum floribundum L.sub sp. *Floribundum* (R.Br. ex. Freesen.)	Oleaceae	*Biluu*	Shrub	Tufa	Leaf	Pounding Leaves of *Jasminum floribundum, Premna schimperi, Searsia glutinosa Ehretia cymosa, G. bicolor,* and roots of *C. spinarum* and wrap over affected body parts
Juniperus procera Hochst. ex Endl.	Cupressaceae	*Gatira abesha*	Tree	Evil spirit	Leaf	Pounding the leaf of *Juniperus procera* with *P. falcatus* leaf and wash the body with the extracts
Myrica salicifolia Hochst ex A. Rich.	Myricaceae	*Macheensoo*	Shrub	Dermatitis	Leaf	Pounding the dried leaves, mix them with water and put them on the affected skin part
Ocimum gratissimum subsp. *Gratissimum*	Lamiaceae	*Hanchabi*	Shrub	Mich	Leaf	Rubbing the leaf on the skin part
Ocimum lamiifolium Hochst. ex Benth.	Lamiaceae	*Daamma kaasee*	Shrub	Mich	Leaf	Squeezing the leaf and cream over the body
Olea europaea L. subsp. *cuspidata* (Wall. Ex G. Don) cif.	Oleaceae	*Ejersa*	Tree	Headache	Stem	Burning of the stem with fire and taking the oil through the nasal cavity
Opuntia ficus-tndioa (L.) Miller	Cactaceae	*Tini*	Shrub	Anemia	Fruit	Chewing and eating the fruit
Periploca linearifolia Quart.Dill. & A.Rich.	Apocynaceae	*Hidda aannannoo*	Liana	Eye disease	Root	Pounding the dried root and fumigating it
Phytolacca dodecandra L.'Hėrit.	Phytolaccaceae	*Handodee*	Climber	Bleeding	Leaf	Squeezing the leaf and putting on the affected nasal part
Podocarpus falcatus (Thunb.) R.B.ex Mirb.	Podocarpaceae	*Birbirsa*	Tree	Heart disease	Seed	Boiling the Seed oil of *P. falcatus* with *Allium sativum* and *Ocimum gratissimum* and mix the juice with coffee and drink it
Premna schimperi Engl.	Lamiaceae	*Urgeessaa*	Tree	Toothache	Leaf	Chewing the leaf with affected teeth
Protea gaguedi J. F. Gmel.	Proteaceae	*Daanisa/ gaarrii*	Shrub	Tapeworm	Seed	Pounding the roasted seed and take it with water
Rhamnus prinoides L.Herit	Rhamnaceae	*Garabagush*	Shrub	Dermatitis	Fruit	Rubbing the ripened fruit over the affected body part
Rosa abyssinica Lindely	Rosaceae	*Qajima/goraa*	Shrub	Ascariasis	Root	Pounding the root and drinking with water
Scolopia theifolia Gilg.	Flacourtiaceae	*Qilisa*	Tree	Ascariasis	Leaf	Pounding the leaf with coffee arabica leaf and drink it with goat milk
Verbascum sinaiticum	Scrophulariaceae	*Gurra harree*	Shrub	Cancer	Root	Pounding the root with coffee and drinking it
Zehneria scabra (L.f.) Sond.	Cucurbitaceae	*Shimbirqoolii*	Climber	Intestinal parasite	Leaf	Pounding the leaf part and drink it with water

Socio-economic contribution of NTFPs

To assess the economic contribution of NTFPs to the local community, we performed several market surveys in the nearby Doba Market. Data was collected by walking through the market and registering the type of NTFPs encountered. We found that the local community living in and around Ades Forest has various income sources from different agricultural activities. Those income sources include crop production, animal production, fruits and vegetables, *Catha edulis*, and NTFPs. The mean annual income generation per household was 24,805.16 ETHB (Ethiopian Birr hereafter). Out of this, the mean annual contribution of income share from NTFPs across all wealth categories was about Birr 3,912.5 per household (Figure 3). On aggregate, NTFPs' income share accounts for about 15.77% of the annual household income. This finding evidences the vital contribution of NTFPs to the livelihood community. However, NTFPs' economic share differs among wealth groups: the rich, medium and poor wealth categories got about 6.16%, 14.78%, and 30.82% economic share, respectively. From the wealth categories, the poor depend more on NTFP utilization than other agricultural activities or wealth groups (Figure 3.) This finding is also supported by studies of Suleiman et al. (2017) from Nigeria and Gonfa et al. (2020) from Ethiopia. The household survey found that fuel wood, household paraphernalia, wild edible plants, honey, smoke wood, and flavoring plants, farm implements were the six most important traded NTFPs for income generation by the community living in and around Ades Forest.

Figure 3. Mean annual income contribution of agricultural activities in wealth categories

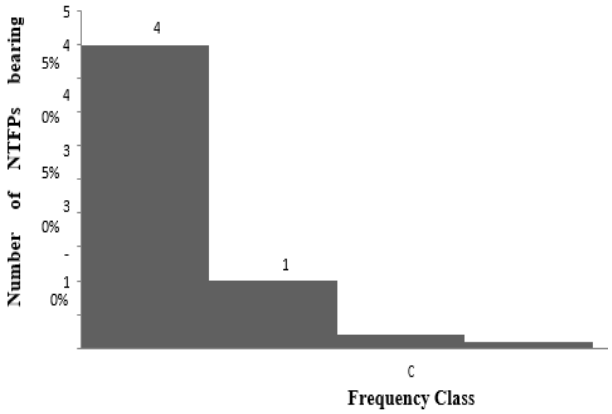

Figure 4. Distribution of NTFPs over frequency classes

Diversity of non-timber plant species in Ades Forest

Species density

The nearest accessible forest resource in the Doba District is Ades Forest, which community members depend on for NTFP collection. A total of 3,770 woody NTFP-bearing plant specimens with an average of 1,450 plants ha^{-1} were evidenced in the forest. This study reveals that *P. falcatus*, *Englerina woodfordioides*, and *Sageretia thea* were the top-three densely populated NTFP-bearing plant species with average densities of 683, 232, and 207 plants per hectare.

Species frequency

Species frequency is defined by Kent (2012) as the probability or chance of finding a species in a given sample area or quadrat. As of occurrence, species were grouped into five percentage frequency classes 0-20, 21-40, 41-60, 61-80, and 81-100, following Kent (2012). The frequency class distribution showed an inverted 'J' shape. The greatest number of species occurring at the lowest frequency class in species composition (Figure 4). The *P. falcatus*, *G. obscura*, and *C. macrostachyus* were the three most frequently occurring plant species found in 67.69%, 49.23%, and 41.54% of sampled quadrats.

Species diversity and evenness

Analysis of species diversity and evenness results shows that Ades Forest has many NTFP-bearing plant species. The Shannon-Wiener's diversity index and evenness of the forest were H' = 3.299 and E' = 0.81, respectively. The high value of Shannon-Wiener's diversity index indicates there were somewhat better representations of NTFP-bearing plant species in Ades Forest.

The phytogeographic comparison of our study site with similar dry Afromontane forests Menagesha Suba, Jello – Muktarand Gelawoldie community forests were made (Table 3). The forest has similar species richness to the Menagesha Suba and Gelawde community forests but is lower than the Jello-Muktar Forest (Table 3). Shannon-Wiener and evenness diversity indices show that the Ades Forest has relatively higher species heterogeneity with a more even plant distribution than Menagesha Suba and Jello-Muktar Forests (Fetene et al. 2010; Reshad et al. 2017). Such high species diversity was possibly ascribed to a preference for environmental/ecological gradients with which the biotic community interacts. Contrarily, the higher diversity and evenness recorded in the Gelawoldie forest reflect better forest management in that community (Mucheye 2020).

Table 3. Phytogeographic comparison of Ades Forest, Ethiopia, with other dry Afromontane forests

Forest	Richness	Shannon-Wiener diversity index (H')	Evenness (E')	Source
Ades Forest	58	3.299	0.81	*Current study*
Gelawoldie community forest	59	3.8	0.9	Mucheye (2020)
Jello –Muktar Forest	97	1.95	0.79	Reshad et al. (2017)
Menagesha Suba Forest	59	1.773	0.816	Fetene et al. (2010)

Our study revealed that the Doba District community endowed a diverse number of NTFP-bearing species for one use or other. From the vegetation and ethnobotanical surveys, 58 NTFP-bearing species were identified and confirmed for their presence in the Ades Forest. The local community has wide experience with the different plant species in diverse use categories. These NTFP-bearing species can be classified into seven NTFP use categories. Many NTFP-bearing plants are used for medicinal plants, Melliferous species, wild food and condiments, farm implements, wood for burning and flavoring plants, energy, household paraphernalia, and construction materials confined in the study area. We also showed that NTFP collection and marketing in the study area have a significant economic contribution to livelihoods.

Ades Forest has a high density and diversity of NTFP-bearing species. However, rather than following a strict protectionist approach in the management of the forest, involving local communities in the management by allowing them to benefit from the forest sustainably may result in lower pressure on these resources. The vegetation survey indicated that, with appropriate forest management activities, Ades Forest could continuously supply NTFPs. However, special attention needs to be given to those plant species with multi-purpose uses, like *P. falcatus.*

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Diversity and traditional ethnozoological uses of ichthyofauna by the Bodo Tribes of Kokrajhar, Assam, Northeast India

GANDOLI BASUMATARY, BICHITRA NARZARY, BRONSON KUMAR KHANGEMBAM♥
Department of Zoology, Bodoland University. P.O. Rangalikhata, Debargaon, Kokrajhar, Assam-783370, India.
Tel./Fax.: +919899046204, ♥email: kbronson173@gmail.com

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Abstract. *Basumatary G, Narzary B, Khangembam BK. 2023. Diversity and traditional ethnozoological uses of ichthyofauna by the Bodo Tribes of Kokrajhar, Assam, Northeast India. Nusantara Bioscience 15: 49-57.* Fish is an important component of ethnomedicine for treating many diseases in many tribal cultures worldwide. Traditional medicine remains the primary healthcare system in most rural populations worldwide, and ethnomedicine is the foundation of many modern-day disease management. The use of fish in traditional healthcare could be a potent source for finding new compounds with therapeutic prospects. Studies on ethnoichthyology have indicated fish as an important component for treating many diseases. However, such studies are yet to be fully documented in the northeastern states of India, especially in Kokrajhar, Assam. The Bodos are one of the largest tribal groups of Assam in Northeast India, with a rich traditional knowledge system. The study explores the traditional uses of fish for various ethnomedicinal properties and health benefits by the Bodos of Kokrajhar, Assam. The study was conducted in eight villages of Kokrajhar District from March 2019 to February 2020 through personal interviews and semi-structured questionnaires with 150 informants. Thirty-four different fish species from 20 different families were identified to be used for their health benefits and therapeutic properties. The highest number of fish species belonged to the family Cyprinidae (20.59%), followed by Channidae (11.76%) and Ambassidae (8.82%). The highest use value (0.58) and relative frequency of citation (0.37) was recorded for *Heteropneustes fossilis.* Anaemia, gastrointestinal and integumentary disorders, and body weakness were the most commonly mentioned ailments treated. The present study also reported some small indigenous fish species for their health benefits. The study also found some unique traditional methods for preparing and applying fish species (*Xenentodon cancila, Chitala chitala, Glossogobius giuris, Leiodon cutcutia,* etc.) not reported earlier. Identification and detailed study of the biochemical profile of these different species may be recommended to develop suitable alternatives to synthetic medicines. This study may be a valuable addition to the rich traditional knowledge of Northeast India.

Keywords: Bodo Tribes, ethnozoology, ichthyofauna, Kokrajhar

INTRODUCTION

Traditional medicine refers to health practices, approaches, knowledge, and beliefs incorporating plant, animal, and mineral-based medicines, spiritual therapies, manual techniques, and exercises, applied singularly or combined to treat, diagnose, and prevent illnesses or maintain well-being (Adnan et al. 2022; WHO 2022). Plants and animals having medicinal properties are being used throughout the world. Traditional medicine remains the most common and affordable form of therapy in low-income countries. About 70-80% of the world's rural population depends on it for primary healthcare, most of which reside in developing countries (Chhetri et al. 2020). Animals and animal-derived products have always been a source of traditional medications and have vital significance in some religions and cultures (Prakash and Prakash 2021). In modern medical science, about half of modern medicines are reported to be derived from biological sources. The traditional knowledge of the ethnic community worldwide has contributed to recognizing living organisms used for treating diseases in livestock and human beings. Consequently, it is important to document the traditional knowledge of different tribal communities on the verge of socioeconomic and cultural deprivation (Laishram and Dey 2021). Though popular worldwide, many of the reported cases of ethnomedicine are restricted to ethnobotany. While compared to medicinal plants, there is insufficient research and data on the use of animals for medicinal purposes (Alves and Rosa 2013). However, recent studies have revealed the use of animals and their products as natural remedies in folk medicinal practices worldwide (Zanvo et al. 2021).

Fish as a cheap protein source also plays a major role in preventing and curing many diseases, including coronary disease, asthma, mental illness, low birth weight, and nutrient deficiencies which underlines the importance of fish in our diet (Naranje and Mishra 2015). Ethnoichthyology focuses on local knowledge, linguistic expressions, nutritious importance, folk practices, material evidence and cognitive perceptions of fish, and the environmental consequences of these interactions (Svanberg and Locker 2020). Some studies have documented the role of ichthyofauna in traditional medicines, mainly in the indigenous rural and fishing community (Vallejo and González 2014; Altaf et al. 2020).

The northeastern region of India is a biodiversity hotspot, and 185 species of fish have been recorded in Assam alone (ASBB 2022). Biodiversity has always been paramount for providing and discovering medical

substances (Neergheen-Bhujun et al. 2017). However, studies to ascertain the magnitude and use of animals and their products in traditional medicine are yet to be fully documented in Northeastern India, including Assam (Kumar et al. 2021). Fish occupy an important place in traditional therapeutic practices in Assam (Rahman et al. 2014). Traditional uses of fish species by ethnic communities of Assam to treat human diseases have rarely been documented (Borah and Prasad 2017). To the best of our knowledge, there is no report available from Kokrajhar, although the district in lower Assam is blessed with diverse fish species (Baro et al. 2015). The Bodos are one of the major tribes of the district, and they have a rich indigenous traditional knowledge of using natural resources for various purposes. Das et al. (2022) reported the diversity and availability of dry fish species used by the Bodos in Kokrajhar, Assam. Fish provide affordable proteins, support livelihood, and have cultural significance in local folklore and traditional medicines. However, no report exists on this rich ethnozoology, especially the ethnoichthyology of the Bodo tribes of Kokrajhar. With increasing urbanization and modern lifestyle, this traditional knowledge faces extinction. Documenting and safeguarding Traditional Knowledge Systems, which is regarded as the unique cultural identity of a community, are an essential part of bioresources management (Wangpan et al. 2019) and for establishing new medicinal prospects and remedial measures for unknown diseases (Borah and Prasad 2017). The present study, therefore, aims to identify and document the vanishing indigenous traditional ethnoichthyological knowledge of the Bodo tribes in Kokrajhar District, Assam, India.

MATERIALS AND METHODS

Study area

The study was conducted in the Kokrajhar District of Assam, India (Figure 1). Kokrajhar is the headquarter of Bodoland Territorial Region (BTR), Assam, India, and is located between 89°46'E and 90°38'E longitude & 26°19'N to 26° 54'N latitude, covering an area of about 3,169.22 km^2. The region is considered the center of Bodo culture. The district is situated on the northern bank of the Brahmaputra River. Bhutan surrounds it in the north, with Chirang and Bongaigaon Districts in the east, Dhubri District in the south, and West Bengal in the western side of the district. The region is rich in biodiversity and natural resources like forests and water. In addition, the district is endowed with several wetlands (known locally as beels) like the Diplai, Dheer, and rivers like Gaurang, Ultapani, Samoka, Gongia, Swrmanga, Sankosh, and Champabati which are sources for a diversity of fish species. Agriculture and allied activities are the main economic activities in the district. The study was conducted in eight villages (Sundrijhora, Bashabil, Landangpara, Tengapara, Harigaon, Kokrajhari, Thulungapuri, and Chautara) of Kokrajhar District, Assam, from March 2019 to February 2020.

Demographics of informants

Information regarding the age, sex, education, and occupation of each informant was collected with the help of predesigned questionnaires. Before any data was collected, the informants' were informed about the study objectives. Their prior consent was taken for the interview and the utilization of the information they provided for publication without disclosing their identity.

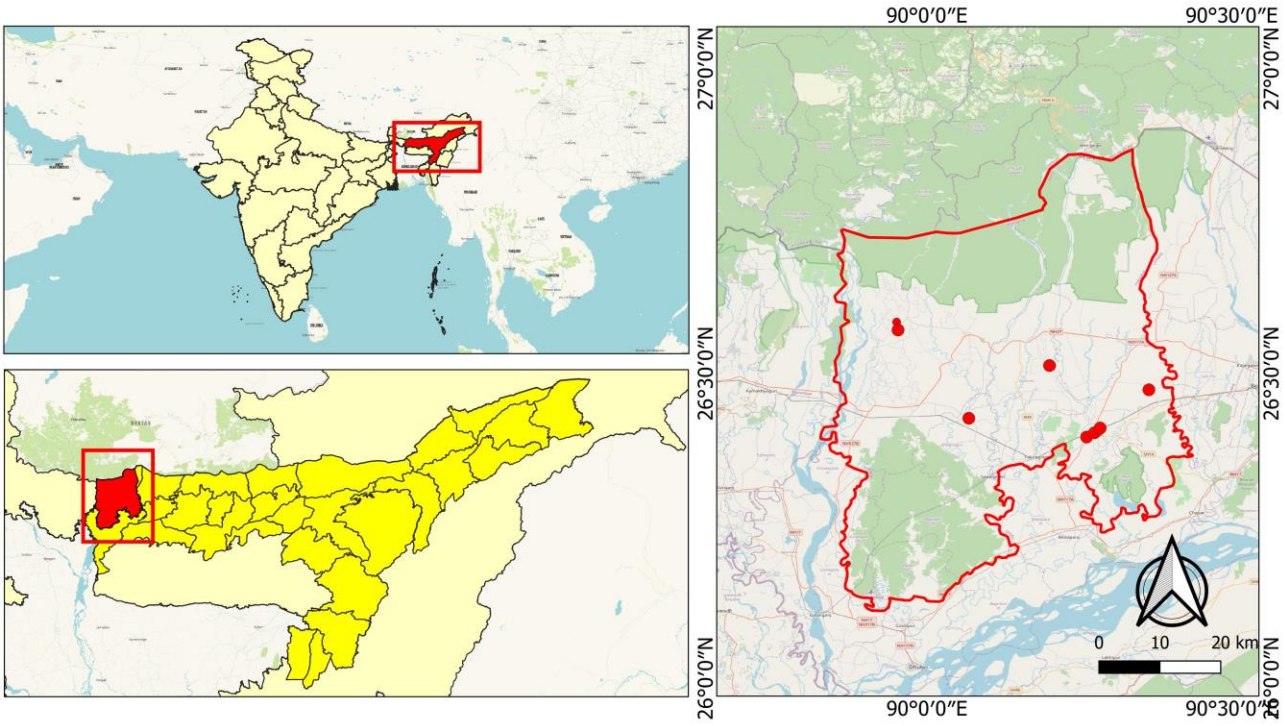

Figure 1. Map of Kokrajhar District, Assam in Northeastern India indicating the study areas

All informants were forthcoming, cooperative, and readily agreed to participate in the survey. None of the informants refused to participate in the survey. Altogether 150 informants participated in the study. A non-probabilistic snowball technique was used to identify potential informants by asking the participants for the location of new respondents fitting the criteria who again indicated the next potential participant. When information about the location of the population of interest is not available or clear, snowball sampling is regarded as effective (Young et al. 2018). All interviews were conducted in the local language of the informants (Bodo), and the interview duration ranged between 40 to 90 mins. Interviews were conducted anonymously, and the information gathered was kept confidential.

Data collection

Information regarding the traditional use of fish by the Bodo community of Kokrajhar, Assam, was obtained through semi-structured questionnaires and personal interviews with the respondents in different villages of Kokrajhar, Assam. The respondents were asked about the name and description of fish used, the mode of their utilization, and the traditional value of health benefits or treating ailments. The fish's local vernacular name and detailed description were used for preliminary identification of the species. Photographs and video clips of probable species matching the respondents' descriptions were shown to verify the species' identity. Whenever possible, fish samples were photographed and collected from the respondents (if they owned the species samples) and preserved (in 10% formalin) for further identification. Identification was done with the help of standard keys and references (Vishwanath 2017; Froese and Pauly 2022). The conservation status of the recorded species was evaluated from the Red List of Threatened Species of the International Union for the Conservation of Nature and Natural Resources (IUCN 2022).

Quantitative ethnozoology

Relative Frequency of Citation (RFC)

The relative frequency of citation (RFC) was calculated to understand the popularity and importance of a fish species. It was calculated following Vitalini et al. (2012) using the formula $RFC = F_c/N$, where F_c is the number of informants who mentioned using the species and N is the total number of informants. The value of RFC lies between 0 and 1, with higher values signifying more importance and citation.

Use value

The relative importance of each fish species was calculated using the use value (UV) Index (Albuquerque et al. 2006). The formula used was $UV = \Sigma U /n$, where, U is the sum of the total number of uses cited by the informants for a fish species, and n is the total number of informants. Higher UV scores usually imply the importance of the species. While low score approaching zero indicates few reports on its usage.

RESULTS AND DISCUSSION

Demographics of informants

A total of 150 informants from the eight villages of the Kokrajhar District of Assam took part in the study (Table 1). All respondents belong to the Bodo tribal community. Altogether, 53 males and 97 females between 25-80 years old participated in the study. The respondents belonged to different job categories: farmers, village elders, traditional healers, housewives, and government servants. The highest frequency was observed in the age groups 51-55 and 45-50 years, with 28 (18.7%) and 26 informants (17.3%), respectively. Most (79.3%) of the informants had at least a primary school education, whereas 21.7% did not receive any formal education. Of all the informants, 16 were practicing traditional healers, 33 were farmers, and 33 were in Government services. Twenty-six informants reported themselves as self-employed, while 38 informants were housewives, and another four were retired Government employees.

Relative Frequency of Citation (RFC) and Use Value (UV)

The UV of all the species ranged between 0.01 and 0.58, whereas the RFC value ranged between 0.01 and 0.37 (Table 2). The highest UV was recorded for *Heteropneustes fossilis* (0.58). *Clarias magur* recorded a UV of 0.48, while that of *M. cuchia* (0.39), *X. cancila* (0.35) and *C. gachua* (0.37) ranged between 0.35 and 0.39. The lowest UV was recorded for *P. sophore* (0.01) and *L. gonius* (0.01). The highest RCF value (0.37) was observed for *H. fossilis*, followed by *M. cuchia* (0.35). While *C. magur, C. gachua*, and *X. cancila* all recorded an RCF value of 0.29, the value was minimal (0.01) for *L. gonius, L. guntea*, *P. sophore*, and *W. attu*.

Diversity and traditional uses of fish

The study revealed 34 fish species belonging to 20 families in 10 orders, which the Bodos utilized for their traditional ethnomedicinal value or other health benefits (Table 2). The majority of the species (26.5% each) belonged to the order Anabantiformes and Cypriniformes (9 species each), while the least (one species each) was observed in the orders Anguilliformes, Beloniformes, Gobiiformes, Osteoglossiformes and Tetraodontiformes (Figure 2).

Order Perciformes and Synbranchiformes recorded three species each, while Siluriformes recorded five species. The highest number of species was recorded in the family Cyprinidae (7 species), which accounted for 20.6% of all the species recorded, followed by Channidae (11.8%) and Ambassidae (8.8%). Two species each were recorded in families Bagridae, Mastacembelidae, and Osphronemidae, while the rest of the families accounted for a different number of species ranging from 1 to 6 species in different families.

In the order Anabantiformes, the species identified were *Anabas testudineus, Badis badis, Channa gachua, Channa punctata, Channa striata, Channa marulius, Trichogaster fasciata, Trichogaster lalius,* and *Nandus nandus*. While, those belonging to order Cypriniformes were

Amblypharyngodon mola, Botia dario, Danio rerio, Labeo rohita, Labeo gonius, Lepidocephalichthys guntea, Puntius sarana, Puntius sophore, and *Rasbora* sp. Only one species each were observed from families Anguilliformes *(Anguilla bengalensis*), Gobiformes (*Glossogobius giuris*), Osteoglossiformes (*Chitala chitala*), and Tetraodontiformes (*Leiodon cutcutia*). Five species *viz. H. fossilis, C. magur, Wallago attu, Mystus tengara,* and *Mystus carcio* were recorded from the order Siluriformes. Other species recorded were *Xenentodon cancila* (Beloniformes), *Chanda nama, Parambassis ranga* and *Parambassis lala* (Perciformes), *Monopterus cuchia, Macrognathus aral,* and *Macrognathus pancalus* from the order Synbranchiformes. The majority of the identified species (30 species, 88.2%) belong to the least concerned (LC) category of the IUCN conservation status, while only two species were found listed in the near threatened (NT) category, and one species each in vulnerable (*W. attu*) and endangered categories (*C. magur*) of the IUCN list (Table 3).

The traditional uses of the different species of fish recorded in the study are listed in Table 2. In our survey, the muscles or the flesh were reported to be most commonly cited for their various health benefits. However, various other parts, such as the head, blood, oil, mucus, bile, viscera, bones, spines, skin, and scales, were also mentioned by some respondents for beneficial properties. The most frequently reported modes of preparation were either cooked fresh or dried before use. However, some species were reported to be used raw. In most cases, whole fish was reported to be used. The usage of specific fish parts was reported in a few species only. For instance: the blood and head of *M. cuchia,* the alimentary canal and bile of *P. sophore,* the skin and air bladder of *W. attu,* the scales of *C. chitala,* the long-pointed beak of *X. cancila* and caudal fin of *C. striata* (Table 2). Some of the ailments treated were associated with: the blood (e.g., anemia) and cardiovascular system, integuments, vision, body weakness, cold and fever, wounds, respiratory system, kidney stones, body pain, and stomachache. Other observed health benefits of fish recorded in this study included lactation in feeding mothers, good vision, improved wound healing, anti-allergy, increased strength, and overall health improvement after an illness.

Discussion

This study finds that the Bodos utilizes diverse fish species for various health benefits and for treating and managing many ailments. Similar studies have reported the use of fish for the treatment and management of a range of health issues, including night blindness, loss of appetite, wounds, skin burns, bronchitis, asthma, tuberculosis, arthritis, earache, cardiac diseases, rheumatism, blood pressure, rickets, calcium metabolism, nervousness, giddiness, smallpox, kala-azar, diarrhea, malaria, body-ache, cancer, and vitamins and minerals deficiency (Naranje and Mishra 2015; Rahman et al. 2018). The importance and popularity of a species for its ethnomedicinal or health benefits are usually estimated by UV and RFC. The UV is an important quantitative analysis that indicates the relative importance of an important ethnobiological species. Higher UV of *H. fossilis* and *C. magur* observed in the study shows that these species were used more widely than others. This also indicates their acceptance among the local population for their benefits. *H. fossilis* and *M. cuchia* recorded high popularity among the informants for their health benefits compared to other fish species, as indicated by their higher RCF values. Low RCF value for *L. gonius, L. guntea, P. sophore,* and *W. attu* indicates limited uses of these species. The highest RCF of *H. fossilis* and *M. cuchia* agreed with the diverse applications mentioned by various informants for these species. Species such as *A. testudineus* and *C. punctata* are considered important species in Kokrajhar for their preference and nutritional value (Devi et al. 2022) .

Table 1. Ethnographic data of informants participating in the study

Variable	Category	Number	%
Age groups	25-30	13	8.7
	31-35	7	4.7
	36-40	14	9.3
	41-45	21	14.0
	46-50	26	17.3
	51-55	28	18.7
	56-60	12	8.0
	61-65	16	10.7
	66-70	9	6.0
	Above 70	4	2.7
Gender	Male	53	35.3
	Female	97	64.7
Education	No formal education	31	20.7
	Primary school	11	7.3
	Secondary school	7	4.7
	High school	28	18.7
	Higher Secondary	33	22.0
	Graduate	36	24.0
	Postgraduates& above	4	2.7
Occupation	Traditional healer	16	10.7
	Farmer	33	22.0
	Housewife	38	25.3
	Government employee	33	22.0
	Self-employed	26	17.3
	Retired Government employee	4	2.7

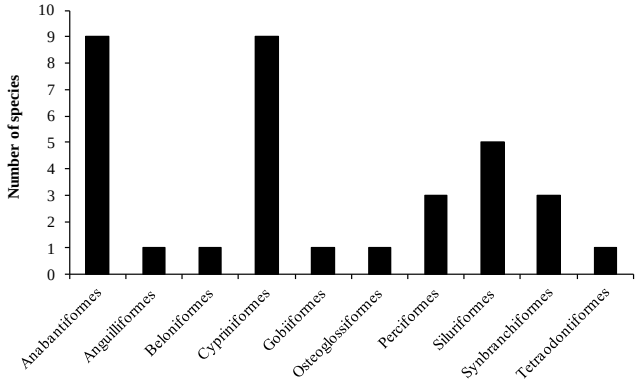

Figure 2. Different fish families were recorded in the study

Table 2. List of traditionally important fish species recorded in Kokrajhar, Assam, India and their traditional uses

Fish species	Local name	Parts used	Traditional uses, health benefits, and mode of usage	RFC	UV
Anabas testudineus	Kaowi	Meat	Provide relief from cold and fever, jaundice treatment, regain strength after any illness	0.07	0.10
Badis badis	Dusumwi	Flesh, head	Cooked with medicinal plants (known locally as Manimuni, Sibru, Jwglaori, and Nwrsing) and consumed to treat postpartum respiratory issues (locally known as Puwati).	0.24	0.28
		Whole fish	Preparation of a traditional fermented product (Napam)		
Channa gachua	Nasrai, nisla	Meat, head	Boiled with medicinal plants and consumed to promote lactation in mothers, improve bone health, relieves joint pain and arthritis	0.29	0.37
		Live fish	They are traditionally believed that the 'beating' or' slapping' by the caudal fin of the live fish on the lower limbs of children induces walking in them (in case of delayed onset of walking in some children).		
Channa marulius	Na sal	Meat, head	Treatment of cold and fever, purify the blood and increase in hemoglobin	0.02	0.03
Channa punctata	Na gwri	Meat, brain	Improve body weakness, provide relief from mild cold and fever, and is good for purifying blood	0.14	0.18
		Head	Treatment of renal stones: soup of ground dry head part in water		
		Whole fish	Preparation of a traditional fermented product (Napam).		
Channa striata	Sol	Meat, head	Good for wound healing, blood purification, and treatment of cold and fever.	0.05	0.22
		Caudal fin	The dry caudal fin is boiled with some medicinal plants and taken as soup to treat typhoid and gastric issues during the postnatal period in women.		
Nandus nandus	Tota	Flesh, head	Treating mild colds and fevers, believed to have anti-microbial properties, good for eyesight, and regaining strength after any illness.	0.11	0.15
Trichogaster fasciata	Bengshi	Flesh, head	Relieves cold and fever, jaundice management.	0.09	0.13
		Meat	Improve health and strength after illness.		
		Whole fish	Preparation of a traditional fermented product (Napam)		
Trichogaster lalius	Bengshi	Whole fish	Dry fish is boiled with vegetables and herbs and consumed during illness and typhoid.	0.07	0.1
Anguilla bengalensis	Nangdor	Head	Treatment of piles: Dry head part is roasted, ground, mixed with mustard oil, and applied. Anticancer properties	0.03	0.04
Xenentodon cancila	Kangkila	Head, snout	Used as a tool to remove dead erythrocytes in bruises or hematoma	0.29	0.35
		Whole fish	Dry fish is boiled and consumed to relieve headache		
Amblypharyngodon mola	Moaya	Flesh, head	Boiled fish improve low blood count, cures stomach ache, and improves eyesight	0.06	0.07
		Whole fish	Preparation of a traditional fermented product (Napam)		
Botia dario	Balabatia	Flesh, head	Provide relief from mild cold, fever, and anemia	0.04	0.04
Danio rerio	Nijou	Whole fish	Believed to be useful in the treatment of anemia	0.03	0.03
			Preparation of a traditional fermented product (Napam)		
Labeo gonius	Kursa	Meat, head	Anti-allergy properties	0.01	0.01
Labeo rohita	Rhou	Head, flesh, viscera	Good cardiovascular health, good for brain development, pain reliever	0.09	0.09
		Bile	Applied for pain relief		
Lepidocephalichthys guntea	Balabatia	Whole fish	Steam cooked wrapped in a special plant leaf, made into a paste, and applied to treat tongue and mouth ulcers.	0.01	0.02
Puntius sarana	Pitikri	Meat	Good for eyesight	0.03	0.03
		Whole fish	Preparation of a traditional fermented product (Napam)		
Puntius sophore	Pitikri	Alimentary canal, bile	Relieves pain caused by *H. fossilis* sting	0.01	0.01
		Whole fish	Preparation of a traditional fermented product (Napam)		
Rasbora sp.	Maoya	Flesh, head	Good for eyesight and brain development	0.01	0.07
Glossogobius giuris	Namutra	Flesh, head	Consumption of boiled or roasted fish is believed to prevent night bed-wetting in children	0.13	0.13
Chitala chitala	Chital	Scales	Remove dandruff in babies: dried scales are grounded, mixed with coconut oil, and applied to remove dandruff in babies	0.03	0.04
Chanda nama	Chandanga	Whole fish	Consumption of boiled fish improves health during illness	0.02	0.02
			Preparation of a traditional fermented product (Napam)		
Parambassis ranga	Chandanga	Whole fish	Treatment of mild cold and fever, consumption of boiled fish improves health during illness	0.08	0.08
			Used for preparation of a traditional fermented product (Napam)		
Parambassis lala	Chandanga	Whole fish	Boiled fish improve health during illness	0.02	0.02
			Preparation of a traditional fermented product (Napam)		

Clarias magur	Magur	Meat	Increase hemoglobin: boiled with some medicinal plants and prescribed for consumption for treating anemia Improve digestion, immunity, and general strength.	0.29	0.48
Heteropneustes fossilis	Singi	Meat	Consumption of boiled fish meat increases hemoglobin, which is useful in treating anemia. In addition, its believed to improve digestion and provide immunity and body strength.	0.37	0.58
Mystus carcio	Tengwna	Meat	Regain strength, especially after illness: the dry or raw flesh is cooked with vegetables or some medicinal plants	0.07	0.07
Mystus tengara	Tengwna	Meat	Regain strength, especially after illness: dry or raw fish is cooked with vegetables or medicinal plants.	0.11	0.16
Wallago attu	Barli	Skin	Cure dry skin	0.01	0.03
		Muscles, bladder	Removes scars on the skin Good nutrition		
		Whole fish			
Macrognathus aral	Turi	Meat	Treatment of mild cold and fever	0.04	0.04
		Whole fish	Preparation of a traditional fermented product (Napam)		
Macrognathus pancalus	Na thuri	Head	Treatment of ingrowing nail causing cellulitis: Roasted head part is ground with the flesh of a freshwater snail species and is applied to provide relief. Anticancer properties	0.07	0.09
Monopterus cuchia	Cuchia	Meat, blood	Increase hemoglobin: meat is boiled or cooked with medicinal plants (like *Lippia geminata*) and eaten to treat low blood or anemic condition. Believed to remove skin scars	0.35	0.39
		Head	Relieves stomach ache: dry head part is roasted, ground, mixed with water, and taken to cure stomach ache.		
Leiodon cutcutia	Na tepa	Whole body	Dry fish is ground and used as a paste in some skin wound types. Roasted fish are ground, mixed with water, and consumed to relieve gastric or stomach issues. Believed to prevent child habit of bed wetting (urination) at night.	0.17	0.24

Table 3. Diversity and IUCN conservation status of the fish species recorded in the study

Fish	Order	Family	IUCN status
Anabas testudineus	Anabantiformes	Anabantidae	LC
Badis badis	Anabantiformes	Badidae	LC
Channa gachua	Anabantiformes	Channidae	LC
Channa marulius	Anabantiformes	Channidae	LC
Channa punctata	Anabantiformes	Channidae	LC
Channa striata	Anabantiformes	Channidae	LC
Nandus nandus	Anabantiformes	Nandidae	LC
Trichogaster fasciata	Anabantiformes	Osphronemidae	LC
Trichogaster Ialius	Anabantiformes	Osphronemidae	LC
Anguilla bengalensis	Anguilliformes	Anguillidae	NT
Xenentodon cancila	Beloniformes	Belonidae	LC
Amblypharyngodon mola	Cypriniformes	Danionidae	LC
Botia dario	Cypriniformes	Botiidae	LC
Danio rerio	Cypriniformes	Cyprinidae	LC
Labeo gonius	Cypriniformes	Cyprinidae	LC
Labeo rohita	Cypriniformes	Cyprinidae	LC
Lepidocephalichthys guntea	Cypriniformes	Cobitidae	LC
Puntius sarana	Cypriniformes	Cyprinidae	LC
Puntius sophore	Cypriniformes	Cyprinidae	LC
Rasbora sp.	Cypriniformes	Cyprinidae	LC
Glossogobius giuris	Gobiiformes	Gobiidae	LC
Chitala chitala	Osteoglossiformes	Notopteridae	NT
Chanda nama	Perciformes	Ambassidae	LC
Parambassis ranga	Perciformes	Ambassidae	LC
Parambassis lala	Perciformes	Ambassidae	LC
Clarias magur	Siluriformes	Clariidae	LC
Heteropneustes fossilis	Siluriformes	Heteropneustidae	LC
Mystus carcio	Siluriformes	Bagridae	LC
Mystus tengara	Siluriformes	Bagridae	LC
Wallago attu	Siluriformes	Siluridae	VU
Macrognathus aral	Synbranchiformes	Mastacembelidae	LC
Macrognathus pancalus	Synbranchiformes	Mastacembelidae	LC
Monopterus cuchia	Synbranchiformes	Synbranchidae	LC
Leiodon cutcutia	Tetraodontiformes	Tetraodontidae	LC

Note: LC: Least Concerned, VU: Vulnerable, NT: Near Threatened

The method of preparation and utilization of the fish was generally simple, and in most cases, the whole fish was reported to be taken either cooked or roasted. Grounded paste, soup, or cooking with some medicinal plants were also common fish preparation methods. Most species recorded were normally used as food fish in most Bodo households. However, it was also observed that some fish species (such as *B. badis*, *C. striata*, *C. gachua*, *A. testudineus*, etc.) were often consumed occasionally in many Bodo households. That was probably due to the variation in seasonal availability and market price. The use of dry fish or its parts was also commonly reported by many informants. Small Indigenous Fish species (SIFs) like *P. sophore*, *P. sarana*, *T. fasciata*, *T. lalius*, *L. guntea*, *C. nama*, *P. ranga*, *P. lala*, *D. rerio*, and *M. pancalus* were the most common species found. Those fishes are also traditionally preserved as a unique fermented product known locally as *napam.* Similar traditional therapeutic uses of species like *G. giuris*, *A. mola*, *C. magur*, *H. fossilis*, *R. daniconius*, *Channa spp.*, *Puntius spp.*, *M. cuchia*, by different tribes and non-tribe indigenous people of Assam have been reported by Barman et al. (2013).

The *H. fossilis*, *C. magur*, and *M. cuchia* were widely mentioned by many informants for improving general health, especially after an illness, and also for increasing hemoglobin. Most recorded fish species were cited to improve general health and provide good nutrition to the body. The *M. cuchia* was also mentioned to be effective in removing scars from the skin. The dried *X. cancila* was boiled and consumed to relieve headaches. The *A. testudineus* was reported to be used to treat jaundice, cold, and fever. The *P. sarana*, *A. mola*, *Rasbora* sp., and *N. nandus* were reported to be good for improving vision. The flesh of *A. mola* was believed to be effective against stomachache. Health benefits of *M. cuchia*, *A. bengalensis*, and *A. japonica* include improvement of blood pressure, lower cholesterol and reduction of the risks of diabetes and arthritis, ease of menstrual pain, and improvement of the health of the skin (Rahman et al. 2014).

The present study also observed some unique uses of fish or its different parts. For instance, the head part of *M. cuchia* and *C. punctata* were found to be exclusively consumed by the Bodos for treating stomach pain and kidney stones, respectively, as prescribed by the local 'traditional healers' known locally as *Ojas*. The *Ojas* are the local traditional ethnomedicinal practitioners in many tribal communities in Assam, including the Bodos (Borah and Prasad 2017). A unique use of the head and long pointed snout or beak of *X. cancila* was also reported in this study. The dry preserved head of this species was generally used as a surgical tool to remove the dead blood cells in bruises or hematomas. This was traditionally believed to be effective in quickly healing bruises. Also, when applied, a special concoction made from the dried and grounded scales of *C. chitala* and coconut oil was reported to remove dandruff in infants. In addition, the Bodos used the muscles and air bladder of *W. attu* to remove scars from the skin. Consuming the muscles of this fish was also reported to be beneficial for removing or reducing scars or dark spots on the skin in this study.

The meat of *C. striata* was reported to be effective in healing wounds, relieving colds and fever, and inducing blood purification. Also, the soup of dried caudal fin of *C. striata* was prescribed for treating typhoid. In many Asian countries, *C. striata* are mostly consumed by people for its good taste and medicinal properties like wound healing and energy booster. Other reported pharmacological benefits of the species include anti-microbial, anti-inflammatory, cell proliferation, and induction of platelet aggregation (Shafri and Manan 2012; Rahman et al. 2018). In addition, the *Channa* species containing: docosahexaenoic acid, high glycine content, arachidonic acids, glycine, polyunsaturated fatty acids, and fatty acids may be responsible for its wound healing properties (Shafri and Manan 2012). In the present study, *C. magur* (magur) and *H. fossilis* (Singhi) were found to be traditionally important fish for consumption among pregnant and lactating mothers in Bodo society. Those fish were believed to induce lactation, provide pre and postnatal nutrition, and promote strength after childbirth. A similar report is available citing the use of the head, flesh, and liver of wels catfish for the treatments for skin, intestines, and throat disorders (Vallejo and Gonzalez 2014).

SIFs are reported to be widely used by the Bodos and other communities for their nutritional and medicinal properties (Roy et al. 2022). This study found that the alimentary tract, including the bile of *P. sophore*, was applied as a pain killer to relieve the pain caused by the poisonous *H. fossilis* fish stings. In the present study, when fed to infants and children, the boiled or roasted meat of *G. giuris* was believed to prevent night bed-wetting. Similar traditional use was also found for another fish species *L. cutcutia.* Also, the paste made of dry *L. cutcutia* was used for healing certain skin wounds by the Bodos in the present study. Another unique traditional belief among the Bodos was that the onset of first walking in children (with suppression/delay of this ability) could be induced by letting/making the caudal fin of live *C. gachua* 'slap' or 'beat' the limbs of such children. The head part of *M. pancalus* was roasted, grounded with the flesh of a local freshwater snail species, and applied to treat ingrowing nails causing cellulitis in the toe finger. The meat of *L. gonius* was known among the Bodos for its anti-allergic properties.

The popularity of animal-based remedies seems to be influenced by cultural aspects and the relations between humans and the surrounding biodiversity. For example, fish, an abundant resource in the region, has been an integral part of the traditional ethnic cuisine and culture of the Bodos of Kokrajhar. Hence, vast diversity in the species and usages of fish was reported in our present study. This indicates that the environment directly influences the choice of zootherapeutic resources, and medicinal use represents a strategy for optimizing the use of resources (Brito et al. 2019). The elaborate use of SIFs and air-breathing fish species for various health-related properties in the present study corroborates these observations.

Interestingly, in the present study, it was observed that some fish species, such as *W. attu*, *P. sophore*, *P. sarana*,

etc., were abstained during certain ailments or health conditions as they are believed to have an adverse effect on the body. For example, the consumption of *W. attu* was avoided in indisposed individuals when the person suffered from fever, chronic body pain, arthritis, etc. Similar reports are available from the Sikuani community in Columbia, where certain fish species are avoided in the traditional diet of Sikuani women during menarche, menstruation, gestation, and postpartum (Cubillos-Cuadrado et al. 2019).

Knowing the therapeutic value of fish increases awareness among people about the health benefits of fish, its essential nutrients, and its role in fighting against diseases and disorders (Naranje and Mishra 2015). The Bodos of Kokrajhar, Assam, inherited a rich ethnoichthyological knowledge through several generations. However, the scientific basis for several claimed health benefits and medicinal value has yet to be established for many species. For example, different species' amino acids, fatty acids, and mineral profiles may be the probable reasons for their health benefits. Nevertheless, proper documentation and mass awareness are important for preserving this rich knowledge as it not only represents the cultural identity of the Bodos, but may also help identify and develop a natural alternative to synthetic drugs in disease management.

In conclusion, the present study has identified and documented the traditional ethnoichthyology of the Bodo tribes of Kokrajhar, Assam. The use of thirty-four different fish species by the Bodos of Kokrajhar, Assam, for their traditional zootherapeutic properties to treat various ailments and for their health benefits indicates the vast diversity of fish species in the region and their importance in the life and culture of the Bodos. Further studies may be recommended to validate and identify the mechanism of action for their therapeutical properties. This information may be useful for innovations in healthcare industries, preventing biopiracy and preserving the rich indigenous traditional knowledge system of the Bodo tribes for future generations.

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Minerals and fatty acids profile of armored catfish *Pterygoplichthys pardalis* from Ciliwung River, Indonesia

FAHMA WIJAYANTI[1], ADE LISDANIYAH[1], MAULIDATUL HASANAH[1], DEWI ELFIDASARI[2,♥]

[1]Department of Biology, Faculty of Sciences and Technology, Universitas Islam Negeri Syarif Hidayatullah Jakarta. Jl. Ir. H. Juanda No. 95, Cempaka Putih, South Tangerang 15412, Banten, Indonesia

[2]Department of Biology, Faculty of Sciences and Technology, Universitas Al Azhar Indonesia. Jl. Sisingamangaraja, Kebayoran Baru, South Jakarta 12110, Jakarta, Indonesia. Tel.:+62-21-72792753, ♥email: d_elfidasari@uai.ac.id

Manuscript received: 26 September 2022. Revision accepted: 12 March 2023.

Abstract. *Wijayanti F, Lisdaniyah A, Hasanah M, Elfidasari D. 2023. Minerals and fatty acids profile of armored catfish* Pterygoplichthys pardalis *from Ciliwung River, Indonesia. Nusantara Bioscience 15: 58-67.* Fish is an important food source in human consumption due to its minerals and fatty acids needed for various body functions. One fish widely eaten by the people around the Ciliwung River, Indonesia is the armored catfish, *Pterygoplichthys pardalis* (Castelnau 1855). It has great economic value and is easily obtained by the residents along this river. Due to its high protein content, *P. pardalis* is a potential source of animal protein for humans. The unavailability of information detailing the minerals content and fatty acids in *P. pardalis* from the Ciliwung River makes it necessary to conduct this research to analyze the fish's minerals content and fatty acid profile. Analyses of minerals, fatty acid contents, and fatty acid profiles were conducted using Atomic Absorption Spectrophotometry (AAS), socletation, and Gas Chromatography-Mass Spectrophotometer (GC-MS) methods, respectively. The mineral content of *P. pardalis* from the highest order was calcium, phosphorus, potassium, magnesium, sodium, iron, and zinc. Based on *P. pardalis* body size, large fish had the highest calcium concentration content, and the lowest calcium content was found in the medium fish. The fat content in this fish was very low (<1%), and the content of Saturated Fatty Acids (SFA) was greater than unsaturated ones. Furthermore, palmitate acid was the dominant fatty acid in the fish, while the biggest ratio of omega-6: omega-3 is fatty acids.

Keywords: AAS, FFA, GC-MS, Indonesia, pleco, *Pterygoplichthys pardalis*

INTRODUCTION

Minerals and fatty acids are important fish nutrients needed by humans. These minerals are inorganic substances the body needs due to their various benefits. Nutrients play an active role in the physiological and structural functions of the body, as well as in preventing nutritional deficiencies (Chen et al. 2018; Muscaritoli 2021; Witkamp 2021). Minerals contained in the body of fish include sodium, potassium, calcium, magnesium, phosphorus, selenium, iron, iodine, cobalt, and manganese (Prabhu et al. 2014; Eti et al. 2019). Sodium and potassium help in regulating osmotic pressure in the body. While, iron helps in transporting oxygen, calcium aids in protecting bone health, and iodine plays a role in controlling normal growth mechanisms, as well as physical and mental development (Prashanth et al. 2015; Goff 2018; Alagawany et al. 2021). Some factors influence the minerals found in fish, such as body size, feeding, species, sex, age, reproduction phase, habitat, and the quality of the fish's waters (Prabhu et al. 2014; Paul et al. 2018).

In addition to the minerals, fatty acids in fish are needed by humans for some essential functions (Pal et al. 2018; Chasanah et al. 2021). For example, the human body needs omega 3; Eicosa-Pentaenoic Acid (EPA); Docosa-Hexaenoic Acid (DHA): Omega 6; and arachidonic acid. These essential fatty acids help form cells, regulate the nervous system, strengthen the cardiovascular system, build the immune system, and help the body absorb nutrients (Citil et al. 2014; Kaur et al. 2014). In addition, these are important for the health of brain and eye functions, improve vision by increasing photoreceptors in the eye, as precursors of several hormones, repair wall tissue of nerve cells, act as anti-inflammatory compounds, and prevent muscle breakdown for bodybuilders (Glick and Fischer 2013; Njinkoue et al. 2016).

The widely eaten fish by the people living around the Ciliwung River, Indonesia is the armored catfish, *Pterygoplichthys pardalis* (Castelnau 1855), which is only one genus of pleco in the river (Elfidasari et al. 2016; Rosnaeni et al. 2017). This fish has been identified in the river since the 1980s as an invasive species originating from South America and could live in various aquatic locations, such as bodies and estuaries of rivers, lakes, and ponds. It also adapts easily to water polluted with waste; hence, it is often called janitor fish. According to Hadiaty (2011), *P. pardalis* dominated the Ciliwung River and caused a decline in the population and species of other freshwater fish present in this river. That could be due to the availability of phytoplankton in the river, serving as natural food in sufficient quantities for *P. pardalis*. In addition, *P. pardalis* has no predators or competitors in this river; therefore, the fish are available in the high population (Elfidasari et al. 2020c).

The fish is used as a raw material for making various processed food products such as dumplings, meatballs,

otak-otak (grilled fish cakes), and crackers in the community. The use of *P. pardalis* as a food ingredient is due to its economic value, and people living along the Ciliwung River easily obtain this fish. *P. pardalis* is a good source of animal protein for humans due to its high protein content (Elfidasari et al. 2019).

Moreover, before this study, there is less data explained the minerals and fatty acids contents of *P. pardalis* from the Ciliwung River. Study in 2018 explained that smaller-sized *P. pardalis* has the highest protein and fat contents (50.0517% and 1.1261%) (Elfidasari et al. 2018). Therefore, the aim of this study was to analyze the mineral contents and fatty acid profiles of *P. pardalis* originating from the Ciliwung River, thus, obtaining information on the potential use of the fish as sources of minerals and fatty acids.

MATERIALS AND METHODS

Study area

The fish sampling locations were at two points along the Ciliwung River Basin, namely the Kalibata area (S1) with coordinates of S 06.25830°-E 106.86040° and the Cawang area (S2) with coordinates of S 06.28599°-E 106.84717° in Jakarta, Indonesia (Figure 1). These two sampling locations are areas with a high *P. pardalis* population of about 58 individuals/m^2 (Elfidasari et al. 2020b).

Procedures

Sampling and sample preparation

The purposive sampling technique was used in this study, which involved choosing a sampling location for fish collection with the assumption that most people consume the fish. Sampling was conducted by catching the fish through a seine net that spread in Ciliwung, and after fishes were caught, they were placed in a container filled with ice cubes to maintain their freshness during transportation. Then, the samples were taken to the laboratory to measure the total length and weight. Based on the body measurements of the fish taken from the Ciliwung River in Jakarta, groupings were conducted concerning the method proposed by Tisasari et al. (2016). A total of 60 *P. pardalis* were measured and grouped into three sizes, large (295-391 mm), medium (193-294 mm), and small (91-192 mm), as shown in Table 1. The fish samples were then dissected, and the flesh separated. Subsequently, the flesh was weighed, placed in a petri dish, and dried in an oven at 105°C for 24 hours. The dried samples were pulverized using mortars and a pestle until smooth. Then, about 1 gram of the powdered samples was subjected to mineral testing with two repetitions per fish size. Finally, *P. pardalis* was compared with the control reared in the Ornamental Fish Research and Aquaculture Center or *Balai Riset dan Budidaya Ikan Hias* (BRBIH), Depok, Indonesia. The fish control reared in an aquarium and fed with pellets.

Minerals content analysis

The mineral content analysis used the Atomic Absorption Spectrophotometry (AAS) method. Hence, the concentration of minerals in the material was calculated using the following formula

$$\text{Mineral concentration (mg/kg)} = \frac{\text{Sample concentration } \left(\frac{mg}{l}\right) x \text{ Sample volume } (L)}{\text{sample weight}} x\ FP$$

Where:
Fp : Dilution factor

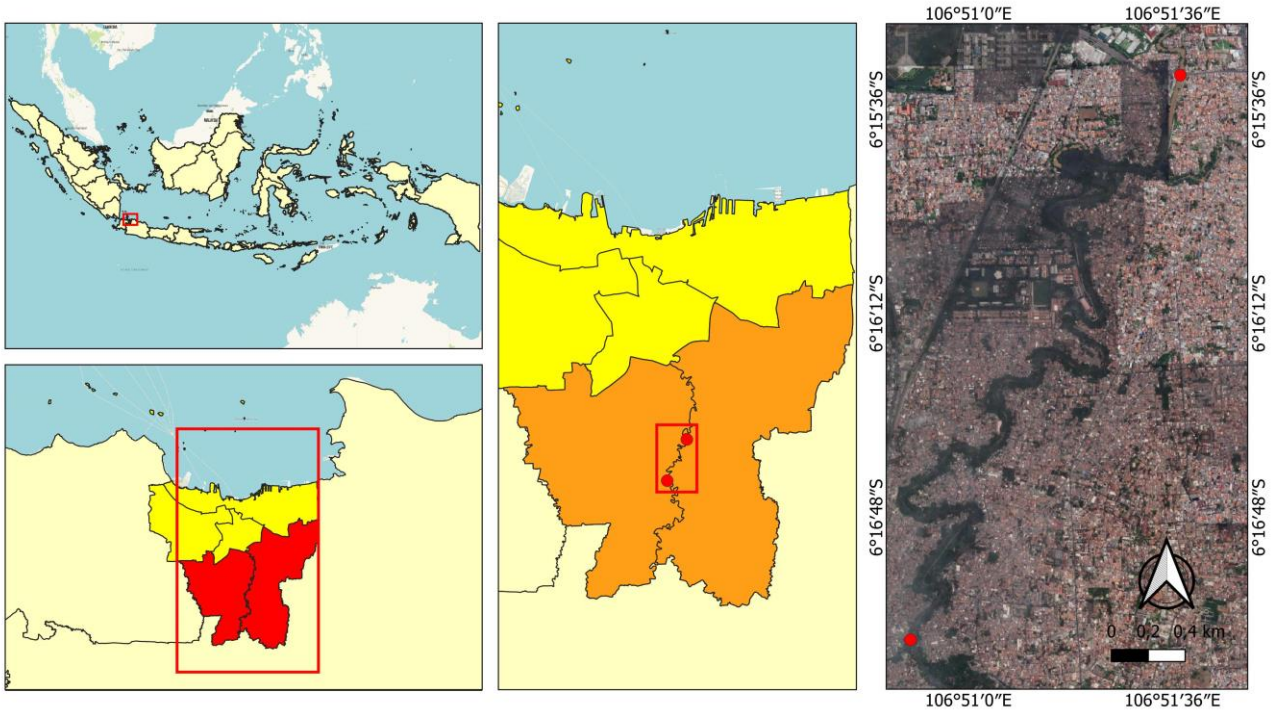

Figure 1. Map of sampling locations for armored catfish *Pterygoplichthys pardalis* in Jakarta, Indonesia

Table 1. Grouping of *Pterygoplichthys pardalis*' body size (Tisasari et al. 2016)

Body size category	Range of body length
Small	91-192 mm
Medium	193-294 mm
Large	295-391 mm

Analysis of fatty acids content

The fatty acid analysis was conducted using the socletation method and yield calculation. Fat content was calculated using the following formula:

$$\text{Fat concentration (\%)} = \frac{W3-W2}{W1} x\ 100\%$$

Where:
W1 : Sample weight (g)
W2 : Weight of an empty erlenmeyer (g)
W3 : Weight of erlenmeyer with fat (g)

Analysis of fatty acid profiles with Gas Chromatography-Mass Spectrophotometer (GC-MS)

This stage involved the extraction of fat from the samples. These were then subjected to the methylation process to form methyl esters. The resultant substances were injected into a chromatographic device (GC-MS) (AOAC 2005).

Analysis of Free Fatty Acids (FFA)

About 1 gram of the sample was placed in 20 mL of 96% alcohol in a 250 mL Erlenmeyer. These were shaken and titrated with 0.1 N KOH until a pink color appeared, which did not disappear in 15 seconds (BSN 1998). The FFA percentage was calculated using the following equation:

$$FFA\ (\%) = \frac{M \times A \times N}{10G}$$

Where:
A : Number of KOH titrations (mL)
N : Normality of KOH
G : Gram of sample
M : weight of fatty acid molecules

Data analysis

The data obtained is entered and grouped or tabulated into a table made under the aims and objectives of the research, then analyzed descriptively and elaborated in the form of charts.

RESULTS AND DISCUSSION

Mineral composition of *P. pardalis*

The highest concentration was calcium at 26,130.96 ppm in large fish, 21,546.44 ppm in medium fish, and 24,463.99 ppm in small fish. This was followed by phosphorus, potassium, sodium, magnesium, iron, zinc, and copper. The highest mineral content was found in the large fish compared with other sizes (Table 2).

The mineral content, such as calcium, magnesium, phosphorus, and iron, of *P. pardalis* from the Ciliwung River was higher than the control fish from the BRBIH pond (Table 3).

Minerals content of *P. pardalis* based on body size

Calcium and phosphorus are the most abundant minerals found in fish. The calcium content was in the range of 21,546.44-26,130.96 ppm (Table 4). Also, the highest calcium content of 26,130.96 ppm was found in the large fish, while the lowest concentration of 21,546.44 ppm was found in the medium fish.

In addition, the calcium content was higher than the phosphorus. The phosphorus content was 6,778.99-8,451.68 ppm, of which the highest content of 8,451.68 ppm was found in large fish, while the lowest concentration was 6,778.99 ppm, found in the small fish (Table 5).

The concentration of potassium in the fish was in the range of 2,942.07 - 4,480.16 ppm. The highest content was found in the small fish at 4,480.16 ppm, while the lowest was in the large fish at 2,942.07 ppm (Table 6).

The sodium content of the fish was in the range of 824-949.99 ppm. The highest concentration was found in the small fish, at 949.99 ppm, while the lowest was found in the medium fish, at 824 ppm (Table 7).

The magnesium concentration of *P. pardalis* was in the range of 748.53 - 913.19 ppm. The highest concentration was found in small fish at 913.19 ppm, while the lowest concentration of 748.53 ppm was found in large fish (Table 8).

The highest concentration of iron was found in the medium *P. pardalis* at 129.82 ppm, while the lowest was found in the large fish at 91.48 ppm (Table 9).

The highest zinc concentration was found in the large fish at 28.32 ppm, while the lowest was found in the small fish at 24.48 ppm (Table 10).

Fatty acids profile of *P. pardalis* from Ciliwung River

The fatty acid profiles for all the fish sizes and control using GCMS showed that the highest fatty acid content was found in the large fish at 84.97% and unidentified fatty acids at 15.03%. On the other hand, the lowest total identified fatty acids were found in medium fish at 62.86% and unidentified fatty acids at 37.14%. Also, the control fish had the second highest fatty acid content at 76.47% and unidentified fatty acids at 25.53% (Table 11).

Analysis of free fatty acids in *P. pardalis* from Ciliwung River

The analysis of free fatty acids from the *P. pardalis* oil sample showed that the highest percentage was found in large fish, while the lowest was in the control (Table 12).

Table 2. The concentration of mineral elements in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia based on the difference in size (ppm)

Element mineral	Mineral concentration in *P. pardalis* from Ciliwung River			
	Control	Large fish	Medium fish	Small fish
Ca	160.52 ± 0.30	26130.96 ± 902.72	2154.44 ± 670.37	24462.99 ± 2346.79
P	5176.02 ± 41.81	8451.68 ± 27.27	7320.75 ± 41.98	6778.99 ± 82.55
K	5258.86 ± 81.04	2942.07 ± 28.82	3450.95 ± 431.17	4480.16 ± 385.82
Mg	510.74 ± 3.40	748.53 ± 23.48	854.85 ± 11.93	913.19 ± 37.36
Na	1293.97 ± 5.13	828.04 ± 7.71	824.00 ± 38.44	949.99 ± 28.21
Fe	21.89 ± 2.51	91.48 ± 4.04	129.82 ± 2.74	94.86 ± 1.42
Zn	16.48 ± 0.69	28.32 ± 1.70	27.29 ± 0.17	24.48 ± 2.05

Table 3. Concentrations of Ca, P, Mg, and Fe minerals in *Pterygoplichthys pardalis* from Ciliwung River and BRBIH, Indonesia

Mineral	Mineral concentration (ppm)	
	P. pardalis from Ciliwung River	Control
Ca	24463.99 ± 2346.79	160.52 ± 0.30
P	6778.99 ± 82.55	5176.02 ± 41.81
Mg	913.19 ± 37.36	510.74 ± 3.40
Fe	94.86 ± 1.42	21.89 ± 2.51

Table 4. The measurement of calcium mineral in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-Repetition Measurement	The Ca content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	25492.63	22020.45	22804.55
2	26769.28	21072.41	26123.42
Total	52261.92	43092.89	48927.98
Average	26130.96	21546.44	24463.99
Standard deviation	902.72	670.37	2346.79

Table 5. The measurement of phosphorus minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-repetition measurement	The P content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	8470.97	6808.68	7257.42
2	8432.39	6749.30	7384.08
Total	16903.35	13557.98	14641.50
Average	8451.68	6778.99	7320.75
Standard deviation	27.27	670.37	2346.79

Table 6. The measurement of potassium mineral in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-repetition measurement	The K content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	8470.97	6808.68	4207.34
2	8432.39	6749.30	4752.98
Total	5884.14	6901.90	8960.33
Average	2942.10	3451.00	4480.20
Standard deviation	28.83	431.18	385.83

Table 7. The measurement of sodium minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-repetition measurement	The Na content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	833.49	796.82	969.94
2	822.58	851.18	930.04
Total	1656.08	1648.00	1899.99
Average	828.04	828.04	949.99
Standard deviation	7.71	38.44	28.21

Table 8. The measurement of magnesium minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-repetition measurement	The Mg content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	765.14	846.41	939.61
2	731.93	863.29	886.87
Total	1497.07	1709.71	1826.39
Average	748.54	854.86	913.19
Standard deviation	23.48	11.93	37.36

Table 9. The measurement of iron minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-Repetition Measurement	The Fe content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	94.34	127.87	93.84
2	88.63	131.76	95.87
Total	182.98	259.64	189.72
Average	91.48	129.82	94.86
Standard deviation	4.04	2.74	1.42

Table 10. The measurement of zinc minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

Nth-repetition measurement	The Zn content in *P. pardalis* (ppm)		
	Large fish	Medium fish	Small fish
1	29,53	27,17	23,03
2	27.12	27.42	25.94
Total	56.65	54.58	48.96
Average	28.32	27.29	24.48
Standard deviation	1.70	0.17	2.05

Table 11. Fatty acid profile of *Pterygoplichthys pardalis*

Fatty acid	Small *P. pardalis* (%) n = 10	Medium *P. pardalis* (%) n = 10	Large *P. pardalis* (%) n = 10	Control *P. pardalis* (%) n=2
Saturated Fatty Acids (SFA)				
Lauric Acid (C12: 0)	0.58	0.51	0.45	0.19
Tridecanoic Acid (C13: 0)	-	-	-	0.11
Myristic Acid (C14: 0)	0.43	0.52	-	0.32
Pentadecanoic Acid (C15: 0)	2.54	2.58	1.89	2.76
Palmitic Acid (C16: 0)	14.27	29.73	24.95	27.75
Heptadecanoic Acid (C17: 0)	-	1.00	0.72	-
Stearic Acid (C18: 0)	13.52	10.10	13.37	-
Behenic Acid (C22: 0)	-	-	-	10.27
Trichosanoic Acid (C23: 0)	-	-	-	0.34
Lignoceric Acid (C24: 0)	-	-	0.66	0.67
Total Saturated Fatty Acids	31.34	44.44	42.04	42.41
Mono Unsaturated Fatty Acids (MUFA)				
Palmitoleic Acid (C16: 0)	6.48	8.61	3.49	1.09
Oleic Acid (C18: 1n9c)	20.69	-	22.87	21.15
Glyceric Acid (C22: 1n9)	2.30	-	2.34	0.54
Nervonic Acid (C24: 1)	-	1.26	-	-
Total Mono Unsaturated Fatty Acids	29.47	9.87	28.70	22.78
Poly Unsaturated Fatty Acids (PUFA)				
Linoleic Acid (C18: 2n6c)	6.83	5.12	7.78	5.42
γ-Linolenic (C18: 3n6)	1.21	1.34	-	-
Linolenic Acid (C18: 3n3)	1.71	1.04	1.49	0.75
Arachidonic Acid (C20: 4n6)	3.42	-	3.22	2.00
EPA (C20: 5n3)	0.89	0.32	0.70	1.11
DHA (C22: 6n3)	1.29	0.73	1.04	2.00
Total Poly Unsaturated Fatty Acids	15.35	8.55	14.23	11.28
Total Fatty Acids	76.16	62.86	84.97	76.47
Total Unidentified Fatty Acid	23.84	37.14	15.03	23.53
Ω6/Ω3	2.94	3.09	3.40	1.92

Table 12. Percentage of free fatty acids of *Pterygoplichthys pardalis* oil

	Body size			
	Large (%) n=10	Medium (%) n=10	Small (%) n=10	Control (%) n=2
	7.15	3.47	5.23	3.73
	1.92	5.13	3.70	3.70
$\bar{x}$	4.53±3.69	4.30±1.17	4.46±1.08	3.71±0.02

Discussion

The mineral content in P. pardalis *from the Ciliwung River*

Variations in the mineral composition of fish could occur due to seasonal and biological differences, such as species, size, dark/white muscle, age, sex, sexual maturity, area of catch, processing method, food source, as well as environmental conditions such as water chemistry, salinity, temperature and contamination (Nurmadia et al. 2013; Debnath et al. 2014)

Based on this study, calcium was the highest mineral content found in the fish (Figure 2). This was suspected to be due to the calcium derived from food sources containing a lot of clam shells and crustaceans on the edge of the Ciliwung River. Also, high calcium levels are only found in the bodies of fish from the sea. Therefore, marine fish could absorb more calcium from their environment, unlike freshwater fish requiring higher calcium content in their feed (Lilly et al. 2017; Monhaty et al. 2017; Islam et al. 2018). The analysis of calcium in mackerel fish (*Scomber scombrus*) and cork fish (*Chana striata*) showed the presence of high calcium content in fish living in water with high salinity. The mackerel habitat is in seawater with a fairly high salinity of 32$^{o}/_{oo}$. Therefore, it has a fairly high calcium content of 29,197.66 mg / 100 g ± 17.77 (Susanti et al. 2016).

However, *P. pardalis* in this study, which is a freshwater fish, has a fairly high calcium content. Also, it is suspected that this fish absorbed more calcium than phosphorus. According to Mogobe et al. (2015), phosphorus absorption could be hindered due to increased calcium in its feed. The feed of *P. pardalis* is a fragment of plants, algae, and detritus. In addition, the clam shells and crustaceans, such as crabs that have died or gone through molting, contribute calcium to water (Samat et al. 2016; Monhaty et al. 2017; Elfidasari et al. 2020c; Ismail et al. 2022).

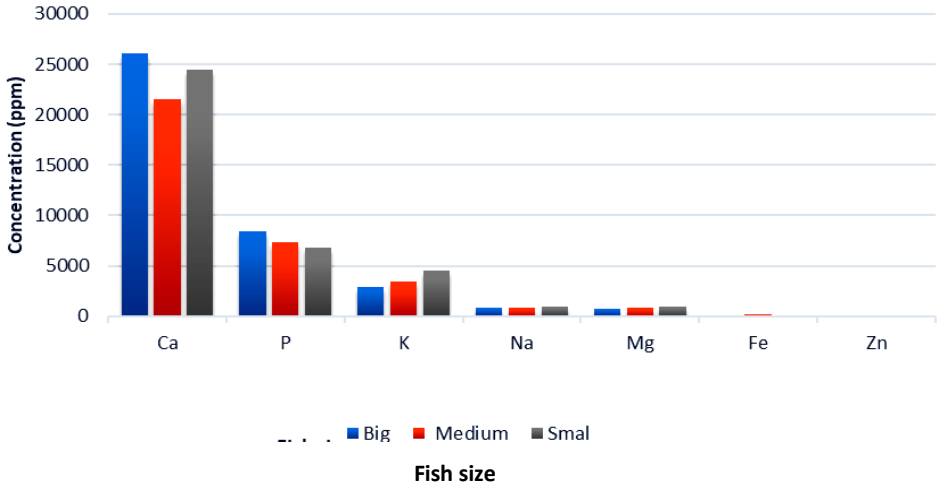

Figure 2. The concentration of minerals in *Pterygoplichthys pardalis* from the Ciliwung River, Indonesia

The high calcium levels in *P. pardalis* could also result from its gills containing chloride secretory cells, also found in other fish. Mitochondrial-rich "chloride" cells in seawater and freshwater fish are the sites for the absorption of Ca^{2+}. These cells in fish which live in a low-calcium freshwater environment adapt by increasing the number or density of cells and increasing cell size to facilitate the optimal transportation of Ca^{2+} (Guh et al. 2015; Leguen et al. 2015; Adam et al. 2019). In addition, the calcium contained in fish helps form bones and scales. According to Ebenstein et al. (2015), the inside of *P. pardalis* scales has a sturdy texture and comprises 58% carbon, 14% oxygen, 7% phosphorus, and 20% calcium.

The second highest mineral content of this fish was phosphorus. High phosphorus levels found in its flesh could result from factors such as food sources. Previous research showed the presence of Loricariidae in the digestive system of fish, a group of algae-eating freshwater fish, consuming several algae living on the bottom surface, such as *Bacillariophyta* algae (Samat et al. 2016; Monhaty et al. 2017; Elfidasari et al. 2020c). The more the presence of phytoplankton in the waters, the higher its phosphorus content. Hence, *P. pardalis* absorbs most of the nitrogen and phosphorus into its body's protective system. In addition, water's phosphorus content affects phytoplankton abundance (Ahmed et al. 2017; Wisha et al. 2018; Marsela et al. 2021; Nindarwi et al. 2021).

Potassium and sodium were the next abundant minerals in the fish. The sodium content of *P. pardalis* in this study was lower than its potassium content. There is a relationship between these two minerals; the higher the sodium, the lower the potassium concentration, and vice versa. According to Debnath et al. (2014) and Ahmed et al. (2017), this is related to the function of both minerals in maintaining the balance of osmotic pressure in the fish's body.

The magnesium content in *P. pardalis* was quite low. This is aligned with previous research, which showed the relatively low magnesium content in fish compared with land animals, and a concentration of ± 65% found in the fish bones (Monhaty et al. 2017; Romharsha and Sarojnalini 2018; Kiliç et al. 2019).

Then, iron content was next abundant. The iron content of *P. pardalis* was quite high compared with other freshwater fish, such as Nile tilapia (*Oreochronis niloticus*), with an iron content of around 0.835-2756 mg (Ramlah et al. 2016). High iron levels in *P. pardalis* flesh could result from the Ciliwung River waters environment. The iron levels in this river exceeded the specified quality standard, set at 2 mg/L. This could be due to the domestic waste produced by the residents on the river's edge (Vincent-Akpu and Obi 2014; Gemaque et al. 2019; Elfidasari et al. 2020a).

Zinc was the lowest mineral content in the samples of *P. pardalis* fish. This is in line with some previous studies which showed the relatively low presence of zinc mineral in several freshwater fish; 0.44 mg in white snapper, 0.45 mg in tilapia, and 0.36 mg in cork fish. That is because zinc is an essential micromineral that catalyzes the work of enzymes. In addition, it plays a role in building the structure of proteins and cell membranes and acts as a transcription factor in the process of gene expression (Sarma et al. 2014; Monhaty et al. 2016; Eti et al. 2019; Paul et al. 2019).

The difference in mineral content in *P. pardalis* from the Ciliwung River and the ones reared in BRBIH (control) could be due to the differences in habitat. The control fish were reared in a pond with the water coming from an underground well at BRBIH, Depok. The water pumping process was channeled to the settling water tank to deposit mud, dirt, parasites, and undesirable organisms, so they do not enter the rearing pond. The hardness of water could also result in the differences in mineral concentrations of fish from both habitats. The water used in rearing the control fish in BRBIH was cleaner than the one from the Ciliwung River, contaminated with various household wastes like soap. Hard water's calcium, magnesium, carbonate, and sulfate contents are usually high (Sengupta 2013; Reksten et al. 2020). Furthermore, differences in mineral content could result from food sources, one of which is the phosphorus mineral. According to Elfidasari et

al. (2020c), Ciliwung waters have abundant phytoplankton, with the discovery of Bacillariophyta algae (82.03%) in the digestive tract of *P. pardalis*. However, the phosphorus concentration of the control *P. pardalis* was low. That could be due to the clean condition of the water in the pond.

The fatty acid content of P. pardalis

The fatty acids obtained from *P. pardalis* through the GC-MS process were classified into: Saturated Fatty Acid (SFA); Mono-Unsaturated Fatty Acid (MUFA); And Poly-Unsaturated Fatty Acid (PUFA). Overall, fish's various sizes contained more SFA than MUFA and PUFA; freshwater fish generally contain more C16 and C18 carbon chain fatty acids, which are included in SFA and MUFA (Kaur et al. 2014; Kandyliari et al. 2020).

In general, SFA is found to be higher in freshwater fish. The results of the study conducted by de Morais et al. (2016) on *P. pardalis* from Brazil showed the presence of palmitic acid (35.71%) as the dominant fatty acid and oleic acid (24.87%) in the fish. According to Bavi and Khodadadi (2017), palmitic and oleic acids are the dominant fatty acids in freshwater fish due to their function as energy ingredients. Therefore, palmitic acid is high in the flesh and liver of freshwater and seawater fish (Babatunde et al. 2020).

Differences in fatty acid content in freshwater fish are influenced by body size, age, sex, habitat, type of food (herbivores, carnivores, omnivores), and other abiotic factors affecting the overall fat content in fish. The high palmitic acid content is a general characteristic of the fatty acid profile of freshwater fish with more saturated fatty acid than unsaturated ones. However, Rodrigues et al. (2017) reported that oleic acid is sometimes the dominant fatty acid in freshwater fish rather than palmitic acid. Furthermore, to maintain the balance of phospholipid membranes in human body cells, there is a need for high levels of palmitic acid, as an SFA, present in freshwater fish flesh, as a daily food portion in the form of omega-3 and omega-6 fatty acids (Carta et al. 2017).

The omega-6 fatty acids (Linoleic Acid, γ-Linolenic acid, and arachidonic acid) from the GC-MS analysis of *P. pardalis* were relatively small. According to Powell et al. (2017), omega-6 fatty acids such as Arachidonic Acid are needed by juvenile fish as an important element during growth and in the immune system. This arachidonic acid also plays an important role in pigmentation and cell growth. Based on previous studies, juvenile *Clarias* given omega-6 fatty acids-rich feed showed a better growth rate (Abaho et al. 2016; Enyidi et al. 2017; Effiong and Yaro 2020). All of these essential fatty acids play a vital role in regulating the osmotic pressure in the fish's body and also in the metabolism process.

The large control of *P. pardalis* contained higher EPA and DHA than large fish from the Ciliwung River, as shown in Table 11. It shows that commercial feed for cultured fish contains high levels of omega-3. That is aligned with the study of Rodriguez-Barreto et al. (2014), which showed that *Seriola dumerili* fish cultivated with commercial feed contained higher EPA in the flesh than wild fish. It also showed that commercial feeds of cultivated fish are generally made from marine ingredients, hence, rich in essential fatty acids.

The ratio between omega-6 and omega-3 fatty acids in the control fish was the smallest, at 1.92, compared with the remaining group. Generally, a greater ratio was produced by cultured fish, but in this case, the opposite results are observed. Therefore, it is assumed that cultured fish given commercial feed contain more complex nutrition than *P. pardalis* from the Ciliwung River. In addition, cultured fish tend to eat feed given regularly (Powell et al. 2017).

The biggest ratio between omega-6 and omega-3 fatty acids was shown by the large *P. pardalis* samples, at 3.4: 1. However, the smallest ratio was shown by the control fish at 1.92: 1. The results of these comparisons are still in the range of the omega-6: omega-3 ratio recommended by the UK Ministry of Health, set at 0.45-4.0 (Sheppard and Cheatham 2018; Shahidi and Ambigaipalan 2018; Djuricic and Calder 2021). The recommended dietary intake ratio needed to reduce obesity in adult humans and prevent coronary heart disease is a balanced ratio of 1 to 2:1 (Eilat-Adar et al. 2013; Liu et al. 2017). Linoleic acid is an omega-6 fatty acid, which after being consumed, parts of it are converted to γ-Linolenic acid and then to Arachidonic Acid (Simopoulos 2016). Therefore, reducing the intake of Linoleic Acid reduces the Arachidonic Acid levels in tissues, which are substrates needed for synthesizing molecules that cause inflammation if taken excessively (Jandacek 2017).

Free fatty acids in P. pardalis

Free fatty acids are an early indicator of bad oil. The content of free fatty acids, even though little, also results in bad taste. Factors responsible for forming free fatty acids include air humidity, light, high temperatures, and destructive bacteria, which cause rancidity (Handayani et al. 2013).

The large *P. pardalis* showed the highest percentage of free fatty acids, while the control fish showed the smallest. Fish cultured with organic food always show fewer free fatty acids. This is because organic food does not change the activity of lipolytic enzymes, which hydrolyze fat. Feed composition plays a very influential role in the fatty acid content of fish. Cultured fish usually eat more uniform feed and fewer microalgae, an important fat source (Balev et al. 2017; Hossain et al. 2018). In addition, they were fed with pellets that are easy to digest for fish. Generally, pellets are made from a mixture of fish meal, plant protein, vitamins, and minerals.

The medium fish showed a small percentage of free fatty acids. This is because fish have the lowest fat content. The free fatty acids content of fish with higher fat content is usually higher than those with lower fat. High levels of free fatty acids in large fish samples were also influenced by the total fatty acids identified by GC-MS (Table 3). This study showed that large fish samples had the largest total fatty acid content, at 84.97%. That contributed to the high percentage of free fatty acids in the large fish samples. The greater the fat content in the fish, the higher the percentage

of free fatty acids (Arai et al. 2015; Rodrigues et al. 2017; Tramice et al. 2021).

The content of free fatty acids in the fish oil sample does not cause nutrient loss but only affects the taste and aroma. The content of free fatty acids also does not affect essential fatty acids, but the amount of fatty acids in the sample determines the percentage of free fatty acids, either high or low (Rodrigues et al. 2017). However, analysis of free fatty acid content is important to determine the quality of the oil (Citil et al. 2014; Suseno et al. 2014; Islam et al. 2018; Reksten et al. 2020). The mineral content of *P. pardalis* from the highest order was calcium, phosphorus, potassium, magnesium, sodium, iron, and zinc. Based on *P. pardalis* body size, large fish had the highest calcium concentration content, and the lowest calcium content was found in the medium fish. The fat content in this fish was very low (<1%), and the content of saturated fatty acids (SFA) was greater than unsaturated ones. Furthermore, palmitate acid was the dominant fatty acid in the fish, while the biggest ratio of omega-6: omega-3 was fatty acids.

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The use of non-medicinal plants by the community of Ayah Village in South Gombong Karst Area, Kebumen, Central Java, Indonesia

ZAHRA HANUN[1], DANASTRI NUR ATHAYA[1], AINI MAR'ATUSH SHOLEKHA[1], CLARA ESTELITA DAMAYANTI[1], IRFAN ABU NAZAR[1], AGUSTINA PUTRI CAHYANINGSIH[2], EDI JUNAEDI[3], INOCENCIO E. BUOT JR.[4], AHMAD DWI SETYAWAN[1,5,♥]

[1]Department of Environmental Science, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia. Tel.: +62-271-663375, ♥email: volatileoils@gmail.com

[2]Graduate Program of Bioscience, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

[3]Program of Biology Education, Faculty of Teacher Training and Education Science, Universitas Kuningan. Jl. Cut Nyak Dhien No. 36 A, Kuningan 45511, West Java, Indonesia

[4]Institute of Biological Sciences, College of Arts and Sciences, University of the Philippines Los Banos. College, Laguna 4031, Philippines

[5]Biodiversity Research Group, Universitas Sebelas Maret. Jl. Ir. Sutami 36A, Surakarta 57126, Central Java, Indonesia

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Abstract. *Hanun Z, Athaya DN, Sholekha AM, Damayanti CE, Nazar IA, Cahyaningsih AP, Junaedi E, Buot JR IE Setyawan AD. 2023. The use of non-medicinal plants by the community of Ayah Village in South Gombong Karst Area, Kebumen, Central Java, Indonesia. Nusantara Bioscience 15: 68-78.* In the homegardens of the people of Kebumen District, Central Java Province, Indonesia, especially in the karst area of Ayah Village, usually planted various plant species with many benefits. However, the knowledge of the people of Ayah Village, Kebumen, about the various benefits of plants is only known from generation to generation, passed on orally and in daily practice habits, so a study is needed to document this information. This study aimed to determine the knowledge of local communities and various types of non-medicinal plants used to fulfill people's daily lives. Data was collected through survey techniques and open interviews with a purposive sampling method with 40 respondents. An inventory of non-medicinal plants resulted in findings of 118 plant species from 59 families. The plants used consisted of 51 species of food plants, 40 species of ornamental plants, 19 species of spices, six species of animal feed, six species of firewood, five species of building materials, and two species of hedges. Some species have more than one use. Our study showed that most of the local community uses plants as food with more diverse plant species compared to other uses.

Keywords: Ethnobotany, karst area, Kebumen, non-medicinal plants

INTRODUCTION

Ethnobotany is the scientific study of indigenous peoples' cultural and ecological practices, including traditional plant knowledge. Ethnobotanical studies provide a repository for the knowledge of indigenous peoples about the medicines and other uses of plants. The study encompasses using plants as food, building materials, medicine, clothing, and traditional ceremonies (Saravanan 2022). In some way, at the very least, for sustenance, every community depends on a different set of plants, each with its own set of regional and cultural features (Potapov et al. 2018). In addition, over a hundred plant species are often recognized as current-day food sources, although historically, thousands of plant species have been used worldwide.

Most homes in rural areas, particularly in the karst mountains of Central Java, Indonesia, have expansive homegardens. The community uses this homegarden to plant various species with different benefits; therefore, the karst region is utilized for agroforestry. Residential homegardens can serve as a habitat for plant growth and maintain a high level of biodiversity (Aronson et al. 2017). A region's plant diversity creates environmental sustainability. Therefore the homegarden plays a significant part in supplying daily demands and providing a comfortable residential. On the other hand, utilizing house homegardens is an alternative to achieving household food self-sufficiency (Suryani et al. 2020).

South Gombong karst is a range of karst mountains located southwest of Kebumen in Central Java, Indonesia. Karst is characterized by caves, surface drainage, and closed depressions (Yanna et al. 2020). As a result, karst has unique qualities and potential that can be utilized. These karst potentials include mineral potential, copious water sources, tourism potential, and scientific potential, all of which will favor the improvement of human welfare in the future (Geekiyanage et al. 2019).

Karst regions offer great potential for living and nonliving natural resources (Kuniansky et al. 2016). The community of Ayah Village, particularly the karst region, has a large homegarden for planting various plant species. Locals cultivate plant varieties they deem helpful in meeting their daily needs. They grow non-medicinal plants in their homegardens, typically utilized by the community as food ingredients, seasonings, ornamental plants, and

firewood. Frequently utilized plant parts include leaves, fruit, flowers, rhizomes, seeds, shoots, tubers, and roots (Fenetahun and Eshetu 2017).

It is feared that the expansion of agricultural, modern industry, urbanization, and, notably, tourism activities in the Ayah village region of Kebumen will negatively impact the local community's ability to meet its fundamental needs as time passes. Access to rural areas has improved with time, which can alter people's habits toward using plants for daily requirements (Wahyuni 2019). People who previously relied on their homegardens to satisfy their daily requirements will begin to rely on the market. Thus they will prefer to purchase products rather than cultivate by themselves. Therefore, it affects the decline of rural homegardens. In addition, there will be direct or indirect local transmission to the younger generation, hindering the transmission of local knowledge from the older generation. It is supplemented by the oral transmission of the local community's knowledge of the various medicinal properties of plants from generation to generation.

As a result, a study should be conducted to determine the current gardening knowledge of the local population. This study intends to assess the local community's knowledge and usage of non-medicinal plants of homegardens in Ayah Village, Kebumen District, Central Java, Indonesia, in fulfilling their daily needs.

MATERIALS AND METHODS

Study area

This study was conducted in the South Gombong karst area, Ayah Village, Kebumen District, Central Java, Indonesia. The South Gombong karst area is a series of karst mountains located southwest of Kebumen, Central Java, Indonesia which includes three sub-districts, namely Ayah, Buayan, and Rowokele. Ayah Village is located at coordinates 7°42′58.7″S, 109°23′19.7″E, and has a karst area that stretches for 277.80 hectares with a height of 335 m above sea level (BPS 2020). The village is located 42.8 km from Kebumen District. A map of the study area can be seen in Figure 1.

Data collection

The study was conducted in November 2022. Data were collected using survey techniques and open interviews using a purposive sampling method (Tongco 2007; Leksikowati et al. 2019) with 40 respondents. The interview was conducted by asking about using various non-medicinal plants grown in the homegardens. The information obtained included the local names of the plants used, the parts of the plants, the benefits, and how the plants were used. In addition, conversations were recorded during the interviews, and the information obtained was recorded. Plants obtained from the survey, including their local names, were identified. The scientific names of these plants were validated using the Plants of the World Online (POWO).

Data analysis

The obtained data were analyzed descriptively and quantitatively and presented using tables and figures (Sholikhah 2016). Use value calculates how much people use a plant species for the same purpose. The index indicates the relative importance of locally known plant species and is determined by the number of use reports described by each informant for each species (Abe and Ohtani 2013).

$$\text{Use Value (UV)} = \frac{\text{Number of citations per species (U)}}{\text{Number of informants (N)}}$$

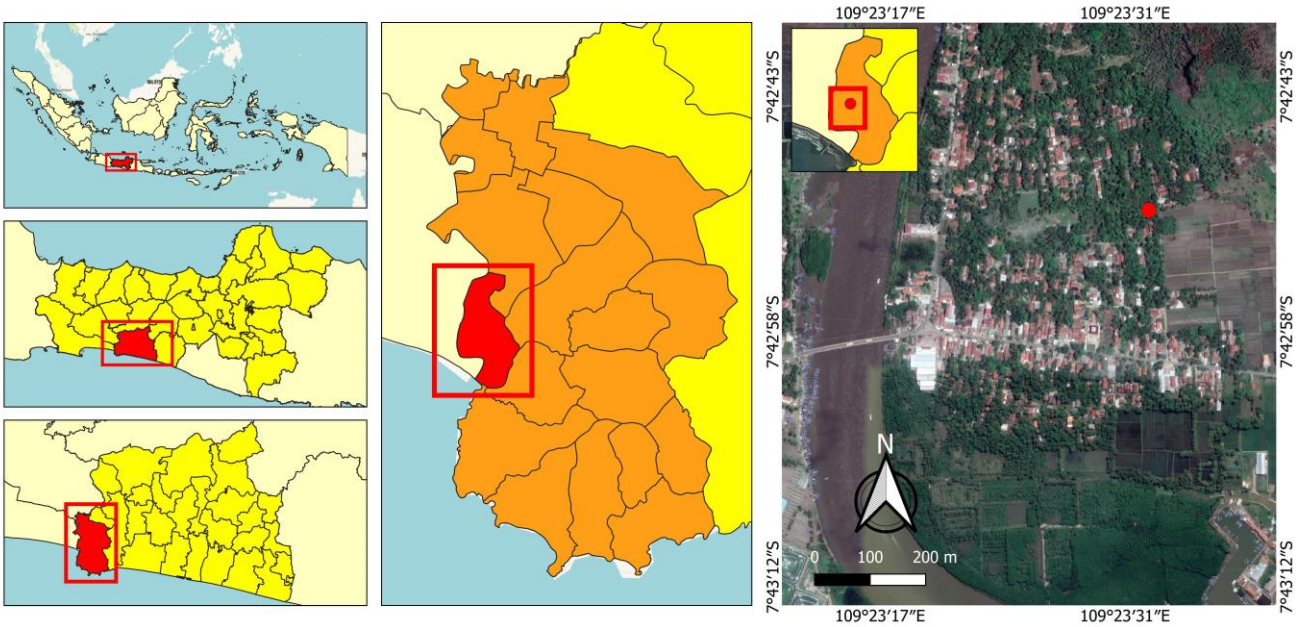

Figure 1. Map of the study area in the South Gombong karst area, Ayah Village, Kebumen, Central Java, Indonesia

RESULT AND DISCUSSION

In this study, 40 respondents were interviewed. Thirty-two of them were female, and eight of them were male. Respondents had varied educational backgrounds, from elementary to college. The junior high school has the largest educational background, with 16 people. The average age of respondents ranges from under 25 to 85 years. However, the highest proportion falls between 25 and 45 years (Table 1). Demographic data shows that most respondents are women, particularly housewives, because they are frequently at home. In addition, despite the broad age range of the respondents, the population's knowledge regarding the usage of non-medicinal plants remained unaffected. That indicated the population's knowledge regarding the use of non-medicinal plants was maintained throughout all age groups.

Table 1. The demographic structure of informants

Parameter	Specification	Frequency
Number of respondents		**40**
Gender	Male	8
	Female	32
Age	<25	2
	25-45	23
	46-65	14
	66-85	1
Education	Elementary school	12
	Junior high school	16
	Senior high school	8
	University	4

Uses of plants

The people of Ayah Village use 118 species from 59 families of non-medicinal plant species for various purposes. They use plants for various purposes, i.e., edible plants, cooking spices, ornamental plants, animal feed, firewood, house-building materials, and hedges. The highest use of plants was for edible plants (42.86%), ornamental plants (33.61%), and cooking spices (15.97%). Utilization with a small percentage includes firewood (5.04%), animal feed (5.04%), house-building materials (4.2%), and hedges (1.68%).

The people of Ayah village still use plants for building materials and household furniture, especially woody plants such as *Tectona grandis* L.f., *Falcataria moluccana* (Miq.) Barneby & J.W.Grimes, and *Cocos nucifera* L. Communities also use plants as animal feed for goats, ducks, and chickens, although with a small percentage. Our study found that the community uses several species for more than one usage, such as *C. nucifera*, which is used as food, firewood, and building materials.

Rural communities generally have large homegardens and use them as home gardens (Figure 2). Home gardens are used to grow various plants such as vegetables, fruit, spices, ornamental plants, and medicinal plants, and even livestock can provide a source of food and income (Galhena et al. 2013). The people of Ayah Village also cultivate more plants in their fields to meet their daily needs. Even though the dubious fields are public lands that the entire community can use on condition that they take care of these fields.

Table 2. Types of use of non-medicinal plants in Ayah Village, Kebumen District, Indonesia

Purpose	Total species	Total genus	Total family	Percentage
Edible plant	51	45	21	42.86%
Ornamental plant	40	39	29	33.61%
Spices	19	17	13	15.97%
Animal feed	6	6	5	5.04%
Firewood	6	6	4	5.04%
Building material	5	5	4	4.20%
Household appliance	2	2	2	1.68%
Fence	2	2	2	1.68%
Firestarter	1	1	1	0.84%
Broomstick	1	1	1	0.84%

Food plants

There are 51 plant species from 21 families that the people of Ayah Village use as food plants, with the highest percentage of uses compared to others (Table 3). Most food crops grown by the community are fruit and vegetable crops they can consume directly or cook. Table 2 shows that the people of Ayah's village use more fruit parts as food plants than other plants. In addition, it indicated that the people of Ayah's village planted more fruit trees in their homegardens.

Ayah Village is very close to the tourist areas of the beaches and mangrove forests. It will indirectly affect the culture and daily life of the villagers in fulfilling their daily needs. The development of the tourism area has caused residential areas to become denser and village facilities more diverse. It impacts the residents' homegardens which are not too large to plant. The people will prefer to buy at the market for their food needs than plant alone. Therefore, the community grows more fruiting trees which can be consumed when they bear fruit. These trees can meet food needs, reducing household expenses and shading. Most respondents were women aged 25 to 65, and most were housewives. Thus, people's knowledge of food crops was also limited to the plants they planted in their homegardens and used for daily consumption. Their needs and habits usually require knowledge of plants used in the local culture (Leal et al. 2018).

Fabaceae is widely used as a food plant in Ayah Village (Figure 3). Plant species from this family include *Parkia speciosa* Hassk., *Pachyrhizus erosus* L., *Vigna unguiculata* (L.) Walp., and *Leucaena leucocephala* (Lam.) de Wit. This family is the world's third most populous plant and includes cosmopolitan plants found in areas with cold to warm temperatures (Anugrah et al. 2022). Overall, the Fabaceae family is also the most widely planted family by Ayah Village people for various purposes, especially food. Of the four species, only *P. erosus* is a fruit plant, while the others are used as vegetables. *L. leucocephala* has various uses and is known as the miracle tree because of its worldwide benefits (Zayed and Samling 2016). Several countries also

use *L. leucocephala* as a food plant, such as Thailand, India, and the USA, which uses young leaves, seeds, and flowers for salads, soups, or other food ingredients (Zayed and Samling 2016). Apart from being used as a vegetable, the old seeds can also be used as a substitute for coffee (Nehdi et al. 2014).

Our study also found that several ornamental plants are consumed by Ayah Village people in their daily lives, such as *Aloe vera* (L.) Burm f., *Polyscias scutellaria* (Burm.f.) Fosberg, and *Portulaca grandiflora* Hook. The *A. vera* is used as a food by eating directly or making it into a salad mix. The *A. vera* grown by the local community does not taste bitter, so it can be consumed raw. The plant is native to the African Continent, especially North and South Africa (Sanchez-Machado et al. 2017; Maan et al. 2018). The *A. vera* has transparent gel, which is the edible part, with around 98-99% of the gel being water (Maan et al. 2018). The main component bears low-calorie carbohydrates (Sanchez-Machado et al. 2017; Maan et al. 2021), protein, fiber, soluble sugars, vitamins, minerals, and phenolic compounds (Maan et al. 2018).

Polyscias scutellaria, also known as *mangkokan*, is one of Indonesia's indigenous vegetable plants, which people in West Java, Central Java, East Java, and Yogyakarta consume (Yurlisa 2016). The people of Ayah village use the leaves of *mangkokan* as food by cooking it. On the other hand, the Sundanese people of West Java usually consume *P. scutellaria* as fresh vegetables. They also eat raw or boiled without additional spices or other ingredients (Hernawati et al. 2022). Whereas *P. grandiflora*, also known as Indian purslane, which is a weed with a high degree of adaptation, is an edible plant and can fulfill most of the nutrients needed by humans. Therefore, it can potentially become a food ingredient supporting nutritional needs (Srivastava et al. 2021). The leaves and stems of *P. grandiflora* are rich in nutrients such as omega-3 fatty acids (five times higher than spinach), alpha-linoleic acid, vitamin A, vitamin C, and vitamin E, electrolytes such as potassium and sodium, minerals such as calcium, magnesium, and phosphorus (Uddin et al. 2014). The people of Ayah's village use these leaves by cooking them. In the Himalayan region, precisely in Pakistan, *P. grandiflora* is a wild plant with young leaves used as food crops. At the same time, other aerial parts are used as animal feed (Abbasi et al. 2015). Apart from that, various countries also use it as a food crop, such as in India, it is used as a vegetable ingredient to make curry, and in Cyprus, it is eaten raw by making processed salads (Iranshahy et al. 2017).

Figure 2. Several homegardens of the people of Ayah Village in Kebumen District, Central Java, Indonesia

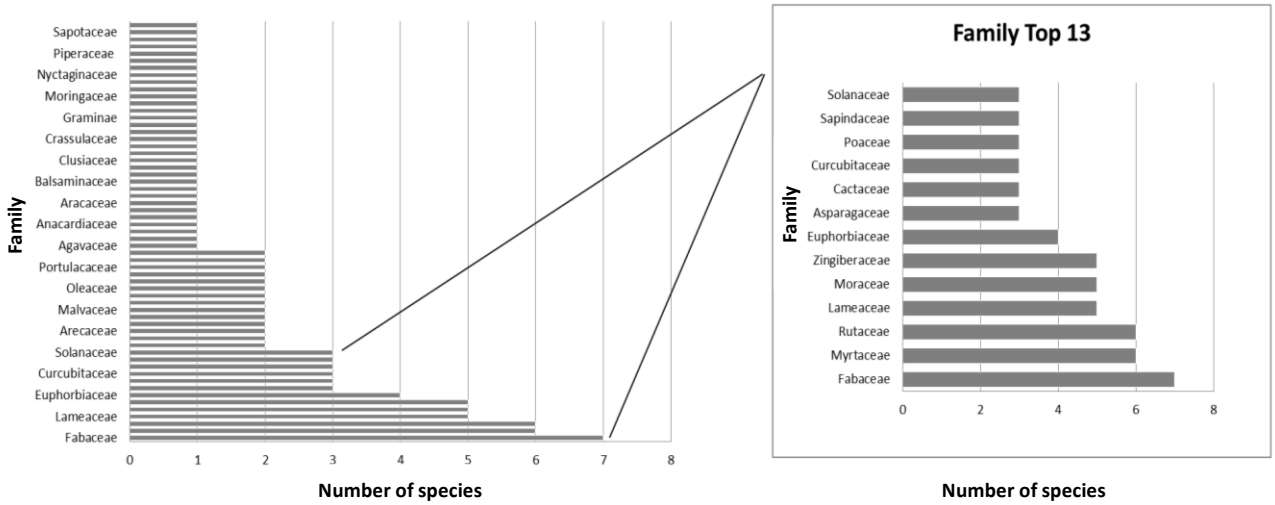

Figure 3. All families and the top 13 families of non-medicinal plants used by the people of Ayah Village, Kebumen District, Indonesia

Table 3. Food plants used by the people of Ayah Village, Kebumen District, Indonesia

Scientific name	Local name	Family	Growth form	Part of used	Method of used	UV
Aloe vera (L.) Burm f.	*Lidah buaya*	Liliaceae	Herbaceous	Leaf	Raw, salads	0.05
Amaranthus sp.	*Bayam*	Amaranthaceae	Herbaceous	Leaf	Cooked	0.12
Ananas comosus (L.) Merr.	*Nanas*	Bromoliaceae	Herbaceous	Fruit	Raw	0.02
Apium graveolens L.	*Seledri*	Apiaceae	Herbaceous	Leaf	Raw, cooked	0.02
Artocarpus altilis (Parkonson) Fosberg	*Sukun*	Moraceae	Tree	Fruit	Steam, fried	0.02
Artocarpus heterophyllus Lam	*Nangka*	Moraceae	Tree	Fruit	Raw, cooked	0.17
Averrhoa carambola L.	*Belimbing*	Oxalidaceae	Tree	Fruit	Raw	0.1
Carica papaya L.	*Pepaya*	Caricaceae	Tree	Fruit	Raw	0.27
Citrus limon (L.) Osbeck	*Jeruk lemon*	Rutaceae	Tree	Fruit	Ingredient for beverage	0.02
Citrus maxima (Burm.) Merr	*Jeruk Bali*	Rutaceae	Tree	Fruit	Raw	0.05
Citrus sinensis (L.) Osbeck	*Jeruk baby*	Rutaceae	Shrub	Fruit	Raw	0.05
Cocos nucifera L	*Kelapa*	Araceae	Tree	Fruit	Raw	0.25
Cosmos Cav.	*Kenikir*	Asteraceae	Herbaceous	Leaf	Raw, boiled	0.02
Cucumis sativus L.	*Timun*	Cucurbitaceae	Climber	Fruit	Raw	0.02
Dimocarpus longan Lour.	*Kelengkeng*	Sapindaceae	Tree	Fruit	Raw	0.05
Durio zibethinus L.	*Durian*	Malvaceae	Tree	Fruit	Raw	0.07
Fragaria L.	*Stroberi*	Rosaceae	Scrub	Fruit	Raw	0.02
Garcinia mangostana L.	*Manggis*	Clusiaceae	Tree	Fruit	Raw	0.12
Gnetum gnemon L.	*Melinjo*	Gnetaceae	Tree	Leaf, fruit, seed	Cooked, fried	0.12
Hylocereus polyrhizus (F.A.C.Weber) Britton & Roses	*Buah naga*	Cactaceae	Climber	Fruit	Raw	0.05
Ipomoea aquatica Forssk.	*Kangkung*	Convolvulaceae	Herbaceous	Leaf	Cooked	0.02
Ipomoea batatas (L.) Lam	*Ketela*	Convolvulaceae	Climber	Tuber, leaf	Steamed, cooked	0.15
Lansium parasiticum (Osbeck) Mabb.	*Duku*	Meliaceae	Tree	Fruit	Raw	0.05
Leucaena leucocephala (Lam.) de Wit	*Klandingan*	Fabaceae	Tree	Seed	Cooked	0.02
Luffa acutangula (L.) Roxb.	*Oyong*	Curcubitaceae	Climber	Fruit	Raw	0.02
Lycopersicon esculentum Mill	*Tomat*	Solanaceae	Herbaceous	Fruit	Raw, cooked, juiced	0.02
Mangifera indica L.	*Mangga*	Anacardiaceae	Tree	Fruit	Raw	0.37
Manihot esculenta Crantz	*Singkong*	Euphorbiaceae	Shrub	Tuber, leaf	Steamed, cooked	0.02
Manilkara zapota (L.) P.Royen	*Sawo*	Sapotaceae	Tree	Fruit	Raw	0.05
Mentha x piperita L.	*Mint*	Lamiaceae	Herbaceous	Leaf	Ingredient for beverage	0.02
Momordica charantia L.	*Pare*	Curcubitaceae	Climber	Leaf	Cooked	0.05
Morinda citrifolia L.	*Mengkudu*	Rubiaceae	Tree	Fruit	Raw	0.07
Moringa oleifera Lam.	*Kelor*	Moringaceae	Shrub	Leaf	Cooked	0.02
Muntingia calabura L.	*Karsen*	Muntingiaceae	Tree	Fruit	Raw	0.02
Musa acuminata Colla	*Pisang kepok*	Musaceae	Herbaceous	Fruit	Raw	0.12
Musa x paradisiaca L.	*Pisang*	Musaceae	Herbaceous	Fruit	Raw	0.32
Nephelium lappaceum L.	*Rambutan*	Sapindaceae	Tree	Fruit	Raw	0.10
Ocimum sanctum L.	*Kemangi*	Lamiaceae	Scrub	Leaf	Raw	0.05
Pachyrhizus erosus L.	*Bengkoang*	Fabaceae	Climber	Tuber	Raw	0.02
Parkia speciosa Hassk.	*Pete*	Fabaceae	Tree	Fruit	Raw, cooked	0.07
Persea Americana Mill.	*Alpukat*	Lauraceae	Tree	Fruit	Raw	0.07
Plinia cauliflora (Mart.) Kausel	*Anggur brazil*	Myrtaceae	Climber	Fruit	Raw	0.02
Polyscias scutellaria (Burm.f.) Fosberg	*Mangkoan*	Araliaceae	Shrub	Leaf	Cooked	0.07
Polyscias filicifolia (c. Moore ex E.Fourn.) L.H.Bailey	*Cakla cikli*	Araliaceae	Shrub	Leaf	Cooked	0.02
Pometia pinnata J.R.Forst.& G.Forst.	*Matoa*	Sapindaceae	Tree	Fruit	Raw	0.02
Portulaca grandiflora Hook	*Krokot*	Portulacaceae	Herbaceous	Leaf	Cooked	0.02
Psidium guajava L.	*Jambu biji*	Myrtaceae	Tree	Fruit	Raw	0.20
Sauropus androgynous (L.) Merr.	*Katuk*	Phyllanthaceae	Shrub	Leaf	Cooked	0.10
Solanum melongena L.	*Terong*	Solanaceae	Herbaceous	Fruit	Cooked	0.12
Syzygium aqueum (Burm.f.) Alston	*Jambu air*	Myrtaceeae	Tree	Fruit	Raw	0.20
Vigna unguiculata (L.) Walp.	*Kacang panjang*	Fabaceae	Climber	Fruit	Raw, cooked	0.02

Ornamental plants

Forty species from 29 families are used by the people of Ayah Village as ornamental plants and are the second most use-category after food (Table 4). Ayah Village is very close to the karst forest area. It caused many villagers to take plants from the forest to be planted in their homegardens as home gardens and used as ornamental plants. Ornamental plants are planted directly in the house's front yard or in pots (Figure 4).

Of the 29 families, the Araceae family has the highest number of species used as ornamental plants. These species are *Aglaonema commutatum* Schott, *Anthurium plowmanii* Croat, *Caladium bicolor* (Aiton) Vent, *Epipremnum aureum* (Linden&Andre) G.S.Bunting, *Monstera adansonii* Schott, and *Colocasia esculenta* (L.) Schott. Apart from the people of Ayah Village, the people of Cisoka Village, Majalengka, West Java, also use the Araceae family as ornamental plants. They are usually planted in the homegarden or used as decorations at certain events, such as weddings and celebrations of Indonesian Independence Day (Mutaqin et al. 2018).

The community widely cultivates ornamental plants to increase aesthetic value around their homegarden and beautify homes (Vivek et al. 2021). For example, the people of Rajegwesi Village, Banyuwangi, Central Java, Indonesia use the same species as those of Ayah Village as ornamental plants. These species include *Codiaeum variegatum* (L.) rumph. Ex A.Juss and *Ixora* sp. The cultural difference between the people of Rajegwesi Village, Banyuwangi, and Ayah Village is in managing their homegardens. The people of Rajegwesi Village houses have three zones, namely *mburitan*, located in the backyard; *iringan*, located on the sides of the house; and *jogan*, located in the house's front yard. That *jogan* zone is explicitly used for ornamental plants (Pamungkas et al. 2013). Like the people of Lampung, Indonesia ornamental plants are placed in the house's front yard (*hadap* or *tengebah*) (Wakhidah et al. 2020).

Spices

The people of Ayah Village use 19 species from 13 plant families as cooking spices (Table 5). These species include *Zingiber officinale* Roscoe, *Ammomum cardamomum* L., *Kaempferia galangal* L., *Alpinia galanga* (L.) Willd., and *Curcuma domestica* Valeton. The part used by the people of Ayah Village is the rhizome for the five species. The community uses it by grinding, bruising, and boiling to make delicious, fragrant, enriching food taste. Aside from being a spice in cooking, several Zingibeaceae plants are mainly used by the community to get rid of the fishy smell of fish or meat, namely *Z. officinale* or *C. domestica*, by grinding it first and then smearing it on fish or meat and letting it sit for a few minutes before processing. The Zingiberaceae family is the most spice plant use. Similarly, there were also found in other areas in Indonesia such as Aceh Tamiang District, Aceh, Indonesia (Navia et al. 2020), Tidung tribe of Kalimantan (Listiani and Abrori 2018), and Saibain Lampung (Wakhidah et al. 2020).

The community's seasoning plants are generally the basic spices used in Javanese dishes. Even so, some people grow *Cinnamomum burmanni* (Nees & T.Nees) Blume as a spice plant even though it is less commonly used in Javanese specialties. The *C. burmanni* is one of the oldest spices, with a sweet and warm sensation that stands out after pepper (Kumar and Kumari 2019). The species is one of Indonesia's endemic plants (Menggala et al. 2019). As the oldest spice, cinnamon has also been used in various traditional dishes in various countries. For example, the people of India generally use cinnamon as a spice in making curry (Bharali et al. 2017), and the people of Bulgaria used it as one of the spices in making *Gornooryahovski Sudzhuk* or traditional sausages (Ivanova et al. 2022).

Figure 4. The *jogan* (front yard) is a zone for the use of ornamental plants by the people of Ayah Village, Kebumen District, Central Java, Indonesia

Table 4. Ornamental plants used by the people of Ayah Village, Kebumen District, Central Java, Indonesia

Scientific name	Local name	Family	Growth form	UV
Adenium obesum (Forssk.) Roem. & Schult.	*Kamboja*	Apocynaceae	Herbaceous	0.05
Adiantum capillus-veneris L.	*Suplir*	Pteridaceae	Herbaceous	0.02
Aeschynanthus Jack	*Bunga lipstik*	Gesneriaceae	Climber	0.02
Aglaonema commutatum Schott.	*Sri rejeki*	Araceae	Herbaceous	0.07
Aloe vera (L.) Burm.f.	*Lidah buaya*	Asphodeloideae	Herbaceous	0.25
Anthurium plowmanii Croat	*Gelombang cinta*	Araceae	Scrub	0.05
Bougainvillea spectabilis Wilid.	*Bunga kertas*	Nyctaginaceae	Shrub	0.17
Caladium bicolor (Aiton) Vent.	*Keladi*	Araceae	Herbaceous	0.07
Cananga odorata (Lam.) Hook f. & Thomson	*Kenanga*	Annonaceae	Shrub	0.07
Chlorophytum comosum (Thunb.) Jacques	*Rekmo putri*	Asparagaceae	Shrub	0.02
Codiaeum variegatum (L.) rumph. Ex A.Juss.	*Puring*	Euphorbiaceae	Shrub	0.07
Coleus scutellarioides (L.) Benth.	*Iler*	Lamiaceae	Scrub	0.07
Colocasia esculenta (L.) Schott	*Talas*	Araceae	Herbaceous	0.02
Cordyline fruticosa (L.) A.Chev.	*Andong*	Asparagaceae	Herbaceous	0.12
Cuphea hyssopifolia Kunth	*Taiwan beauty*	Lythraceae	Scrub	0.02
Dendrobium sp.	*Anggrek*	Orchidaceae	Herbaceous	0.12
Dracaena sanderiana Mast.	*Bambu cina*	Asparagaceae	Herbaceous	0.07
Drynaria sparsisora (Desv.) T.Moore	*Simbar*	Polypodiaceae	Herbaceous	0.02
Epiphyllum anguliger (Lem.) G.Don	*Wijaya kusuma*	Cactaceae	Herbaceous	0.05
Epipremnum aureum (Linden&Andre) G.S.Bunting	*Sirih gading*	Araceae	Climber	0.02
Ficus coreana	*Dolar*	Moraceae	Herbaceous	0.05
Hibiscus tiliaceus L.	*Waru*	Malvaceae	Tree	0.02
Impatiens balsamina L.	*Pacar air*	Balsaminaceae	Herbaceous	0.05
Ixora javanica (Blume) DC.	*Soka*	Rubiaceae	Shrub	0.02
Jasminum sambac (L.) Aiton	*Melati*	Oleaceae	Shrub	0.05
Kalanchoe pinnata (Lam.) Pers.	*Cocor bebek*	Crassulaceae	Herbaceous	0.02
Lantana camara L.	*Lantana*	Verbenaceae	Shrub	0.05
Michelia x alba DC	*Kantil*	Magnoliaceae	Shrub	0.02
Michelia champaca (L.)	*Cempaka*	Magnoliaceae	Shrub	0.02
Monstera adansonii Schott	*Janda bolong*	Araceae	Climber	0.02
Opuntia monacanthos (Willd.) Haw	*Kaktus*	Cactaceae	Herbaceous	0.02
Oxalis triangularis A.St.-Hill	*Bunga kupu-kupu*	Oxalidaceae	Herbaceous	0.02
Phalaenopsis amabilis (L.)	*Anggrek bulan*	Orchidaceae	Herbaceous	0.02
Polyscias scutellaria (Burm.f.) Fosberg	*Mangkokan*	Araliaceae	Shrub	0.05
Portulaca grandiflora Hook	*Cantik manis*	Portulaccaceae	Herbaceous	0.05
Rosa multiflora Thunb.	*Mawar*	Rosaceae	Shrub	0.1
Sansevieria trifasciata (Prain) Mabb.	*Lidah mertua*	Agavaceae	Herbaceous	0.15
Streblus asper Lour.	*Serut*	Moraceae	Shrub	0.05
Syzygium paniculatum Gaertn.	*Pucuk merah*	Myrtaceae	Shrub	0.1
Tradescantia pallida (Rose) D.R.Hunt	*Adam hawa*	Commelinaceae	Herbaceous	0.05

Table 5. Cooking plant spices used by the people of Ayah Village, Kebumen District, Central Java

Species name	Local name	Family	Growth form	Part of used	Method of used	UV
Allium sativum L.	*Bawang*	Amaryllidaceae	Herbaceous	Tuber	Crushed, mashed	0.03
Alpinia galanga (L.) Willd.	*Laos*	Zingiberaceae	Herbaceous	Rhizome	Crushed	0.15
Amomum cardamomum L.	*Kapulaga*	Zingiberaceae	Herbaceous	Rhizome	Boiled	0.03
Capsicum annuum L.	*Cabai*	Solanaceae	Shrub	Fruit	Cut, mashed	0.23
Cinnamomum burmanni (Nees & T.Nees) Bl.	*Kayu manis*	Lauraceae	Tree	Bark, leaf	Cooked, brewed	0.03
Citrus x aurantifolia (Christm.) Swingle	*Jeruk nipis*	Rutaceae	Tree	Fruit	Squeezed	0.03
Citrus hystrix DC.	*Jeruk sambal*	Rutaceae	Tree	Fruit	Squeezed	0.03
Citrus limon (L.) Osbeck	*Jeruk lemon*	Rutaceae	Tree	Fruit	Squeezed	0.08
Cocos nucifera L.	*Kelapa*	Araceae	Tree	Flower sap	Boiled	0.10
Coriandrum sativum L.	*Ketumbar*	Apiaceae	Herbaceous	Seed	Mashed	0.03
Curcuma domestica Valeton	*Kunyit*	Zingiberaceae	Herbaceous	Rhizome	Mashed, boiled	0.38
Cymbopogon citratus (DC.) Stapf	*Serai*	Poaceae	Herbaceous	Stem	Crushed, cut	0.28
Kaempferia galanga L.	*Kencur*	Zingiberaceae	Herbaceous	Rhizome	Mashed, boiled	0.05
Myristica fragrans Houtt.	*Pala*	Myristicacea	Tree	Seed	Boiled	0.03
Pandanus amaryllifolius Roxb. Ex Lindl	*Pandan*	Pandanaceae	Herbaceous	Leaf	Boiled	0.13
Piper nigrum L.	*Merica*	Piperaceae	Climber	Fruit	Mashed, cooked	0.03
Syzygium polyanthum (Wight) Walp	*Salam*	Myrtaceae	Tree	Leaf	Dried, boiled	0.13
Tamarindus indica L.	*Asam jawa*	Fabaceae	Tree	Fruit	Cooked	0.10
Zingiber officinale Roscoe	*Jahe*	Zingiberaceae	Herbaceous	Rhizome	Crushed, boiled	0.40

Other uses

The people of Ayah Village use 23 plant species from 12 families for other uses, such as animal feed, building materials, firewood, and hedges (Table 6). There are several plant species with many use-category. For example, *C. nucifera* is used by the community as a material for broomsticks, building materials, firewood, and lighters. It is also used by the people of Rajegwesi Village, Banyuwangi, Central Java, Indonesia for their household needs, such as food, firewood, building materials, and furniture (Pamungkas et al. 2013). Another plant with multiple usage categories is *P. scutellaria* which is used as an animal feed and hedge plant.

The people of Ayah Village use three species from the Poaceae family. This family has the highest number of species for other uses. The community uses *Oryza sativa* L. and *Pennisetum purpureum* Schumach. as animal feed for cattle and goats and *Dendrocalamus asper* (Schult. & Schult.f.) Backer as a building material. Some people in Ayah's village still use the leaves of *Areca catechu* L. and *Nypa fruticans* Wurmb to make *welit*. *Welit* is a collection of leaves arranged in rows and strung together, usually used as a roof on traditional buildings. Currently, *welit* (Figure 5) as a roof for houses is limited to specific uses. In some areas, *welit* is no longer applied to residential buildings and is only used as a tradition. For example, the people of Cirebon, West Java, use *welit* in the *memmayyu* ceremony to replace the old *welit* roof at the Trusmi Mosque once a year (Lestari 2013).

The part of the plant used

Our study revealed that the people of Ayah Village strongly used fruiting plants (40%). The fruiting plant parts can be utilized and processed using various methods. The most common method is being eaten directly, i.e., mango (*Mangifera indica* L.), dragon fruit (*Hylocereus polyrhizus* (F.A.C.Weber) Britton & Roses), and crystal guava (*Psidium guajava* L.). The other uses are cooked, such as tomatoes (*Lycopersicon esculentum* Mill), *petai* (*P. speciosa*), and eggplant (*Solanum melongena* L.). The second largest part is the leaves, with a percentage of 27%, and most of them are used as vegetables. The community also uses wood (7%), rhizomes and stems (5% each), tubers (4%), branches and seeds (2% each), and leaf bones, dry leaves, flower sap (1% each) (Figure 6). Based on the percentage of plant parts that are widely used, it is known that the community preference is practical and easy to get. It may be related to the study area being in a village in a tropical karst area, so local people prefer to grow food plants that are easy to grow in their homegardens (Suhendar et al. 2018).

The life form of the plants used

The plants planted and used by the people of Ayah Village have various life-form characteristics. Figure 7 is the percentage of how plants grow. The community's most plant growth uses are herbaceous, trees, shrubs, scrubs, and climbers. In the karst area of Ayah Village, the characteristics of plants are dominated by trees at 39 of 118 species (33%).

The second largest form of plants' characteristic growth is occupied by 32% or 38 species of herbaceous. The life-form of shrubs, scrub, and climbers are 17, 15, and 10 plants from 118 species, respectively. Tree life-form includes teak (*T. grandis*) and acacia (*Acacia auriculiformis* A.Cunn. ex Benth.). The *T. grandis* can grow optimally at 700 m above sea level. The plant has shallow roots and can grow well on soil with a thin layer, like in karst areas. Apart from *T. grandis*, another dominant species is *A. chinensis*, a member of the Fabaceae family. It was the dominant plant because it can grow in almost all soil conditions, including dry land (Tabun et al. 2020). In addition, its roots are shallow patterns, suitable to grow in karst areas. Therefore, these two species are abundant and dominate the South Gombong Karst Area.

Figure 5. Leaves used as *welit*

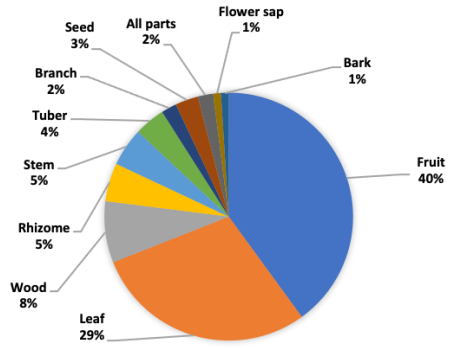

Figure 6. Part of the plant used by the community of Ayah Village, Kebumen District, Central Java, Indonesia

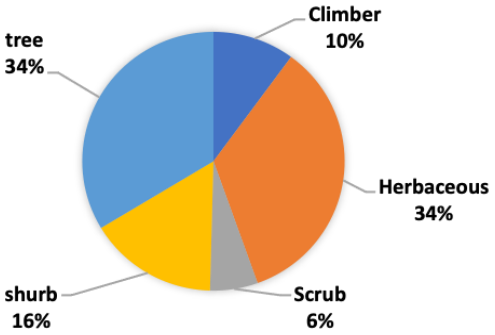

Figure 7. The life-form plants used by the people of Ayah Village, Kebumen District, Central Java, Indonesia

Table 6. The use of plants for various purposes by the people of Ayah Village, Kebumen District, Central Java, Indonesia

Scientific name	Local name	Family	Growth form	Part of used	Uses	UV
Acacia auriculiformis A.Cunn. ex Benth.	*Akasia*	Fabaceae	Tree	Wood	Firewood	0.15
Areca catechu L.	*Jambeh*	Aracaceae	Tree	Leaf	Building material	0.03
Artocarpus heterophyllus Lam.	*Nangka*	Moraceae	Tree	Leaf	Animal feed	0.05
Carica papaya L.	*Pepaya*	Caricaceae	Tree	Leaf	Animal feed	0.03
Cocos nucifera L.	*Kelapa*	Araceae	Tree	Bone leaves	Broomstick	0.03
Cocos nucifera L.	*Kelapa*	Araceae	Tree	Stem	Building material	0.03
Cocos nucifera L.	*Kelapa*	Arecaceae	Tree	Stem	Firewood	0.08
Dendrocalamus asper (Schult. & Schult.f.) Backer	*Bambu*	Poaceae	Tree	Stem	Building material	0.03
Falcataria moluccana (Miq.) Barneby & J.W.Grimes	*Sengon*	Fabaceae	Tree	Wood	Building material	0.05
Falcataria moluccana (Miq.) Barneby & J.W.Grimes	*Sengon*	Fabaceae	Tree	Wood, branch	Firewood	0.05
Lagistrum ovalifolium Hassk.	*Teh-tehan*	Oleaceae	Shrub	All parts	Fence	0.03
Manihot carthaginensis subsp. *Glaziovii*	*Singkong*	Euphorbiaceae	Shrub	Leaf	Animal feed	0.03
Nephelium lappaceum L.	*Rambutan*	Sapindaceae	Tree	Wood	Firewood	0.05
Nypa fruticans Wurmb	*Daonan*	Arecaceae	Tree	Leaf	Building material	0.03
Oryza sativa L.	*Padi*	Poaceae	Scrub	Leaf	Animal feed	0.03
Pennisetum purpureum Schumach.	*Rumput gajah*	Poaceae	Scrub	Leaf	Animal feed	0.08
Polyscias scutellaria (Burm.f.) Fosberg	*Mangkokan*	Araliaceae	Shrub	Leaf	Animal feed	0.03
Polyscias scutellaria (Burm.f.) Fosberg	*Mangkoan*	Araliaceae	Shrub	All parts	Fence	0.03
Tectona grandis L.f.	*Jati*	Lamiaceae	Tree	Branch	Firewood	0.18
Vitex pinnata L.	*Laban*	Lamiaceae	Tree	Wood, branch	Firewood	0.03

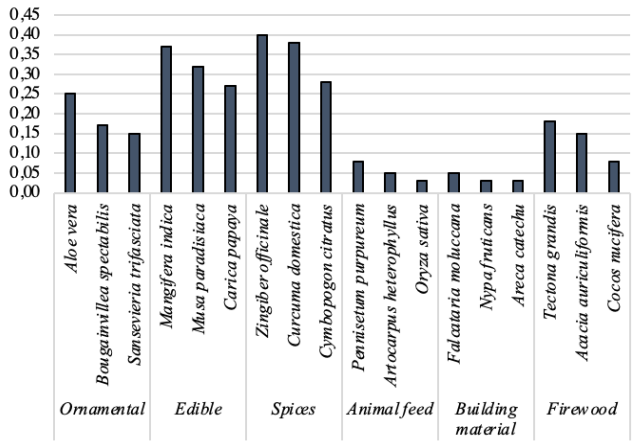

Figure 8. Top five use values for each use of plants by the people of Ayah Village, Kebumen, Central Java, Indonesia

Use Value (UV)

The villagers of Ayah village use the plants as food, ornamental, cooking spices, animal feed, firewood, household furnishings, building materials, and hedges (Figure 8). The *M. indica*, with a UV of 0.37, is the most common plant used for food. *A. vera*, with a UV of 0.25, is commonly planted as an ornamental plant. The *Z. officinale*, with a UV of 0.4, is the most valuable plant for cooking spices. Finally, with a UV of 0.08, *P. purpureum* has the highest value as an animal feed. *M. indica* is native to tropical and subtropical regions, where its fruit can be consumed directly. Mango fruit is edible when ripe or unripe and can be processed into food and beverages. In comparison, *A. vera* is widely cultivated and used as an ornamental plant and food. In addition to their an ornamental plant, *A. vera* has aother application such as a hair cosmetic (García et al. 2019). In addition, the species does not require a large growing area and is commonly grown in containers.

Studies have shown that many plants serve multiple purposes; one is the coconut (*C. nucifera*), in which every part of the species can be used, from the roots and stems to the leaves, flowers, fruit, and beyond. The coconut is called the "tree of a thousand benefits" (Kappally et al. 2015).

In karst regions, particularly the tropics, *Z. officinale* is widely used as a cooking spice to stimulate appetite (Rahayu et al. 2021). In addition, this species can be used to remove fishy odors from processed fish, chicken, and meat and as an ingredient in hot beverages. Moreover, villagers in Ayah use *P. purpureum* (elephant grass) as animal feed due to the plant's excellent adaptation to the dry season, particularly in karst regions. Elephant grass is characterized by its easy and rapid growth and high nutrient content (De Conto et al. 2016). In addition, the species can be utilized as animal feed for grazing. The *P. purpureum* is also the most widely utilized plant species in the Pacitan karst region of East Java for livestock forage (Cahyaningsih et al. 2022).

In conclusion, the people of Ayah Village in the Kebumen District of Central Java, Indonesia use plants as food, spices, ornamentals, animal fodder, firewood, furniture, building materials, and even hedges, according to the study's results. Villagers cultivate the plants in their homegardens and the surrounding area to serve their needs. The leaves and fruit are the most commonly consumed parts, explaining why people prefer cultivating them. In addition, trees and herbs of various sizes are planted and

used extensively throughout the neighborhood. Community knowledge regarding the use of non-medicinal plants is also well maintained across all age groups, and there is a relatively high diversity of plants used for various uses.

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Vegetation composition, diversity, stand structure, and carbon storage of Lolkisale Village Land Forest Reserve in the Northeastern part of Tanzania

EZEKIEL EDWARD MWAKALUKWA[1,♥], ANDREW MWAKISU[2], SAMI MADUNDO[3], SALIM MOHAMMED SALIM MALIONDO[1]

[1]Department of Ecosystems and Conservation, College of Forestry, Wildlife and Tourism, Sokoine University of Agriculture. P.O Box 3010 Chuo Kikuu, Morogoro, Tanzania. Tel.: +255-23-2604944, ♥email: ezedwa@sua.ac.tz
[2]Private consultant. P.O. Box 215, Babati, Manyara, Tanzania
[3]Department of Forest Engineering and Wood Sciences, College of Forestry, Wildlife and Tourism, Sokoine University of Agriculture. P.O Box 3009, Morogoro, Tanzania

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Abstract. *Mwakalukwa EE, Mwakisu A, Madundo S, Maliondo SMS. 2023. Vegetation composition, diversity, stand structure, and carbon storage of Lolkisale Village Land Forest Reserve in the Northeastern part of Tanzania. Nusantara Bioscience 15: 79-90.* Little is known about the effects of human activities on the condition of the Lolkisale Village land Forest reserve located in Monduli District, Arusha region, in the northeastern part of Tanzania. This study assessed the status of woody species diversity, composition, structure, and available potential of the forest on carbon storage. The vegetation data were collected from 33 concentric sample plots of 5 m, 15 m, and 20 m radius laid systematically across the forest area of 960 ha. A total of 58 plant species belonging to 30 families were identified. Diversity indices have indicated a high woody species diversity in the forest reserve. The most important species were *Commiphora schimperi* (O.Berg) Engl., *Dombeya rotundifolia* (Hochst.) Planch., *Acacia tortilis* (Forssk.) Hayne, and *Combretum molle* R.Br. ex G. Don. Stand structure comprises 190 ± 117 stems ha^{-1}, basal area of 7.68 ± 5.17m^2ha^{-1} and standing volume of 64.04 ± 45.85 m^3ha^{-1}, while the mean above-ground carbon stocks and the mean below-ground carbon stocks were 19.55 ± 13.38 Mg C ha^{-1} and 3.91 ± 2.68 Mg C ha^{-1} respectively. Generally, the reserve was found to be in good condition. The observed high diversity of woody species signifies the importance of legally protecting this area as a village land forest reserve. In addition, quantifying other carbon pools, such as soil, dead wood, and surface litter, should be considered for estimating this forest's total carbon stock potential. In this regard, measures to control the use of the forest as a grazing area would be useful to allow new regrowth and young trees to attain maturity stages without being interfered with by the livestock.

Keywords: Community forest, dry evergreen forest, game controlled area, human activities, montane forest, REDD+

INTRODUCTION

The effects of deforestation and forest degradation on the quality and condition of forest resources have been well studied (Foley et al. 2007; Kideghesho 2015; Newmark and McNeally 2018; Doggart et al. 2020; FAO 2020; FAO and UNEP 2020; Gizachew et al. 2020; Wade et al. 2020; Shapiro et al. 2021; Wolf et al. 2021; Fritz et al. 2022; Mammides et al. 2022). Deforestation reduces the ability of the forest to offer its various ecosystems services, including carbon sequestration, amelioration of climate for rainfall formation, conservation of watershed services, soil fertility, biodiversity, and habitats for other living organisms (Lele 2009; Betts et al. 2017; Karki et al. 2017; Houghton and Nassikas 2018; Popkin 2019; Qin et al. 2021; Njora and Yilmaz 2022). For example, FAO (2020) estimated that between 1990 and 2020, around 420 million ha of forest has been deforested worldwide and converted to other land uses. More specifically, about 10 million ha of forest were lost annually between 2015-2020. According to TEEB (2010), conserving forests could avoid greenhouse gas emissions worth US$ 3.7 trillion globally. This is the amount the world will save by avoiding deforestation.

According to FAO (2020), the rate of forest loss is greater in Africa than anywhere else in the world. For example, from 2010 to 2020, the African continent experienced a net forest loss of 3.9 million ha annually, compared to 2.6 in South America, 0.1 in North and Central America, and 0.0 in Asia (FAO 2020; Wolf et al. 2021). Burning and clearing land for agriculture are the most important causes of forest loss hence carbon emissions (Doggart et al. 2020; FAO 2020). Currently, Tanzania is losing 469,420 ha of forest area annually (URT 2017). Therefore, using a deforestation rate of 372,816 ha (MNRT 2015), and considering on other provisioning services apart from those which are usually reflected in the Gross Domestic Product (GDP) accounting, including non-timber forest products, regulating services such as water, and supporting services such as biodiversity, the present value of net economic losses from deforestation to the Tanzanian economy in 2013-2033 is estimated to be TSh 5,588 billion (US$ 3.5 billion) (UNEP 2015).

Different strategies have been suggested and practiced globally to help reduce or curb the effects of deforestation, such as establishing new protected areas, especially in areas found to be rich in biodiversity (Watson et al. 2014; Bebber

and Butt 2017; Miller and Nakamura 2018; CBD 2020; Wade et al. 2020; Wolf et al. 2021; Daba et al. 2022). Recently, Tanzania has set strategies to bring about 16 million hectares of forests found in village land areas that have constantly been facing serious threats of deforestation (i.e., Doggart et al. 2020) to effective protection by 2031 as one way of reducing the effects of deforestation. (URT 2021; MNRT 2022a). In this case, Participatory Forest Management (PFM) through Community Based Forest Management (CBFM) approach has been implemented to assist villagers in conserving forests found within their reach. According to MNRT (2022b), the total area of declared and gazetted CBFM forests is estimated to be 1,917,224 ha out of the total area of 2,202,335 ha under CBFM in Tanzania mainland. Some villages are still under different stages of declaration or gazettement of their village forest area.

The Lolkisale Village Land Forest Reserve (LVLFR) is believed to harbor distinct diversity of micro-habitats. It is rich in flora and fauna, like any other isolated mountain with forest on top of hills found in northern Tanzania's Monduli District, Arusha region. LVLFR is known to preserve some water sources for the nearby villages and act as a corridor for animals migrating from nearby national parks. However, the forest has not been declared nor gazetted due to a lack of data on forest conditions for preparing management plans (URT 2002). LVLFR is also among the forest reserves that face various levels of human interference (Sitati et al. 2014; Sitati et al. 2016; Mwakalukwa et al. 2023a; Mwaluseke et al. 2023a). This uncontrolled use of forest resources could cause a massive biodiversity loss. However, the biodiversity assessment has not been conducted. This study, therefore, specifically aims to; (i) assess the status of woody species diversity, composition, and structure in the LVLFR, (ii) assess the effects of anthropogenic activities in the condition of the LVLFR, and (iii) assess the potential of the LVLFR in carbon storage.

MATERIALS AND METHODS

Study area

The Lolkisale Village Land Forest Reserve (LVLFR) is located within Lolkisale village in Lolkisale ward, Monduli District, about 50 km off the road from Arusha town along the road leading to Babati or Lake Manyara National Park, Tanzania (Figure 1). The Lolkisale village is bordered by seven other villages: Meserani, Nalalan, Lobosoi, Tukusi, Mbuyuni, Makuyuni, and Naitoliaa. Land uses in Lolkisale village include livestock keeping, farming, forest reserve, settlement, infrastructures, e.g., roads, and social services such as dams, schools, etc. The village government owns LVLFR, although the reserve is not yet gazetted. LVLFR covers about 960 hectares. Elevation ranges from 1,491 - 2,097 masl (mean 1,724 ± 32). The district where the LVLFR belongs is generally semi-arid with average rainfall between 400 and 900 mm per annum while the average temperature ranges from 11.5°C (July) to 29°C (December). The slope ranges from 14-75% (mean 39.1 ± 2.8%). The vegetation is described as dry evergreen montane forests.

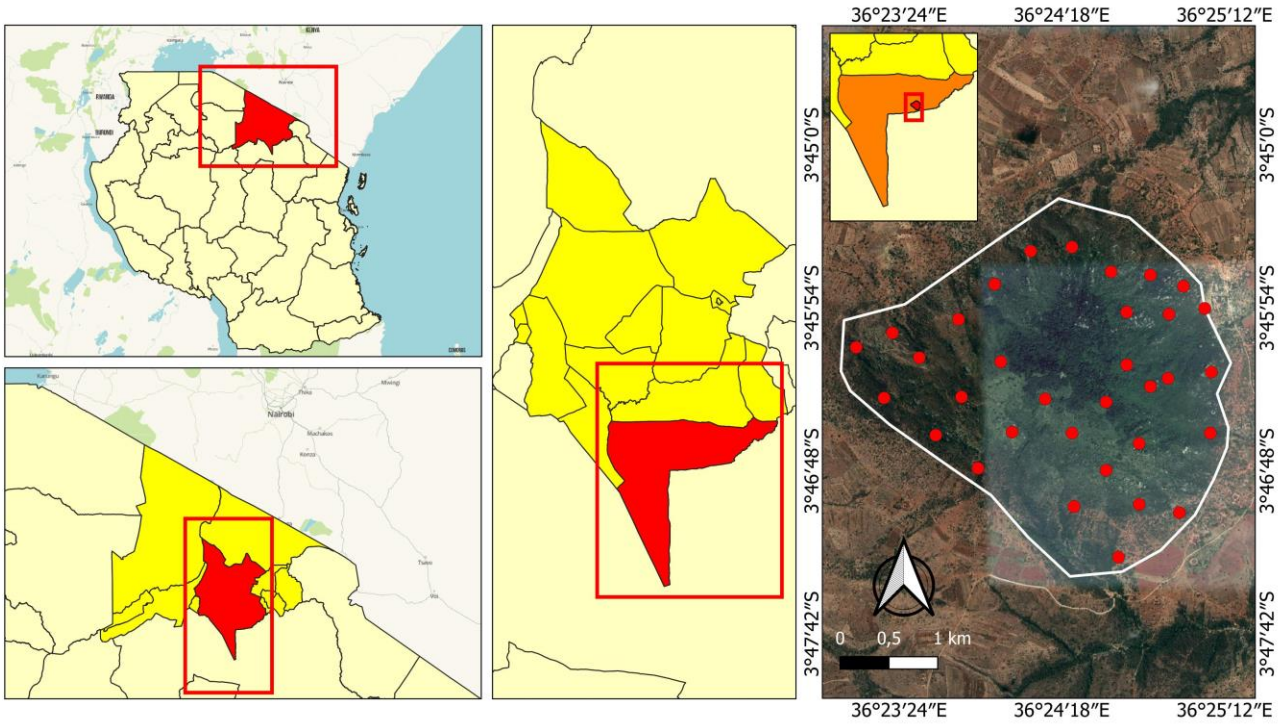

Figure 1. The map of Monduli District, Tanzania, showing the location of Lolkisale Village Land Forest Reserve (LVLFR) and sample plots layout in the reserve

Data collection

The field survey was conducted in August and September 2014 and involved systematically establishing 33 concentric sample plots of 5 m, 15 m, and 20 m radius across the LVLFR of 960 ha. The following parameters were recorded within each of the 33 plots: within the 5m radius, all small trees and shrubs with DBH < 1 cm were counted, and their species were identified, and medium-size trees and shrubs (≥ 1cm DBH but < 5cm DBH) were identified and measured concerning diameter. The species were identified within a 15 m radius, and the diameter was measured for all large trees and shrubs with DBH ≥ 5cm. Stumps of trees and shrubs were measured for Basal Diameter (BD) at 10 cm above ground within a 20 m radius plot. The Diameter at Breast Height (DBH) was measured 1.3 m above ground using diameter tape or caliper. In addition, three stems with small, medium, and large DBH in a plot were selected and measured for heights using a Suunto hypsometer. Altitude was recorded at the plot center using GPS, and the slope was measured from the plot's center facing the slope's direction using a Suunto clinometer.

Data analysis

The collected data were analyzed for species richness, the number of stems/ha, basal area/ha, volume/ha, and biomass/ha. Total species richness was computed as the total number of species across all 33 plots. Species diversity was computed using the Shannon-Wiener diversity (H′) index and Simpson's diversity index (D), whereas the Importance Value Index (IVI) was determined as the sum of relative density and dominance (basal area) and expressed in percent (Kent 2012; Mwakalukwa et al. 2014). Forest structure was expressed through stem density, basal area, and volume for species against diameter classes. Data on Diameter at Breast Height (DBH) and Height (Ht) were used to estimate volume and biomass using the developed equations and hence estimate the forest's stand volume, above-ground, and below-ground carbon stocks potential. The models developed by Mwaluseke et al. (2023b) for dry evergreen montane forests were used to estimate the volume and biomass content of the forest; after that converted to carbon content per ha of the forest. Below-ground biomass of each species was estimated as 20% of the total above-ground biomass:

$$\text{Height (m)} = 2.3249 + 6.6101/\text{DBH} + 0.2847\text{DBH} \ (R^2 = 0.78, \text{RMSE} = 1.79, \text{AIC} = 164.37).$$

$$\ln(\text{Volume, m}^3) = -9.845 + 1.915\ln(\text{DBH}) + 1.089\ln(\text{Ht}) \ (R^2 = 0.97, \text{RMSE} = 0.296, \text{AIC} = -144.18).$$

$$\ln(\text{Biomass, kg}) = -1.666 + 0.853\ln(\text{WD} \times \text{DBH}^2 \times \text{Ht}) \ (R^2 = 0.95, \text{RMSE} = 0.324, \text{AIC} = 224.13).$$

Where:

DBH : Diameter at Breast Height (cm)
Ht : total tree height (m)
WD : basic Wood Density (g/cm^3)
RMSE : Root Mean Square Error
AIC : Akaike's Information Criterion
R^2 : coefficient of determination

Wood basic density values for each species were extracted from various sources (Bryce 1967; Goldsmith and Carter 1981; Drichi 1992; Brown 1997; Suzuki 1999; Ishengoma et al. 2000; Hamza et al. 2001; Mbwambo et al. 2006; Mwaluseke et al. 2023b). Carbon stock was estimated by multiplying with a conversion factor of 0.49 (Manyanda et al. 2020) and presented per hectare (Mg Cha^{-1}). All data analyses were performed using Excel spreadsheet and R (version 4.2.0).

RESULTS AND DISCUSSION

Species richness

The results for species richness of all size categories (small individuals of DBH< 5 cm and large individuals of DBH ≥ 5 cm) that were identified in the LVLFR are found in Table 1. A total of 58 species (30 plant families) of trees and shrubs were identified. Trees contributed 84% (26 plant families), and shrubs 16% (8 plant families) of the species. Generally, tree and shrub species from the family Mimosoideae contributed the most (17%) to the total number of species, followed by those from the families Anacardiaceae (10%) and Rutaceae (9%). For trees alone, the greatest number of species was found in Mimosoideae family (18%), followed by Rutaceae family (10%) and Anacardiaceae (8%), whereas for shrub species alone were from Anacardiaceae family (22%).

Therefore, a total of 49 species (28 families) were found among large sizes (DBH ≥ 5cm), with Mimosoideae (18%), Anacardiaceae (10%), Rutaceae (8%), and Burseraceae (6%) being the most species-rich plant families. While among small sizes (DBH < 5 cm), a total of 26 species (17 families) were observed, with Mimosoideae (16%), Rutaceae (16%), Anacardiaceae (16%), Oleaceae (16%), and Papilionoidea (16%) contributing most of the species (Table 1). Generally, the average number of species per plot was 4 species (range 1 - 8 per plot). The species accumulation curve indicates the rate of encountering new species (Figure 2). Species initially increased rapidly to the 20th plot and slowly up to the 33rd plot. However, since only 33 plots were sampled, the later result implies that any further increase in sample size might have included additional new species. Nevertheless, the sample size was sufficient to provide the baseline information necessary for understanding the composition and diversity of the species in LVLFR.

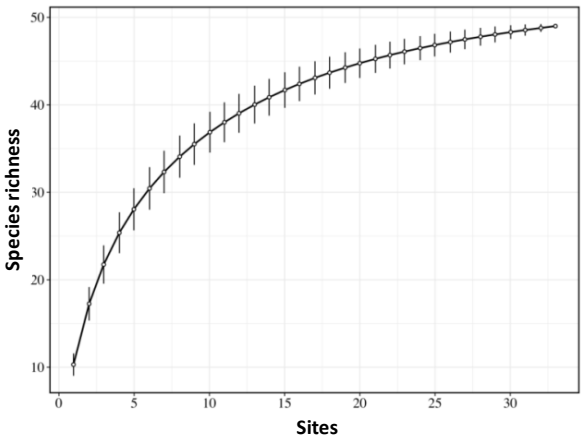

Figure 2. Species accumulation curve of tree species in Lolkisale Village Land Forest Reserve (LVLFR), Tanzania

The species richness of 58 different trees and shrubs and 30 plant families reported in this study using 33 sample plots of 0.071 ha is lower when compared to other studies from other tropical forests. For instance: Sitati et al. (2014) found a total of 75 tree and shrub species from 100 plots of 0.02 ha established in a dry evergreen forest of Gelai Forest Reserve in Tanzania; Mwaluseke et al. (2023a) found a total of 79 tree and shrub species from 56 concentric sample plots of 0.071 ha established in a dry evergreen forest of Lendikinya Forest Reserve in Tanzania; Kayombo et al. (2022) found a total of 84 tree species from 60 plots of 20 m × 20 m established in a dry evergreen forest of Monduli Mountain Forest Reserve in Tanzania; Boz and Maryo (2020) from Ethiopia reported a total of 76 woody species representing 40 families in a dry semi-evergreen Afromontane forest from 64 sample plots (0.04 ha); Masresha and Melkamu (2022) reported 13 values of different species richness ranging from 62-122 tree species from dry evergreen Afromontane forest patches in Ethiopia and Erenso et al. (2014) found a total of 95 species from a dry evergreen forest in Ethiopia.

However, compared to other studies, the species richness of 58 was relatively higher despite the smaller sample size used in this study (33 plots). For instance: Sitati et al. (2016) found a total of 43 tree and shrub species from 77 plots of 0.071 ha established in a dry evergreen forest of Ketumbeine Forest Reserve in Tanzania; Mwakalukwa et al. (2023b) found a total of 54 tree and shrub species from 23 plots of 0.071 ha established in a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania; Masresha and Melkamu (2022) in Ethiopia reported 13 different values of species richness ranging from 34-57 tree species; Mialla (2002) reported species richness of 42 trees and shrubs from 48 sample plots of 0.071 ha; Dugilo (2009) reported species richness of 42 species from 28 sample plots of 0.071 ha; Feroz et al. (2016) reported 40 species (in 0.16 ha) in tropical wet evergreen forest in Bangladesh; and Kacholi et al. (2015) reported six different values of species richness ranging between 17 - 52 from seven individual tropical wet forests of Uluguru forests in Tanzania.

The species richness in this study falls within the range of species commonly found in miombo woodland of 40 - 229 species (Mwakalukwa et al. 2014; Jew et al. 2016). That shows LVLFR has a relatively large number of forest plant species, stressing the significance of its conservation. The higher values found in other studies could be attributed to greater sampling effort (total area, number of sample plots, and sizes) employed by other studies compared to this study.

Species diversity

The results for species diversity in the LVLFR according to Shannon-Wiener diversity indices for large (DBH ≥ 5 cm) and small (DBH < 5 cm) individuals were 3.41 and 2.78, respectively. Species of large individuals (DBH ≥ 5 cm) that were observed to have the greatest contributions were: *Commiphora schimperi* (O.Berg) Engl. (0.24), *Dombeya rotundifolia* (Hochst.) Planch. (0.18), *Acacia tortilis* (Forssk.) Hayne (0.17), *Dracaena usambarensis* Engl. (0.17), *Teclea nobilis* Del. (0.17), and *Combretum molle* R.Br. ex G.Don (0.16). While for smaller ones (DBH < 5 cm) were: *Albizia petersiana* (Bolle) Oliv. (0.32), *C. molle* (0.29), *D. rotundifolia* (0.22), *Grewia bicolor* Juss. (0.20), *Vepris simplicifolia* (Verd.) Mziray (0.18), *Cassipourea malosana* (Baker) Alston (0.13), and *T. nobilis* (0.13). According to the Simpson index, species diversity for large individuals was 0.04, and that of small individuals was 0.09. The index of dominance (1-D) for large individuals was 0.96, and for smaller individuals was 0.91, while the index for evenness or equitability (J) for large individuals was 0.88 and for smaller individuals was 0.85.

In terms of frequency of occurrence for standing individuals (DBH ≥ 5 cm) in the LVLFR, *C. schimperi* was the most frequent species (42% of plots), followed by *D. rotundifolia* (36%), *C. molle* (30%) and *Acacia nilotica* (L.) Willd. ex Delile (21%). In comparison, for small sizes (DBH < 5cm), *C. molle* (21%), *D. rotundifolia* (15%), and *A. petersiana* (12%) were the most frequent species (Table 1). The Importance Value Index (IVI) for large individuals (DBH ≥ 5 cm) shows that *C. schimperi* (30.77), *D. rotundifolia* (23.86), *A. tortilis* (14.49), and *C. molle* (13.72) were the most important species among standing individuals (Table 1).

The values of the Shannon-Wiener index (H'= 3.41) for trees and shrubs in the present study are lower than those reported by other researchers. For example, Mwaluseke et al. (2023a) reported an H' value of 3.46 from a dry evergreen forest of Lendikinya Forest Reserve in Tanzania; Kacholi et al. (2015) found an overall H' value of 4.03 from the Uluguru forests in Tanzania, and Tynsong et al. (2022) reported an H' values ranging from 3.74 - 3.95 (mean 3.85 ± 0.06) from the tropical evergreen forests in India. However, H' values in this study are much higher than those documented by other researchers. For example, Masresha and Melkamu (2022) reported 18 different H' values ranging between 1.31- 3.35 from dry evergreen Afromontane forest patches in Ethiopia; Dugilo (2009) reported H' value of 1.30 from Tanzania; Kayombo et al. (2022) reported an H' value of >1.5 from Tanzania; Erenso et al. (2014) reported H' value of 1.79 from Ethiopia; Sitati et al. (2016) from a dry evergreen forest of Ketumbeine Forest Reserve in Tanzania (H' value of 2.36); Mwakalukwa et al. (2023b) from a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania who reported an H' value of 2.70; Sitati et al. (2014) reported an H' value of 2.85 from a dry evergreen forest of Gelai Forest Reserve in Tanzania; and Boz and Maryo (2020) from Ethiopia reported an average H' value of 3.38. However, the H' value of 3.41 in this study falls in the *H'* value commonly found in miombo woodland, where values range from 1.05 - 4.27 (Shirima et al. 2011; Mwakalukwa et al. 2014; Jew et al. 2016). According to Magurran (2004) and Mwakalukwa et al. (2014), the H' values normally vary between 1.5 and 4.5 and rarely exceed 5. A threshold of 2 is the minimum value, above which an ecosystem can be regarded as medium to highly diverse. Therefore, the value of 3.41 found in this study implies that the LVLFR has high diversity in tree and shrub species. High diversity might be attributed to relatively low levels of disturbance experienced in the forest, as very few stumps were observed during the survey.

Table 1. Checklist of tree and shrub species identified in the Lolkisale Village Land Forest Reserve (LVLFR), Tanzania, showing frequency (%); density (mean ± SE); basal area (mean ± SE); Wood basic density; Importance Value Index (IVI); Stand volume (mean ± SE); Above-ground Carbon (mean ± SE); and Below-ground Carbon (mean ± SE) for trees and shrubs with a minimum DBH 1 cm (plot size = 15 m radius)

Species/botanical name	Family	Habit / Life form	Wood basic density (kg/m^3)	Frequency (%)	Density (stems/ha)	Basal area (m^2/ha)	IVI	Stand volume (m^3/ha)	AGC (Mg/ha)	BGC (Mg/ha)
Commiphora schimperi (O.Berg) Engl.	Burseraceae	Tree	640	42	21±6	0.88±0.26	30.77	5.93±1.85	2.07±0.63	0.41±0.13
Dombeya rotundifolia (Hochst.) Planch.	Sterculiaceae	Tree	640	36	13±4	0.17±0.06	23.86	0.82±0.28	0.35±0.12	0.07±0.02
Acacia tortilis (Forssk.) Hayne	Mimosoideae	Tree	640	15	12±6	0.67±0.34	14.49	5.18±2.62	1.67±0.84	0.33±0.17
Combretum molle R.Br ex G. Don	Combretaceae	Tree	758	30	10±3	0.32±0.14	13.72	2.06±0.94	0.85±0.37	0.17±0.07
Lannea schimperi (Hochst.ex A. Rich.) Engl.	Anacardiaceae	Tree	640	9	2±1	0.09±0.05	8.07	0.61±0.35	0.21±0.12	0.04±0.02
Acacia nilotica (L.) Willd. ex Delile	Mimosoideae	Tree	797	21	8±4	0.11±0.06	7.68	0.55±0.27	0.28±0.14	0.06±0.03
Albizia gummifera (J.F.Gmel.) C.A.Sm.	Mimosoideae	Tree	640	9	8±4	0.87±0.51	6.40	9.12±5.78	2.52±1.55	0.50±0.31
Commiphora mossambicensis (Oliv.) Engl.	Burseraceae	Tree	370	9	9±6	0.18±0.12	6.38	0.94±0.61	0.24±0.16	0.05±0.03
Teclea nobilis Del.	Rutaceae	Tree	849	18	12±7	0.32±0.27	6.34	2.06±1.83	0.94±0.81	0.19±0.16
Albizia petersiana (Bolle) Oliv.	Mimosoideae	Tree	640	12	6±3	0.09±0.07	5.32	0.49±0.40	0.20±0.15	0.04±0.03
Nuxia congesta R.Br.ex Fresen.	Loganiaceae	Tree	640	9	7±5	1.35±1.07	5.08	15.59±12.52	4.12±3.28	0.82±0.66
Lannea triphylla (Hochst.ex A.Rich.) Engl.	Anacardiaceae	Tree	450	6	3±3	0.23±0.20	4.48	1.81±1.58	0.43±0.37	0.09±0.07
Calodendum capense (L.f.) Thunb.	Rutaceae	Tree	583	9	5±3	0.15±0.09	3.85	0.92±0.51	0.31±0.18	0.06±0.04
Cassipourea malosana (Baker) Alston	Rhizophoraceae	Tree	785	12	7±4	0.28±0.16	3.79	2.41±1.67	0.88±0.54	0.18±0.11
Olea europaea L.	Oleaceae	Tree	1169	3	1±1	0.10±0.10	3.69	0.84±0.84	0.06±0.06	0.01±0.01
Commiphora africana (A.Rich.) Engl.	Burseraceae	Tree	276	18	3±1	0.09±0.04	3.61	0.55±0.23	0.10±0.04	0.02±0.01
Acacia thomasii Harms	Mimosoideae	Tree	503	3	1±1	0.07±0.07	3.56	0.49±0.49	0.13±0.13	0.03±0.03
Acacia mellifera (Vahl) Bosc	Mimosoideae	Tree	947	9	5±3	0.09±0.06	3.51	0.53±0.39	0.29±0.20	0.06±0.04
Boscia angustifolia Harv.	Capparidaceae	Tree	640	6	3±3	0.09±0.07	3.11	0.72±0.59	0.23±0.18	0.05±0.04
Steganotaenia araliacea Hochst.	Araliaceae	Tree	370	9	3±2	0.01±0.01	2.89	0.04±0.03	0.01±0.01	0.00±0.00
Acacia hockii De Wild.	Mimosoideae	Tree	720	12	3±2	0.05±0.03	2.88	0.28±0.17	0.12±0.07	0.02±0.01
Erythrina abyssinica Lam. ex DC.	Papilionoidea	Tree	426	3	1±1	0.35±0.35	2.82	4.87±4.87	0.83±0.83	0.17±0.17
Cassipourea gummiflua Tul.	Rhizophoraceae	Tree	720	3	2±2	0.02±0.02	2.74	0.11±0.11	0.06±0.06	0.01±0.01
Cordia monoica Roxb.	Boraginaceae	Tree	830	9	4±3	0.03±0.02	2.55	0.17±0.13	0.09±0.06	0.02±0.01
Rhus natalensis Bernh.ex Krauss	Anacardiaceae	Shrub	606	3	2±2	0.01±0.01	2.48	0.02±0.02	0.01±0.01	0.00±0.00
Ormocarpum kirkii S.Moore	Papilionoidea	Tree	742	6	1±1	0.01±0.00	2.17	0.03±0.02	0.02±0.01	0.00±0.00
Obetia radula (Baker) Baker ex B.D.Jacks.	Urticaceae	Tree	640	6	2±1	0.08±0.07	2.09	0.74±0.69	0.22±0.20	0.04±0.04
Dracaena usambarensis Engl.	Agavaceae	Tree	640	3	12±12	0.19±0.19	2.01	0.95±0.95	0.39±0.39	0.08±0.08
Acacia gerrardii Benth.	Mimosoideae	Tree	816	6	1±1	0.04±0.03	1.92	0.21±0.18	0.10±0.08	0.02±0.02
Turraea robusta Gürke	Meliaceae	Tree	640	6	1±1	0.05±0.05	1.80	0.38±0.37	0.13±0.12	0.03±0.02
Lannea humilis (Oliv.) Engl.	Anacardiaceae	Tree	640	3	1±1	0.03±0.03	1.76	0.15±0.15	0.06±0.06	0.01±0.01
Drypetes gerrardii Hutch.	Ephorbiaceae	Tree	703	3	2±2	0.03±0.03	1.45	0.14±0.14	0.06±0.06	0.01±0.01
Terminalia brownii Fresen.	Combretaceae	Tree	640	6	1±1	0.06±0.06	1.45	0.54±0.51	0.16±0.15	0.03±0.03
Adenium obesum (Forssk.) Roem. & Schult.	Apocynaceae	Tree	881	3	1±1	0.04±0.04	1.40	0.29±0.29	0.13±0.13	0.03±0.03
Grewia bicolor Juss.	Tiliaceae	Shrub	670	9	2±1	0.01±0.00	1.32	0.02±0.01	0.01±0.01	0.00±0.00
Azanza garckeana (F.Hoffm.) Exell & Hillc.	Malvaceae	Tree	640	6	1±1	0.03±0.03	1.02	0.19±0.17	0.07±0.06	0.01±0.01

Fagaropsis angolensis (Engl.) H.M.Gardner	Rutaceae	Tree	689	3	0±0	0.05±0.05	1.00	0.42±0.42	0.14±0.14	0.03±0.03
Ozoroa insignis Delile	Anacardiaceae	Tree	529	3	1±1	0.02±0.02	0.87	0.08±0.08	0.03±0.03	0.01±0.01
Maesa lanceolata Forssk.	Myricinaceae	Tree	676	3	4±4	0.08±0.08	0.76	0.39±0.39	0.17±0.17	0.03±0.03
Rytigynia sp.	Rubiaceae	Shrub	689	3	3±3	0.14±0.14	0.69	1.03±1.03	0.36±0.36	0.07±0.07
Vepris simplicifolia (Verd.) Mziray	Rutaceae	Tree	800	3	3±3	0.03±0.03	0.67	0.15±0.15	0.08±0.08	0.02±0.02
Balanites aegyptiaca (L.) Delile	Balanitaceae	Tree	630	3	0±0	0.03±0.03	0.64	0.17±0.17	0.06±0.06	0.01±0.01
Pappea capensis Sond.	Sapindaceae	Tree	640	3	0±0	0.01±0.01	0.62	0.08±0.08	0.03±0.03	0.01±0.01
Ximenia americana L.	Olacaceae	Shrub	640	3	1±1	0.00±0.00	0.57	0.01±0.01	0.01±0.01	0.00±0.00
Ziziphus mucronata Willd.	Rhamnaceae	Tree	640	3	0±0	0.02±0.02	0.55	0.17±0.17	0.06±0.06	0.01±0.01
Ekebergia capensis Sparrm.	Meliaceae	Tree	609	3	0±0	0.06±0.06	0.41	0.55±0.55	0.16±0.16	0.03±0.03
Ochna sp.	Ochnaceae	Tree	640	3	1±1	0.02±0.02	0.37	0.12±0.12	0.05±0.05	0.01±0.01
Albizia amara (Roxb.)Boivin	Mimosoideae	Tree	677	3	0±0	0.01±0.01	0.32	0.04±0.04	0.02±0.02	0.00±0.00
Mytenus senegalensis (Lam.) loes.	Celasteraceae	Tree	685	3	0±0	0.01±0.01	0.09	0.08±0.08	0.03±0.03	0.01±0.01
Acacia brevispica Harms	Mimosoideae	Shrub	640	+						
Cadaba farinosa Forssk.	Capparidaceae	Shrub	640	+						
Claucena anisate (Willd.) Hook.f. ex Benth.	Rutaceae	Tree	704	+						
Croton scheffleri Pax	Euphorbiaceae	Tree	721	+						
Harissonia abyssinica Oliv.	Simaroubaceae	Shrub	640	+						
Lonchocarpus eliocalyx Harms	Papilionoidea	Tree	758	+						
Rhus vulgaris Meikle	Anacardiaceae	Shrub	760	+						
Schrebera alata (Hochst.) Welw.	Oleaceae	Shrub	607	+						
Schrebera trichoclada Welw.	Oleaceae	Tree	801	+						
Total (all species)				**415**	**190 ± 117**	**7.68 ± 5.17**	**200**	**64.04 ± 45.85**	**19.55 ± 13.38**	**3.91 ± 2.68**

Notes: + indicates species identified among smaller individuals within 5 m radius plots (DBH<5cm). Mg/ha = Megagram per hectare

Stand density

The total mean stem density for large individuals with DBH ≥ 5 cm in the LVLFR was 190 ± 117 stems ha^{-1} (Table 1, Figure 3), and that of small individuals with DBH < 5 cm (including individuals with DBH < 1 cm) was 486 ± 346 stems ha^{-1}. Among large individuals, the most abundant species were *C. schimperi* (10.8% of 190 stems ha^{-1}), *D. rotundifolia* (6.8%), *A. tortilis* (6.1%), *T. nobilis* (6.1%), and *D. usambarensis* (6.1%). Among small individuals, the most abundant species were *C. molle* (16.7% of 486 stems ha^{-1}), followed by *G. bicolor* (11.9%), *A. petersiana* (11.1%), *D. rotundifolia* (9.5%), and *A. tortilis* (9.5%). Generally, the distribution of trees to size classes showed the usual reverse J shape (Figure 3).

The stem density of 190 ± 117 stems ha^{-1} for the woody species with DBH ≥ 5 cm reported in this study is lower than that documented by Mwakalukwa et al. (2023b) from a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania, who reported a mean density of 288 ± 173 stems ha^{-1}; Dugilo (2009), from dry evergreen forest of Selela village forest reserve in Tanzania, reported a mean density of 310 stems ha^{-1}; Sitati et al. (2014), from a dry evergreen forest of Gelai Forest Reserve in Tanzania, reported a mean density of 377 stems ha^{-1}; Sitati et al. (2016) from a dry evergreen forest of Ketumbeine Forest Reserve in Tanzania reported a mean density of 435 stems ha^{-1}; and Gebeyetu et al. (2019) from five forests in Ethiopia reported a range of 365.6 - 664.1 stems ha^{-1} with a mean of 636.5 stems ha^{-1}; Kacholi et al. (2015), from seven tropical wet forests in the Uluguru forests in Tanzania, reported an overall mean density of 390 stems ha^{-1}.

The stem density of 190 ± 117 stems ha^{-1} is ten times lower than those reported by Mialla (2002) from Monduli Forest Reserve, a dry evergreen mountain forest in Tanzania, reported a mean density of 1,822 stems ha^{-1}. Mwaluseke et al. (2023a) from a dry evergreen forest of Lendikinya Forest Reserve in Tanzania reported a mean density of 1,398 ± 679 stems ha^{-1}; Atomsa and Dibbisa (2019) reported a mean density of 1,453 stems ha^{-1} from Ethiopia; Boz and Maryo (2020) reported the total density of 1,745.3 stems ha^{-1} from Ethiopia. Whereas Tynsong et al. (2022) reported a mean density of 2,005 ± 48.01 trees ha^{-1} with a range from 1,944 to 2,100 trees ha^{-1} in the tropical evergreen forests of North-East India. Furthermore, the mean stems density values of 190 ± 117 stems ha^{-1} from this study are also lower than the density value range found in miombo woodland of 281-1,521 stems ha^{-1} (Shirima et al. 2011; Mwakalukwa et al. 2014). That implies LVLFR is among the lowest-stocked dry evergreen montane forests in Tanzania and other tropical countries. The higher density reported in other studies might be attributed to microclimate influence, which creates favorable conditions for the growth of more species. The presence of wildlife animals such as Elephants could have affected the density of species in the LVLFR. The density distribution indicated a dominance of small trees depicting the normal reversed "J" shape, which indicates strong regeneration status and recruitment of the forest, a tendency normally observed in the natural mixed species of different ages (Figure 3).

Basal area

The mean basal areas for large individuals (≥ 5 cm DBH) and small individuals (<5 cm DBH) were 7.68 ± 5.17 m^{2}ha^{-1} and 0.16 ± 0.12 m^{2}ha^{-1}, respectively (Table 1, Figure 4). The species contributing most to the basal area of large individuals were *Nuxia congesta* R.Br.ex Fresen. (17.6%), *C. schimperi* (11.5%), *Albizia gummifera* (J.F.Gmel.) C.A.Sm. (11.3%), and *A. tortilis* (8.7%). In comparison, those contributing most to the basal area of smaller individuals were *A. petersiana* (24.0%), *D. rotundifolia* (11.8%), *T. nobilis* (11.0%), *G. bicolor* (10.7%), and *C. molle* (10.0%).

The mean basal area of 7.68 ± 5.17 m^{2}ha^{-1} determined in this study is much lower than that documented in other mountain forests, which normally range between 20 - 60 m^{2}ha^{-1} (Burke 2005; Sitati et al. 2016). For instance, Mwaluseke et al. (2023a) from Tanzania reported a mean basal area of 11.42 ± 5.41 m^{2}ha^{-1}; Mwakalukwa et al. (2023b) from Tanzania reported a mean basal area of 11.47±7.23 m^{2}ha^{-1}; Sitati et al. (2014) reported a mean basal area of 26.87 m^{2}ha^{-1}from Tanzania; Sitati et al. (2016) from Tanzania reported a mean basal area of 30.49 ± 2.3; Mialla (2002) reported a mean basal area of 69.3 ± 1.6 m^{2}ha^{-1}from Tanzania; Kacholi et al. (2015) from Uluguru mountain forests reported a mean basal area of 24 m^{2}ha^{-1}; and Tynsong et al. (2022) reported a range from 52.26 to 68.05 m^{2}ha^{-1} (mean 61.72 ± 4.82 m^{2}ha^{-1}) in the tropical evergreen forests in India. The basal area determined in this study is ten times lower than the mean basal area of 114.64 m^{2}ha^{-1} reported by Erenso et al. (2014) from Ethiopia, and a mean basal area of 126.47 m^{2}ha^{-1} from lowland dry semi-evergreen forest in Ethiopia (Boz and Maryo 2020). Siraj and Zhang (2018) recorded a total basal area of 454.52 m^2·ha^{-1} from a dry Afromontane forest in Ethiopia.

The mean basal area found in this study is within the range of values commonly found in other forests, including miombo woodland of 3.9 - 16.7 m^{2}ha^{-1} (Backéus et al. 2006; Dugilo 2009; Mwakalukwa et al. 2014). Therefore, the low basal area obtained in this study could be due to the low stem density observed in reserve. On the other hand, the higher basal area observed in other studies could be associated with the high stem density of individuals in the higher DBH classes compared to other forests.

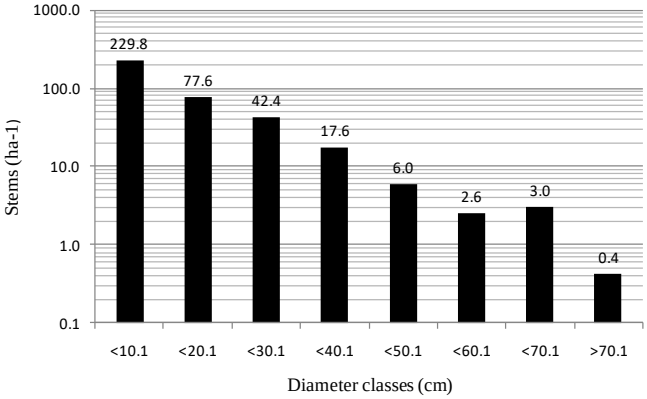

Figure 3. The density of trees ≥ 1 cm DBH by diameter class in the Lolkisale Village Land Forest Reserve, Tanzania (n = 33). NB: logarithmic scale on the vertical axis

Stand volume

The mean standing volume ha^{-1} for individuals with a diameter (≥ 5 cm DBH) was 64.04 ± 45.85 m^3ha^{-1} (Table 1, Figure 5). The species contributing most to the standing volume of large individuals were *N. congesta* (24.3% = 15.59 ± 12.52 m^3ha^{-1}), *A. gummifera* (14.2%), *C. schimperi* (9.3%), *A. tortilis* (8.1%), and *Erythrina abyssinica* Lam. ex DC. (7.6%). Their distribution in terms of diameter classes is presented in Figure 5 below. Generally, the distribution of standing trees to size classes showed that trees with a diameter of 20.1 - 70.1 cm contributed higher to the mean total standing volume in the forest.

The mean standing volume of 64.04 ± 45.85 m^3ha^{-1} reported in this study for trees and shrubs with DBH ≥ 5 cm was considered lower than 395.07 ± 14 m^3ha^{-1} reported by Sitati et al. (2016) from a dry evergreen forest of Ketumbeine Forest Reserve in Tanzania. However, the mean standing volume of 64.04 ± 45.85 m^3ha^{-1} reported in this study is much higher than those reported by Mwaluseke et al. (2023a) from a dry evergreen forest of Lendikinya Forest Reserve in Tanzania with a value of 54.47 ± 24.1 m^3ha^{-1}; Mwakalukwa et al. (2023b), who reported a value of 27.3 ± 16.3 m^3ha^{-1} from a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania; and Dugilo (2009) who reported a value of 40.03 ± 11.21 m^3ha^{-1} from Selela village forest reserve in Tanzania. The volume reported in this study is within the range of 16.7 to 155.9 m^3ha^{-1}, commonly reported in other forests, including miombo woodland (Mwakalukwa et al. 2014; Masota et al. 2016). The relatively higher volume reported by this study might be caused by the few large-sized trees and shrubs in the forest, contributing higher to the total volume.

Harvested stems

The mean stems ha^{-1} for stumps in Lolkisale VLFR was found to be 3 ± 3 stems ha^{-1}. The most harvested tree species were *A. tortilis* (0.86 ± 0.86 stems ha^{-1}), *Cordia monoica* Roxb. (0.43 ± 0.30 stems ha^{-1}), *Commiphora africana* (A.Rich.) Engl. (0.43 ± 0.43 stems ha^{-1}), *C. schimperi* (0.43 ± 0.30 stems ha^{-1}), *A. nilotica* (0.22 ± 0.22 stems ha^{-1}), *Commiphora mossambicensis* (Oliv.) Engl. (0.22 ± 0.22 stems ha^{-1}), and *Lannea schimperi* (Hochst.ex A. Rich.) Engl. (0.22 ± 0.22 stems ha^{-1}). Regarding of basal area of the harvested stems, the mean basal area ha^{-1} was 0.06 ± 0.05 m^2ha^{-1}. Harvested tree species with the highest basal areas were *C. schimperi* (0.02 ± 0.01 m^2ha^{-1}), *C. africana* (0.01 ± 0.01 m^2ha^{-1}), *A. nilotica* (0.01 ± 0.01 m^2ha^{-1}), and *L. schimperi* (0.01 ± 0.01 m^2ha^{-1}). Their distribution per diameter class falls within one diameter class of 1.0-10.0.

The mean stems ha^{-1} for stumps of 3 ± 3 stems ha^{-1} is lower than that reported by Mwaluseke et al. (2023a) from a dry evergreen forest of Lendikinya Forest Reserve in Tanzania, who reported a value of 63 ± 37 stems ha^{-1} with *Diospyros abyssinica* subsp. *abyssinica* being the most contributing species (12.7% of the total), followed by *Teclea simplicifolia* (11.1%), while *Drypetes natalensis* (Harv.) Hutch. and *D. rotundifolia* contributed 9.5% each. According to Mwaluseke et al. (2023a), stumps distribution showed the expected reversed "J" shape with higher stem density in DBH class ≤ 10 cm, but no stumps with DBH > 50 cm was found. In the basal area, the mean basal area ha^{-1} for stumps of 0.06 ± 0.05 m^2ha^{-1} found in LVLFR was also lower than that reported by Mwaluseke et al. (2023a), who reported a value of 1.12 ± 0.63 m^2ha^{-1}. This is true because no large stumps were observed in the LVLFR. That means trees harvested were within a diameter size class (≤10 cm), unlike those reported by Mwaluseke et al. (2023a), which were within a diameter size class (≤ 10 to 50 cm), implying that larger size trees were overexploited in Lendikinya Forest Reserve.

Biomass and carbon storage

The mean above-ground biomass and carbon stocks potential of Lolkisale VLFR for tree individuals with a diameter ≥ 5 cm were 39.90 ± 27.30 Mg ha^{-1} and 19.55 ± 13.38 Mg C ha^{-1}, respectively. At the same time, the mean below-ground biomass and carbon stocks potential of the forest reserve for tree individuals with a diameter ≥ 5cm were 7.98 ± 5.46 Mg ha^{-1} and 3.91 ± 2.68 Mg C ha^{-1}, respectively (Table 1, Figure 6). Tree species that made a high contribution to the observed above-ground carbon density were *N. congesta* (21.1% = 4.12 ± 3.28 Mg C ha^{-1}), *A. gummifera* (12.9%), *C. schimperi* (10.6%), *A. tortilis* (8.5%) and *T. nobilis* (4.8%). On the other hand, species that made a high contribution to the observed below-ground carbon density were *N. congesta* (21.1% = 0.82 ± 0.66 Mg C ha^{-1}), *A. gummifera* (12.9%), *C. schimperi* (10.6%), *A. tortilis* (8.5%), and *T. nobilis* (4.8%).

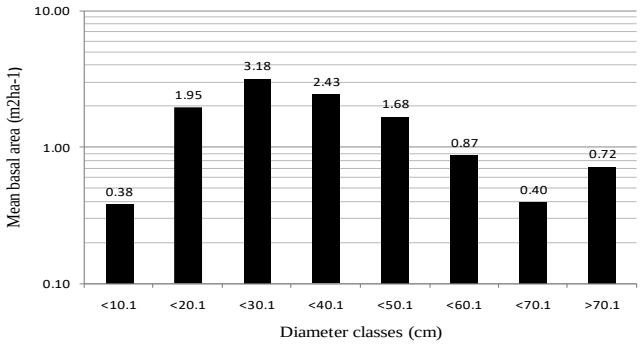

Figure 4. Distribution of basal area per hectare for trees ≥ 1 cm DBH by diameter classes in the Lolkisale VLFR, Tanzania (n = 33). NB: logarithmic scale on the vertical axis

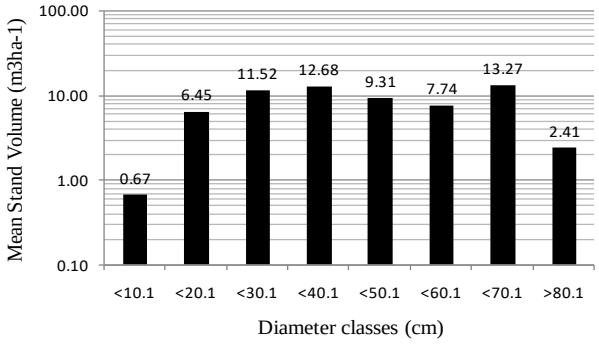

Figure 5. Distribution of mean volume per hectare for trees ≥ 5 cm DBH by diameter classes in the Lolkisale VLFR, Tanzania (n = 33). NB: logarithmic scale on the vertical axis

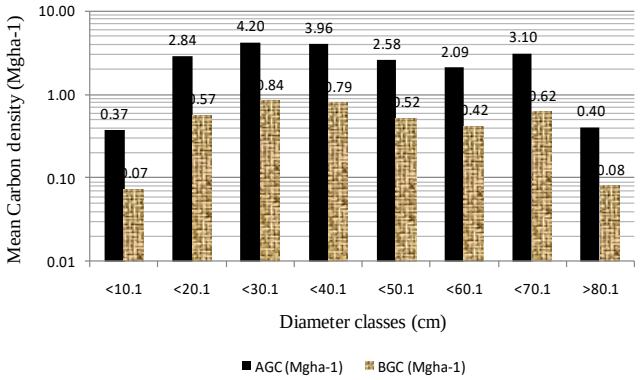

Figure 6. Distribution of both above-ground and below-ground mean carbon density of tree species with diameter ≥ 5 cm by diameter classes in the Lolkisale VLFR, Tanzania (n = 33). NB: logarithmic scale on the vertical axis

The mean above-ground carbon stocks of the trees and shrubs with DBH ≥ 5 cm of 19.55 ± 13.38 Mg C ha^{-1} determined in this study is lower than that documented from other tropical forests. For instance, Swai et al. (2014) reported a mean carbon stock of 48.4 ± 8.0 t C ha^{-1} from the Hanang mountain forest in Tanzania; Mwakalukwa et al. (2023b) reported a mean carbon stock of 56.93 ± 34.60 Mg C ha^{-1} from a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania; Asrat et al. (2022) reported two values of 180.18 ± 17.19 t·C ha^{-1} and 106.71 ± 7.64 t·C ha^{-1} from dry evergreen Afromontane forests in Ethiopia; Gebeyehu et al. (2019) reported a mean value of 191.6 ± 19.7 Mg C ha^{-1} from five different dry Afromontane forests in Ethiopia; Wondimu et al. (2021) reported a value of 332.69 ± 37.42 t C ha^{-1} from a dry evergreen Afromontane forest in Ethiopia; Rawal and Subedi (2022) reported two values of mean carbon stock of 51.86 t C ha^{-1} and 59.55 t C ha^{-1}from two community forests in Nepal; and Naveenkumar et al. (2017) from a tropical dry forest in India reported a range of 99 to 216 t C ha^{-1}. In contrast, the mean above-ground carbon stocks found in this study are higher than that reported by Mwaluseke et al. (2023a), from a dry evergreen forest in Tanzania reported a value of 16.04 ± 7.7 t C ha^{-1}; and Biadgligne et al. (2022) who reported two values of 14.84 ± 1.27 t C ha^{-1} and 3.49 ± 0.66 t C ha^{-1} from two community forests from Ethiopia. With regards to the below-ground carbon density, the value reported in this study of 3.91 ± 2.68 Mg C ha^{-1} is much lower than that reported by Mwakalukwa et al. (2023b) from a dry evergreen montane forest of Essimingor Nature Forest Reserve in Tanzania who reported a value of 34.71 ± 19.72 Mg C ha^{-1}.

The low value reported in this study could be due to many small and few large trees, which contributed less to the total mean carbon density than the presence of many large trees reported in other studies. On the other hand, the low value of below-ground carbon density could be due to a lack of allometric models for site-specific and dry Evergreen Mountain forests in Tanzania (Mwaluseke et al. (2023b). We used a ratio of 20% of above-ground carbon density to represent the below-ground components; this could not have been the best approach than using the site-specific allometric models.

In conclusion, the LVLFR has high species diversity (H'= 3.41) and is relatively rich in diversity of woody species (58 species) compared to many of the dry evergreen montane forests of Tanzania and other tropical forests. The mean stand volume is relatively higher, although tree density and basal area are lower than in other tropical forests. The above-ground and below-ground carbon stocks are also lower than those reported in other studies from dry areas. The reported data on carbon stock provides baseline data for the possibility of future payment schemes for REDD+ project implementation in Tanzania.

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Response of various growth regulators and shade intensity to the growth of arabica coffee seedlings of Sigarar Utang variety

REVANDY ISKANDAR MUDA DAMANIK♥, CHAIRANI HANUM, LOKO JEREMIA SEMBIRING
Department of Agrotechnology, Faculty of Agriculture, Universitas Sumatra Utara. Jl. Dr. A. Sofian No.3, Padang Bulan, Medan 20155, North Sumatra, Indonesia. Tel.: +62-61-8213236, ♥email: revandy.iskandar@usu.ac.id

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Abstract. *Damanik RIM, Hanum C, Sembiring LJ. 2023. Response of various growth regulators and shade intensity to the growth of arabica coffee seedlings of Sigarar Utang variety. Nusantara Bioscience 15: 85-89.* The productivity of Arabica coffee (*Coffea arabica* L.) of the Sigarar Utang variety in Indonesia is still relatively low, especially in Simalungun District, North Sumatra Province, Indonesia. Growth regulators and shade are some factors that must be considered in nurseries. This research aims to determine the best growth regulators type and shade intensity for Arabika coffee seedling growth. The experiment was conducted from March to June 2022 in Nagori Ujung Bawang, Simalungun District, and the Central Laboratory of Agriculture Faculty, Universitas Sumatera Utara, Medan, North Sumatra. The study used a Split Plot design of 2 factors and 3 replications. The first factor (main plot) consists of 4 levels, namely 0, 50, 70, and 90% shade intensity. The second factor (sub-plot) consists of the three growth regulator treatments, i.e., auxin, cytokinin, and the combination of auxin and cytokinin. The results showed that the interaction between shade intensity and growth regulators had a significantly different effect on increasing root length. The shade treatment significantly affected plant height, leaf area, and total chlorophyll content of leaves. However, it did not affect the stem diameter. A shade intensity of 90% significantly increased plant height, leaf area, and total chlorophyll content of leaves.

Keywords: Auxin, coffee, cytokinin, light intensity

INTRODUCTION

Coffee is one of the plantation crops to produce coffee. Coffee is a popular and favorite beverage for many people and has relatively high economic value. Coffee can be processed into a delicious productive drink with a distinctive aroma as a refresher for the body and mind. For Indonesia, Coffee is a commodity that contributes significant foreign exchange after palm oil, rubber, and timber in the non-oil and export of gas (FAO 2003).

Coffee is one of the most important economic commodities in the province of North Sumatra, Indonesia (Sitompul et al. 2018). Simalungun District is the second number largest Arabica coffee-producing area in North Sumatra, Indonesia according to statistical data from the Directorate General of Plantations (2018), with production reaching 9,744 t/year in a total area of 7,843 ha with an average annual production of only 1,443 kg/ha of fresh fruit or around approximately 481 kg grain/ha. Compared with coffee-producing countries such as China and Vietnam, the productivity of coffee fields in Indonesia is only a quarter of the productivity of these countries, which are capable of producing more than 2.2 t/ha of grain annually (FAO 2014). The plantation area and productivity of arabica coffee in Indonesia still need to be improved due to the market potential and a high selling price. Some farmers still use seeds from the natural seedling because have no knowledge to factors affecting to the seed quality, so coffee productivity is not optimal (Harahap et al. 2015).

Coffee is a plant that requires shading throughout its life cycle. The degree of shading varies according to the growth phase of the coffee. The shade level in the seeding phase or young age is higher than in the generative phase. Improper shade levels in the seedling phase could produce low-quality coffee seedlings (Arief et al. 2011).

Coffee breeding is a comprehensive activity in preparing seeds for distribution to be planted in the field. Although the seeds used come from superior seeds, if the seeding is not following the correct operational standards, it will produce low-quality seedlings ready for distribution (Anita et al. 2016). One of the critical things in coffee nurseries, besides watering, fertilizing, and controlling pests/diseases, is adjusting the light intensity as needed. A previous study by Muliasari et al. (2016) showed that the optimum shade intensity for the growth of arabica coffee seedlings of the Catimor variety is 66%. Meanwhile, Artina et al. (2021) recommended 80% shade as the best shade intensity used in Gayo variety arabica coffee varieties in Gayo coffee nurseries.

Another factor that influences the growth of coffee seedlings is growth regulators. Growth regulators are non-nutrient organic compounds that can support, prevent and change plant physiological processes in small amounts. Auxins support cell extension, and gibberellin stimulates cell division, elongation or both. Cytokinin supports cell division, ethylene plays a role in fruit ripening process, and the mechanisms of quality generation by abscisic acid. Plant growth regulators are likened to coordinators in growth and development processes (Asra et al. 2020).

A study by Hidayati and Subroto (2018) showed that 2 ppm of auxin and cytokinin at a concentration significantly affects the number of leaves and roots, root length, and growth rate of seedlings from hypocotyl responses during development. The highest number of leaves is obtained from cytokinin, while the root length and the highest number of roots are obtained from auxin. Besides that, the highest growth rate was obtained from a combination of auxin and cytokinin. Cytokinin and auxin synergize for plant tissue differentiation (Febriyanti 2016). Therefore, this research aims to determine the best type of growth regulators and shade intensity to improve the growth and quality of Arabica coffee seedlings of the Sigarar Utang variety.

MATERIALS AND METHODS

Sampling site

This research was conducted from March to June 2022 in Nagori Ujung Bawang, Simalungun District and the Central Laboratory Agriculture Faculty, Universitas Sumatera Utara, Medan, North Sumatra, Indonesia.

Materials and equipment

The materials used in this research were coffee seedlings of Sigarar Utang variety, growth regulators (auxin and cytokinin), net (50%, 70%, 90%), soil planting medium with a ratio of 1:1:1 (top soil andosol soil, sand, and compost), rice husks, polybags of size 20 x 20 cm, bamboo, rope, water, chemical fertilizer. The equipment used in this research were stationery, paper, bars, cameras, buckets, drills, lux meters, micrometers, calipers, sprayers, machetes, knives, hoes, scales, spectrophotometers UV/VIS, Whatman 40 filter paper, and other tools that support research.

Design experiment

This research was conducted experimentally using a split-plot design consisting of 12 treatments, and each treatment had 3 replications, so there were 36 experimental units. Each unit consisted of 5 seedlings, and there were 180 seedlings. Observations were made for all seedlings. The shade intensity uses paranet as the main plot, i.e., N0: no shade, N1: 50% shade, N2: 70% shade, and N3: 90% shade. Growth regulators were used as a sub-plot, i.e., Z1: 2 ppm auxin, Z2: 2 ppm cytokinin, and Z3: 2 ppm auxin + 2 ppm cytokinin.

Data analysis

Data were analyzed by Analysis of Variance, followed by Multiple Range Test (MRT) at a level of 5%.

RESULTS AND DISCUSSION

Plant height

The results showed no interaction between growth regulators and shade intensity to the height of coffee seedlings. The application of various growth regulators has no significant effect on increasing the growth of coffee seedlings. In contrast, shade intensity significantly increased the growth of coffee seedlings at 8 Weeks After Planting (WAP). The average height of coffee seedlings with the treatment of various growth regulators and shade intensity was presented in Table 1.

Leaf area

The results of growth regulators and shade intensity treatments on the leaf area of coffee seedlings (Table 2), showed no interaction between the treatment of various growth regulators and shade intensity. The application of different growth regulators has no significant effect on increasing the leaf area of coffee seedlings. In contrast, shade intensity significantly affects the leaf area of coffee seedlings.

Table 1. Plant height of coffee seedlings at 2-8 WAP with the treatment of growth regulator and shade intensity

Time	Growth regulators	Shade				Average
		N_0 (0%)	N_1 (50%)	N_2 (70%)	N_3 (90%)	
				(cm)		
2 WAP	Z_1 (auxin)	14.80	14.33	14.17	14.27	14.39
	Z_2 (cytokinin)	14.67	13.93	14.27	14.13	14.25
	Z_3 (auxin + cytokinin)	14.20	14.07	14.13	14.27	14.17
	Average	14.56	14.11	14.19	14.22	14.27
4 WAP	Z_1 (auxin)	15.40	14.77	15.40	16.03	15.40
	Z_2 (cytokinin)	14.80	14.10	15.73	16.23	15.22
	Z_3 (auxin + cytokinin)	14.67	14.90	15.23	15.63	15.11
	Average	14.96	14.59	15.46	15.97	15.24
6 WAP	Z_1 (auxin)	16.17	17.13	17.43	18.50	17.31
	Z_2 (cytokinin)	15.80	16.03	18.13	19.10	17.27
	Z_3 (auxin + cytokinin)	15.50	18.60	16.87	18.13	17.28
	Average	15.82	17.26	17.48	18.58	17.28
8 WAP	Z_1 (auxin)	17.40	21.57	21.40	22.83	20.80
	Z_2 (cytokinin)	18.30	19.57	22.73	22.80	20.85
	Z_3 (auxin + cytokinin)	17.30	21.93	22.03	23.17	21.11
	Average	17.67d	21.02c	22.06b	22.93a	20,92

Note: The number followed by the same letter indicates no significant difference on Multiple Range Test of 5%

Root length

The treatment of various growth regulators and shade intensity on root length showed a significant difference between the treatment of different growth regulators and the intensity of shade to the root length of coffee seedlings. The average root length of coffee seedlings with the treatment of various growth regulators and shade intensity can be seen in Table 2.

Stem diameter

The results showed no interaction between the growth regulators type and shade intensity on the stem diameter of the coffee seedlings. The application of the growth regulators type had no significant effect on increasing the diameter of the stem of coffee seedlings, and the shade intensity treatment also had no significantly effect on the stem diameter of the coffee seedlings. The average stem diameter of coffee seedlings treated with treatment of various growth regulators and shade intensity was presented in Table 2.

Total chlorophyll content of leaves

The results showed no interaction between the treatment of growth regulators and shade intensity to the total chlorophyll content of coffee seedling leaves. Different growth regulators did not significantly affect the total chlorophyll content of coffee seedling leaves. At the same time, shade intensity significantly increased the total chlorophyll content of coffee seedling leaves. The average total chlorophyll content of coffee leaves with the treatment of various growth regulators and shade intensity can be seen in Table 2.

Discussion

The results showed that applying 90% shade increased the highest height of coffee seedlings up to 22.93 cm at 8 WAP. Meanwhile, the lowest growth in coffee seedlings was found in the treatment of no shading, which was 17.67 cm. There was a slow increase in plant height at 2 WAP - 6 WAP, where the shade intensity did not significantly affect plant height. It is due to the process of transferring plant seedlings on different planting media so that they require acclimatization (Sudrajat and Siagian 2014). At the end of the study (8 WAP), shading intensity significantly affect plant height. The treatment of 90% shading intensity resulted in the best plant height, and it shows that 10% sun intensity can produce the best plant height of 22.93 cm. Artina et al. (2021) showed that 80% shade intensity improved the size of coffee seedlings than the no-shade treatment. The higher height of seedlings on the shade treatment was due to the etiolation of the plant seedlings (Alridiwirsah et al. 2015). A previous study by Arisandi (2015) showed that coffee at the beginning of the growth phase required higher shading intensity than the adult or generative growth phase.

Leaves have an essential role in photosynthesis. The results showed that 90% shading intensity results in the highest leaf area of coffee seedlings (24.85 cm) at 8 WAP, and no-shading treatment results in the lowest leaf area (16.35 cm). It shows that the low light intensity in coffee seedlings causes a higher leaf area than coffee seedlings with full light intensity. A study by Ferita et al. (2009) also showed that seedlings planted at low light intensity had wider and longer leaves than seedlings planted at 100% light intensity (no shade). Bote and Struik (2011) also stated that coffee plants grown under shade with lower light intensity had a higher photosynthetic rate and had relatively higher leaf area and growth rate than higher light intensity.

Table 2. Growth characteristics of coffee seedlings at 8 WAP with the treatment of growth regulator and shade intensity

Growth regulators	Shade				Average
	N_0 (0%)	N_1 (50%)	N_2 (70%)	N_3 (90%)	
Leaf area			(cm^2)		
Z_1 (auxin)	12.93	22.13	22.23	21.92	19.80
Z_2 (cytokinin)	19.17	17.82	28.40	24.34	22.43
Z_3 (auxin + cytokinin)	16.95	25.60	22.60	28.30	23.36
Average	16.35c	21.85b	24.41a	24.85a	21.87
Root length			(cm)		
Z_1 (auxin)	18.13ab	17.13c	16.03d	17.80abc	17.28
Z_2 (cytokinin)	18.30ab	17.63bc	15.37e	15.33e	16.66
Z_3 (auxin + cytokinin)	17.80abc	17.63bc	16.37d	18.40a	17.55
Average	18.08	17.47	15.92	17.18	17.16
Stem diameter			(mm)		
Z_1 (auxin)	2.59	2.81	2.70	2.64	2.68
Z_2 (cytokinin)	2.75	2.78	2.78	2.72	2.76
Z_3 (auxin + cytokinin)	2.68	2.83	2.77	2.72	2.75
Average	2.67	2.81	2.75	2.69	2.73
Total chlorophyll content			(mg/g)		
Z_1 (auxin)	13.97	13.91	14.50	16.25	14.66
Z_2 (cytokinin)	13.71	13.72	13.50	17.75	14.67
Z_3 (auxin + cytokinin)	13.88	14.73	15.13	13.84	14.39
Average	13.85c	14.12bc	14.38b	15.95a	14.57

Note: The number followed by the same letter indicates no significant difference on Multiple Range Test of 5%

Based on Table 2, shows that the highest growth rate of coffee seedling root (18.40 cm) was obtained from the treatment combination of 90% shading intensity and the combination of auxin and cytokinin. It was followed by no shade + cytokinin treatment of 18.30 cm, no shade + auxin treatment of 18.13 cm and the lowest as obtained in 90% shade + cytokinin treatment of 15.33 cm. It shows that the root length of coffee seedlings is related to plant height. The highest plant height (Table 1) is obtained with 90% shading intensity and the combination of auxin and cytokinin. Ferita et al. (2009) stated that well-shoot growth results in well-root growth. Good root growth, would affect the balance of the seedlings. The excellent growth of the upper part influences the number of photosynthesis results translocated to all parts of the plant, including the roots. A relationship exists in plant tissues between root formation, endogenous auxin, and cytokinin in plant tissues. Low light intensity stimulates endogenous growth regulators to be more effective in root growth and development (Widiastoety 2014).

Roots are essential in the metabolism of the plant to form new organs. The formation of new organs is influenced by the interaction between endogenous growth regulators (natural) and exogenous growth regulators (synthetic) in plants. The combination effect of auxin and cytokinin on root growth showed that it causes cell division and stimulates the formation and development of roots in arabica coffee seedlings of the Sigarar Utang variety. Mahadi et al. (2015) stated that the combination of auxin and cytokinin causes the concentration of endogenous growth regulators in cells to increase because these two growth regulators are factors that trigger the process of growth and development of tissue. Auxin and cytokinin act synergistically. Cytokinin enhances cell numbers and cell division through mitosis, while auxin plays a role in cell elongation (Hidayati and Subroto 2018).

The treatment of the growth regulators type and various shade intensities did not affect the stem diameter (Table 2). Growth regulators, light intensity, and other factors influence the growth of coffee seedling stems. A previous Suherman and Kurniawan (2015) study showed that sufficient nutrients and good soil media enhance plant stems. Due to the age of coffee seedlings, the application of growth regulators did not affect the stem diameter. In line with Sudrajat and Siagian (2014), seeds require acclimatization in transferring to planting media.

The 90% shade intensity treatment resulted in the highest chlorophyll content in coffee seedling leaves (15.95 mg/g) at 8 WAP. No-shade treatment yielded the lowest total chlorophyll content (13.85 mg/g). The leaf chlorophyll results were similar to the leaf area (Table 2), significantly affected by low light intensity. It shows that the low light intensity in coffee seedlings (90% shade treatment) significantly increased chlorophyll content compared to the coffee seedlings with full light intensity (no shade). Chlorophyll serves as a light catcher that plants need for the process of photosynthesis to take place. Sholikhah et al. (2015) stated that the high chlorophyll content indicates high photosynthesis. Bote and Struik (2011) also reported that coffee plants under shade had higher photosynthesis than those under no shade. The results are similar to the results of Soleh et al. (2021), that the treatment of 80% shade intensity resulted in the highest leaf chlorophyll index of coffee seedlings at 12 weeks after seeding (46.73 mg/g) and the lowest was in no shade (35.97 mg/g).

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Identification of metallothionein protein in *Anodonta woodiana* as a biomarker of mercury (Hg) contamination

SATA YOSHIDA SRIE RAHAYU[1,♥], AZAHRA FADILA[1], MELTA RINI FAHMI[2,3]

[1]Program of Biology, Faculty of Mathematics and Natural Sciences, Universitas Pakuan. Jl. Pakuan No. 1, Ciheuleut, Bogor 16143, West Java, Indonesia. Tel.: +62-251-8375547, ♥email: sata_rahayu@unpak.ac.id
[2]Innovation Centre for Tropical Sciences. Jl. Komp. Bogor Raya Permai No. 24, Bogor 16113, West Java, Indonesia
[3]Research Institute for Ornamental Fish Culture. Jl. Perikanan No. 13, Pancoran Mas, Depok 16364, West Java, Indonesia

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Abstract. *Rahayu SYS, Fadila A, Fahmi MR. 2023. Identification of metallothionein protein in* Anodonta woodiana *as a biomarker of mercury (Hg) contamination. Nusantara Bioscience 15: 90-94.* Heavy metal contamination can affect the survival of aquatic biota and will accumulate in the bodies of organisms. Moreover, contamination identification at the molecular level can be analyzed using biomarker analysis. Biomarkers are responses measured individually, ranging from enzymes and xenobiotic measurements to organ and overall conditions. Biomarker analysis can be done by checking the metallothionein protein, this expression can be induced by Reactive Oxygen Species (ROS). Metallothionein (MT) has a thiol group with nucleophilic properties. As a result, this group can make Metallothionein able to find metals and free radicals. Therefore, prevention that can be done to reduce contamination at a higher trophic level requires monitoring the molecular level by observing the metallothionein protein. For example, *Anodonta woodiana* (Rea, 1834) or *kijing taiwan* induced by $HgCl_2$ aims to characterize their absorption ability in the environment through metallothionein protein. That was conducted by the SDS-PAGE (Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis*)* method. SDS-PAGE electrophoresis results showed that the size of the metallothionein protein was 7 kDa, 15 kDa, and >30 kDa. Size >30 kDa is a group of proteins related to stress control or Heat shock protein (Hsp). The presence of Hsp is due to the body increasing stress protein synthesis and metallothionein to reduce normal protein synthesis. Based on the results obtained, this study revealed that *A. woodiana* could absorb $HgCl_2$, as evidenced by the metallothionein protein characterization results.

Keywords: *Anodonta woodiana*, environment, heavy metals, molecular SDS

INTRODUCTION

Heavy metals can accumulate in aquatic organisms by bioconcentration (pollutants entering directly from the water into aquatic organisms), bioaccumulation (pollutants entering through mechanisms), and biomagnification (increasing pollutant concentrations in tissues) organisms based on the food chain (Adhani and Husaini 2017). Dewi (2017), stated that mercury heavy metals (Hg) effectively bind to sulfhydryl groups (-SH) and can replace metal ions such as Zn, inhibiting organism's growth and development process, heavy metals that accumulate in aquatic organisms will generally be stored in the body, so these heavy metals will continue to be in the food chain and will cause detrimental effects. One of them is *Anodonta woodiana* (Rea, 1834) or *kijing taiwan*.

The *A. woodiana* removing metals based on a concentration gradient from high to low. Therefore, pollution prevention at a higher trophic level also requires treatment at a lower trophic level firstly, this can be done using organisms as biomarkers. Biomarker analysis could be examined Reactive Oxygen Species (ROS), which can attack various substrates in the body. For example, proteins, lipids, and nucleic acids, because this makes it possible to carry out biomarker analysis on mercury (Hg)-induced *A. woodiana.*

The *A. woodiana* species used the biomarkers in this study to view, identify, and study the uptake of heavy metals by analyzing the size of Metallothionein (MT) protein bands. The *A. woodiana* has the property of non-selective filter feeders, which allows it to filter out particles that enter the body from the water. Therefore, *A. woodiana* cannot select particles that enter the body. The *A. woodiana* belongs to the class Bivalvia which can adapt to polluted environments. That's could be caused by several factors, one of which is that heavy metals have high bioavailability, so they quickly enter the body. Some Bivalvia animals have poor metal metabolism or have the excretory capacity, soft metal, resulting in the accumulation of heavy metals in their body. The response given by aquatic organisms is by a physiological detoxification process and a molecular response that can be carried out by identifying metallothionein protein synthesis.

Metallothionein protein is a compound that can bind heavy metals. metallothionein can bind heavy metals in previous research because it contains many cysteines. In addition, metallothionein has a lot of thiol/sulfhydryl (-SH) groups. Therefore, it can bind heavy metals. Metallothionein generally increases with excessive heavy metal exposure and will increase cellular toxicity if the heavy metal entry rated exceed the metallothionein synthesis rate (Khati et al. 2012). Metallothionein protein expression can be induced by Reactive Oxygen Species (ROS), Glucocorticoids (GC), and

cytokines (Kimura et al. 2008). Preventive measures that can be taken to reduce contamination at a higher trophic level require monitoring at the molecular level by observing metallothionein proteins. This study aims to identify the protein metallothionein in *A. woodiana* by investigating the size of the associated protein band as a biomarker in mercury (Hg) contamination to preventing contamination at a higher trophic level. $HgCl_2$ induction can affect the characterization of metallothionein protein in *A. woodiana* so that it can be applied in the ground to reduce pollution by its ability to absorb heavy metals.

MATERIALS AND METHODS

Sample *Anodonta woodiana* collection

The samples of *A. woodiana* were collected using the dredges (rake) method by sweeping the bottom of the sandy and muddy waters. This was carried out at a maximum of 1 day (long day trip) for each capture by seser catching tools, namely sweeping the bottom of the waters in a straight line using active fishing gear (WWF Indonesia). As many as 36 mussels were taken with a random sampling technique. The samples were treated with different concentrations of $HgCl_2$ in 5 repetitions, while the controls were untreated. Finally, the mussel were put in a cool box the laboratory. As many as five mussels were put into each aquarium containing mercury with different concentrations (0.5 ppm, 1 ppm, 1.5 ppm).

Making Hg solution

A stock solution of Hg was made by weighing 0.13 g of Hg powder. Then the powder was dissolved in distilled water with up to 1 Liter Dilutions. This process was carried out for 3 treatments (0.5 ppm, 1 ppm, and 1.5 ppm).

Acclimatization

The collected mussels must acclimate to laboratory conditions to reduce stress on the experimental animals. Therefore before acclimatization, the clams were put into a transitional aquarium containing fresh water to clean the mussel from the mud. After which, they were transferred to the acclimatization aquarium. Acclimatization was carried out for seven days using water from their natural habitat, and this was done to prevent changes in metallothionein protein bands in mussel. The aquarium has a circulation pump, fed with fine pellets daily ad libitum (Prihatini 2013). This pellet has a composition of microalgae and phytoplankton which has the form of small granules. The pellet is given as much as one gram with a count twice daily in the morning and evening.

Metallothionein protein analysis

Anodonta woodiana *induction*

The mussels acclimatized for seven days, they were transferred to the treatment aquarium and treated with $HgCl_2$ at a concentration of 0.5, 1, 1.5 ppm, and the control five liters of water, pure from its habitat. Five mussels were used for each treatment aquarium. On the 30th day after $HgCl_2$ induction, two individuals were collected for the hepatopancreatic tissue on *A. woodiana* and the rest as reserves. This method refers to previous research by Rahayu et al. (2019).

Protein extraction

After induction with $HgCl_2$ (0.5 ppm, 1 ppm, 1.5 ppm), hepatopancreas were taken, mashed using a mortar dish, and diluted with PBS to a pH of 7.4. The mashed samples were stored in a refrigerator at 4°C. Next 1,500 µL was added to a microtube and centrifuged at 300 rpm for 15 minutes at 4°C. The supernatant taken from that centrifugation the protein supernatant. First, the protein concentration was determined by preparing a 1000 µL blank using 800 µL of distilled water and adding 200 µL bio rad reagent. Then using supernatant of *A. woodiana* sample for 1000 µL of sample and taking 2 µL of water and adding 798 µL of distilled water and 200 µL of bio rad reagent, the homogenizing and incubating for 5 minutes. After that, it could be read using a spectrophotometer with a wavelength of 95 mm to produce the protein absorbance. This method refers to Diaman's research (2016).

Metallothionein protein separation

Metallothionein protein analysis was carried out by SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis) electrophoresis method. This method refers to the Laemmli method (1970) in Diaman (2016) and previous research conducted by Rahayu et al. (2019). Therefore to prepare for gel printing, the glass plate, comb, and spacer were cleaned with detergent and 70% alcohol. Once the gel printer is prepared, the separating gel made in the gel printer is inserted and waits for polymerization to occur. Then put the stacking gel above the separating gel, put a comb on it, and waiting for polymerization. The comb is slowly lifted from the top of the stacking gel. After that, it was put into the electrophoresis apparatus, and the running buffer was inserted and heated using electricity from the SDS-PAGE tool for 15 minutes. Buffer gel with Tris-Tricine-SDS pH 8.25 composition and electrophoresis buffer gel using Tris HCL solution at pH 8.9. The sample was inserted into the well by 20 mL, and given an electric current with a voltage of 40 volts (bromophenol blue). After bromophenol blue reaches the bottom of the stacking gel and a voltage of 200 volts is added, the flow is stopped after bromophenol blue reaches the bottom of the separating gel. The gel is removed from the printer slowly, and Commasie Brilliant Blue R-250 is put into a dye solution with 0.1% concentration for 30-60 minutes until the protein bands are stained. Then, a destaining solution is added, which removes the color of the gel that does not contain protein. The destaining solution was changed 3-4 times until the gel was clean. The protein's molecular weight is calculated Rf and plotted on a logarithmic graph of the Rf marker protein whose molecular weight is known.

RESULTS AND DISCUSSION

Results

Metallothionein protein characterization. Before performing metallothionein protein characterization. Mercury analysis was first performed on the hepatopancreas of *A. woodiana*. The first analysis was carried out when the mussels were just taken from the river to determine the original mercury level contained in the mussel bodies. The second analysis was carried out after the mussels were induced by mercury in the aquarium for 30 days. The second analysis was carried out to see how much mercury absorbed in mussel body and whether mussels have great potential as bioindicators in heavy metal absorption (Table 1).

From the second analysis conducted to check mercury levels in *A. woodiana*'s hepatopancreas, it is evident that the mussel can absorb mercury. After that, characterization was carried out through Metallothionein protein to see how much mercury exposure affected the size of metallothionein characterization.

Table 1. Mercury (Hg) Level in Hepatopancreas of *A. woodiana*

Date	Sample	Hg (mg/kg)
June 3, 2022	Hepatopancreas of *A. woodiana*	0.011
July 4, 2022	Hepatopancreas of *A. woodiana*	0.015

Discussion

This research was conducted of prevent pollution to a higher trophic level, because, at lower trophic levels, it has been detected that there is pollution through the biomarker method. In this study, metallothionein induction by Hg was carried out on the *A. woodiana* to observe the absorption of heavy metals that can affect metallothionein protein characterization. metallothionein analysis was conducted on mussels with Hg induced at 1.5 ppm treatment, This was done because none of the samples survived in the treatment of 0.5 ppm and 1 ppm. Several factors could affect this, such as feed deposits turning into ammonia is toxic to animals. If the metallothionein protein does not function normally or the amount of heavy metals exceeds the heavy metal binding capacity, then metallothionein also does not function normally (Prihatini 2013). The difference in tolerance of a species to heavy metal toxicity can be determined by the effect of the species-specific ability to regulate heavy metals and the bioavailability of the metal itself (Ramzy et al. 2021). This can be interpreted as the percentage and speed of the active substance in heavy metal when it reaches the body's systemic circulation; the body of the mussels responds differently. This can result in some mussels surviving and some not surviving because the response of each mussel's body is different in regulating heavy metals. Metals Pb, Cd, and Hg are toxic because they effectively bind with the enzyme system's sulfhydryl group (SH) to forming metalloenzymes and metalloproteins. As a result, the cells' enzyme activity cannot be processed. Prihatini (2013) states that in the group that can survive, mussels can respond to heavy metal content through growth dilution, namely dilution of metal concentrations in the body by increasing volume and fluid for toxic metal effects during growth in mussel. The presence of metallothionein serves to bind essential heavy metals needed by aquatic organisms, but while metallothionein binds too much metal, there will be an increase metallothionein.

This study uses the SDS-PAGE electrophoresis method (Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis) to separate proteins based on the molecular weight in kDa (Kilo dalton) units. The organ used for analysis is the hepatopancreas section. Based on references from previous studies, it is stated that the hepatopancreas absorbs more metals. Therefore, the hepatopancreas, is responsible for detoxification.

The results of SDS-PAGE electrophoresis showed the size of the metallothionein protein in hepatopancreas tissue, with a size of 7 kDa and 15 kDa, and there was a large protein size of >30 kDa (Figure 1). Metallothionein generally has a size of 6-7 kDa (Dewi 2017), and in the results of this study, there were 9 metallothionein that had a value above 6-7 kDa. This indicates more heavy metal uptake than usual because the amount and duration of heavy metal exposure can influence the amount of MT. In the results of SDS-PAGE electrophoresis with a size of >30 kDa, which is considered a group of proteins related to stress control or Heat shock protein (Hsp). The presence of Hsp is due to the body increasing stress protein synthesis and metallothionein reducing normal protein synthesis in response to stress. This result is corroborated by previous research by Butet (2013), which showed protein synthesis of 70 kDa by mercury induction in blood clams (*Anadara granosa* Linnaeus 1758).

Metallothionein has a thiol or sulfhydryl group with nucleophilic properties. The presence of this group makes Metallothionein able to bind metals and free radicals (Lee and Jae 2010). Therefore the metallothionein protein can protect cells from deadly compounds with this function. Thirumoorthy et al. (2007) stated that metallothionein protein consists of MT-1 and MT-2 isoforms and is very sensitive to the presence of heavy metals in animals. Metallothionein protein expression is induced by Reactive Oxygen Species (ROS) or free radicals, glucocorticoids (GC), and several cytokines (Kimura et al. 2008). Metallothionein protein synthesis generally involves the Metal Transcription Factor-1 (MTF-1) protein (Dray et al. 2008) which is a Zn sensor and is located in the cytoplasm (Formigari et al. 2008). It is associated with functioning genes in Zn homeostatic processes. Based on Langmade et al. (2000) state that MTF-1, which is active after the accumulation of Zn, will then translocate into the nucleus, where it will bind to the Metal Response Element (MRE) contained in the promoter of the metallothionein gene, which triggers the expression of this protein. Zn^{2+} ion is an essential component in protein regulation and is included in the control of intercellular homeostasis (Smith et al. 2008). Zinc is included in the essential metals needed by 300 enzymes in biological activity, for example, if a zinc deficiency in cells will inhibit growth (Prasad and Bin 2019).

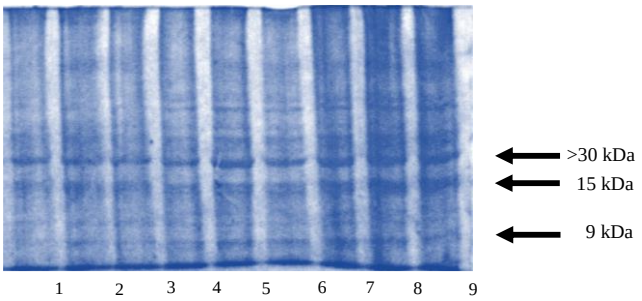

Figure 1. Results of protein characterization by SDS-PAGE electrophoresis

The metallothionein classification is based on four classes based on metallothionein location in the organ and metal type binding (i) MT-1, an metallothionein protein that has a cysteine site and is closely related to the cysteine site in mammals, for example, in mollusks, (ii) MT-2, an metallothionein protein that has nothing in common with mammalian metallothionein, (iii) MT-3, consists of non-protein metallothionein known as phytochelatin.

Metallothionein has the following protein structure: (i) Thionein, a protein with many groups, (ii) Metalation is a chemical reaction because a hydrogen atom is replaced with one of the metals. For example, in Cu, which Hg replaces, the increase occurs in the thiol/sulfhydryl (-SH) group, (iii) Dimerization is the joining of two monomer molecules to form a dimer.

When heavy metal pollution occurs in waters, aquatic organisms will experience three stages of response. The first is the shock recovery stage along with an increase in biosynthetic processes (mitosis and protein synthesis) to repair the damage and restore physiological disorders; the second stage is the damage stage and restores the disturbance. Physiologically, the production of metal-binding proteins such as metallothionein will bind at this stage. Finally, the upregulation of ions occurs to counter the damaging effects of heavy metals. Ultimately, the internal organism physiology will return to its initial state, forming a new balance. This process increases the tolerance of organisms to heavy metals (Soegianto et al. 2018).

The metallothionein method protects cells from lethal compounds, and metals enter the cells due to a passive diffusion process (metals are categorized into cells based on a high concentration gradient to low concentration). The increased concentration of heavy metals in the body stimulates the formation of Reactive Oxygen Species (ROS) free radicals that cause oxidative damage. The body will respond by synthesizing metallothionein protein which acts as an antioxidant. Heavy metals bound by metallothionein protein will be stored in amorphous intracellular granules called "chocolate vesicles" in detoxification. Therefore it will increase the number of tubular epithelial cells, hemocytes, or brown vesicles in the connective tissue. This process involves detoxification and tissue recovery (Prihatini and Mulyati 2013). Detoxification can also be observed from the filtration rate and increased feeding.

The chemical effects of heavy metals can impact the number of heavy metals that accumulate in the body of aquatic organisms. These heavy metals will generally bind to lipids and proteins in living tissue. Proteins that contain metal in their protein structure are the main protein target for heavy metal binding (Rumahlatu et al. 2012). The induction of metallothionein synthesis comes from regulating metallothionein gene expression originating from transcription and translation processes. Metallothionein genes will convey the increasing metals in cells through metal activation of transcription factors to bind certain metals (Soegianto et al. 2018). Heavy metals are known to stimulate the transcription of the metallothionein gene so that its production speed will increase. The kinetic stability of the metallothionein structure is shallow; because of that, metallothionein can bind metal very strongly (Dewi 2017). The presence of metallothionein protein in this study showed that *A. woodiana* was produced as a biological response to an increasing concentration and duration of mercury (Hg) exposure.

In conclusion, this research shows that the metallothionein protein in *A. woodiana* has sizes of 7 kDa, 15 kDa, and > 30 kDa, indicating a biological response to an increasing concentration and duration of mercury (Hg) exposure. Induction of $HgCl_2$ can affect the characterization of metallothionein protein. This research concluded that *A. woodiana* could be used as a bioindicator in an environment. This research also concluded that cleaning pollutants in polluted ecosystems is necessary.

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Diversity of plant species for food coloring in Vietnam

NGUYEN THI LUONG[1], NGUYEN VAN HOP[2,♥], NGUYEN VAN QUY[2], VO MINH HOAN[2]

[1]Faculty of Economics, Vietnam National University of Forestry. Dong Nai Campus, No. 98 Tran Phu, Trang Bom Town, Trang Bom District, Dong Nai 76000, Vietnam

[2]Faculty of Natural Resources and Environment, Vietnam National University of Forestry. Dong Nai Campus, No. 98 Tran Phu, Trang Bom Town, Trang Bom District, Dong Nai 76000, Vietnam. Tel./Fax.: +84-977-672338, ♥email: nvhop@vnuf2.edu.vn

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Abstract. *Luong NT, Hop NV, Quy NV, Hoan VM. 2023. Diversity of plant species for food coloring in Vietnam. Nusantara Bioscience 15: 95-104.* Using natural colors of plants for food processing is an inevitable trend for the safety of consumers' health. It also provides essential vitamins, minerals, and nutrients to the body. This study aimed to systematize indigenous knowledge about food coloring plants of 11 ethnic groups in North, Central, and South Vietnam. Methods of ethnobotanical investigation, field investigation under the instruction of local people, and inheritance of documents combined with data analysis were employed. Therefore, 110 species of vascular plants belonging to 54 families of food coloring were discovered in Vietnam. As a result, 28 species were used with high frequency, and 15 species were identified as conservation values domestically and globally. Besides, the richness of folk knowledge of local people was also recorded. Five plant life forms were identified, i.e., shrubs, wood, vines, herbaceous, and bamboo. Eleven plant parts were used; leaves accounted for the largest proportion (36.36%), followed by fruit, wood, flowers, bark, seeds, tubers, rhizomes, young tops, sap, and roots. Ten different colors were created from plants for food dyeing; red accounted for the highest percentage (28.18%), followed by yellow, green, black, and gray was the lowest (0.91%). Most plants can produce monochromatic colors (94/110 species). Of the species recorded, 39.09% were wild plant species, 40.00% were cultivated, and 20.91% of species could be found in the wild or cultivated. The number of species that gave color to cook rice was dominant (68.18%), followed by cakes, drinks, and soups, soaked in alcohol and sticky. This study shows the diversity of species composition, the abundance of traditional knowledge, and the potential of plants for food coloring in Vietnam. In the future, in-depth studies on the species' nutritional composition, chemistry, vitamins, and extracts should be proposed, contributing to the food and beverage industry and especially maintaining and developing a culinary culture imbued with national identity.

Keywords: Diversity, dye plant, folk knowledge, food coloring, Vietnam

INTRODUCTION

Plant-based colorants are becoming globally significant as a potential source of natural food coloring because of their versatility and for avoiding many synthetic colors' health hazards (Shamina et al. 2007). So, artificial colorants have gradually been replaced by natural pigments, which are becoming increasingly important in Vietnam and other parts of the world due to the potential noxiousness of artificial food dyes to human health (Ung et al. 2018). Natural dyes are less toxic, less polluting, less hazardous to health, non-carcinogenic, and non-toxic. They harmonize in color, are light, soft, and delicate, and create a tranquil effect and product aesthetics (Das and Kalita 2016; Hop et al. 2022). Best of all, they are environmentally friendly and can be recycled afterward (Das and Kalita 2016; Hop et al. 2022). Therefore, using natural colors created from plants that are not harmful to human health is inevitable.

Colorant's applications include papers, printing, plastics, leather, textile, cosmetics, indicators in analytical chemistry, and food (Brudzynska et al. 2021). Especially food coloring plants have high applicability in people's daily life and the food and beverage processing industry. Food coloring plants are one, group, or more species capable of providing natural food coloring from any part of the plant. They are added to food to create or improve its color, increase the product's attractiveness, and provide essential nutrients to consumers. They are extracted from natural sources and artificially synthesized. Natural colorants are extracted or processed from organic materials available in nature. It is safe for human health and easy to create natural colors using manual methods. Many species also add vitamin and mineral content to the body (Hop et al. 2022). In this context, artificial colors used in food, if used regularly or in high concentrations, can cause allergic reactions such as itching, rash, or swelling of a body part (Shamina et al. 2007). They are even dangerous to the consumer's health. Therefore, using these derived from plant species to create food coloring is an inevitable trend now and in the future.

Food is an integral part of every culture. It has always traveled alongside the history and development of every ethnic group in Vietnam (Luu-Dam et al. 2016). Vietnam's culinary culture is a place of cultural interference of 54 ethnic groups residing in 63 provinces and cities from plains and midlands to mountainous areas, rural to urban areas, and from the North to the South. That creates a culinary culture imbued with national identity. Vietnam's culinary culture is formed from productive labor activities and daily life. The King Hung legend mentions Chung cake wrapped in La dong leaves (*Phrynium* spp.) as green represents earth and Day cake made of sticky rice as white

represents heaven. Prince Lang Lieu created these two types of cake, and thanks to his unique idea, Lang Lieu became the next Viet King (Luu-Dam et al. 2016).

Vietnamese dishes are often harmonious in color and flavor, making the overall dish reasonable and increasing the irresistible attraction. These colors and flavors are extracted from plants according to folk experience and are passed down from generation to generation in various ways. Several studies on coloring plants have been conducted in Northern Vietnam (Luu-Dam et al. 2016; Hop et al. 2022). However, there is still a lot of indigenous knowledge about the use of plants for coloring by ethnic groups in other regions that have not been discovered. On the other hand, traditional knowledge about the species of plants used to dye food is disappearing due to the use of artificial dyes, the number of people who have experience with the use of coloring plants is decreasing, and the younger generation is less interested. There is no transfer of knowledge on using plant species for food coloring.

Moreover, rapid socio-economic change in Vietnam threatens the persistence of plant-derived dyes and associated cultural practices and traditional knowledge (Luu-Dam et al. 2016). Therefore, preserving traditional knowledge about food coloring plants is very important. This study provides periodic updates on various aspects of food coloring plants utilized in Vietnam, such as species composition, life form, part-used, color composition, origin, and intended use. In addition to recording the indigenous knowledge of ethnic groups about using plants to create food coloring, this study is expected to provide an important database to improve people's awareness. It also understands local people about the role and value of food coloring plants and culinary culture in general, contributing to preserving and developing the unique indigenous knowledge of local people.

MATERIALS AND METHODS

Study site

Vietnam is located in the Southeast of Asia, stretching across many different latitudes (8°34'-23°23' North latitude and 102°109' - 109°24' East longitude) (Figure 1), covering an area of about 331,212 km², the sea 3200 km, the border with China, Laos, and Cambodia with more than 4200 km. Characterized by quite a diverse terrain, including plains, plateaus, and mountains, mountainous areas account for 3/4 of the territory. Besides, the diversity of tropical climates, i.e., the north is characterized by four seasons: spring, summer, autumn, and winter; the south has two seasons: rainy and sunny. These features have created a diversity of ecosystems such as mangroves, dipterocarp forests, semi-evergreen, evergreen, and other land uses. Furthermore, there is a diversity of species composition, with about 20,000 plant species recorded (Ban 2005), of which many taxa are endemic and have economic and use values. Not only known as one of the biodiversity centers of the world. Vietnam is also known for its diversity of ethnic groups, creating a unique and diverse culinary culture. The intersection between indigenous culinary knowledge and the diversity of plant resources throughout the calendar period has formed knowledge about using plant species for food coloring. This knowledge is especially interesting in the traditional festivals of the local people. This study was conducted from November 2021 to November 2022 in eight provinces of Vietnam, which are three northern provinces (Son La, Cao Bang, Tuyen Quang Province), two central provinces (Quang Nam and Quang Tri Province), and three provinces in the South (Dong Nai, Lam Dong, and Dak Nong Province) (Figure 1).

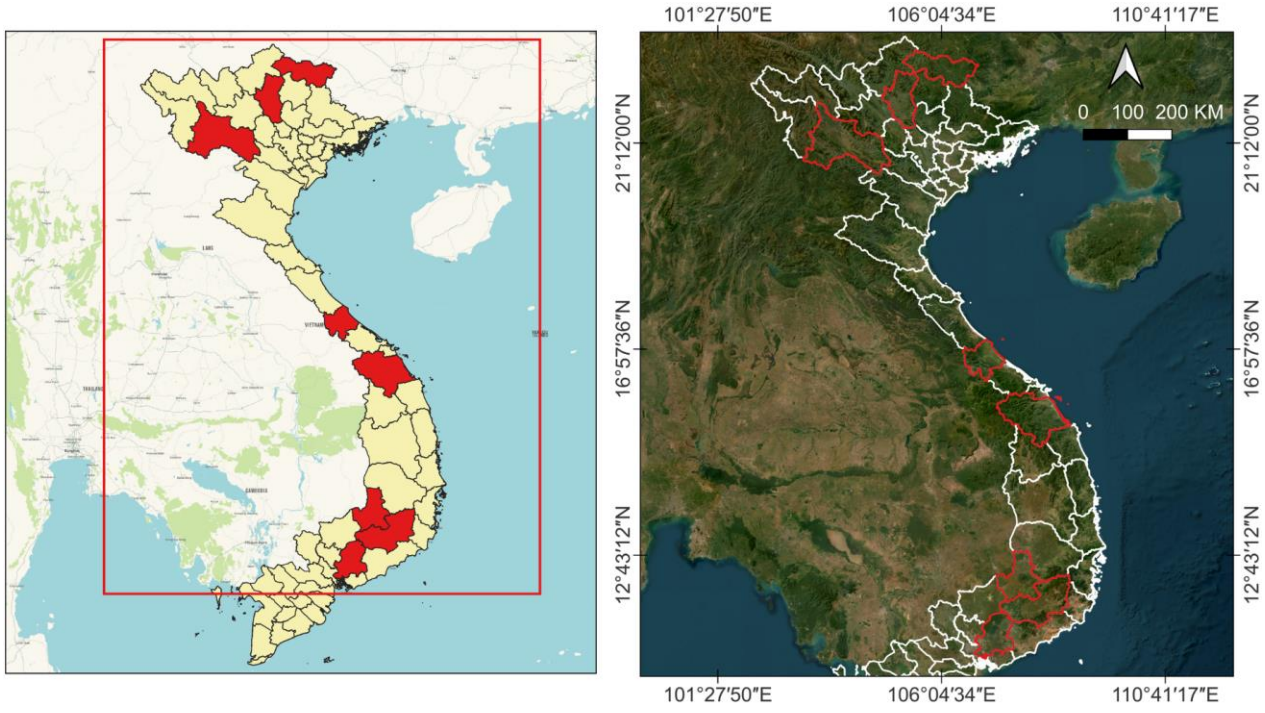

Figure 1. Map of the study area in Vietnam (red)

Data collection

Related documents, articles, websites, and reports on colorant plants for food coloring domestically and globally are collected. Information was collected through structured and semi-structured questionnaire interviews. The interviewees were experienced in collecting and coloring from local plant species in Northern Vietnam, i.e., White Thai peoples in Son La City, Son La Province; H'Mong people in Van Ho District, Son La Province; Tay, Nung people in Quang Uyen District, Cao Bang Province; Dao people in Son Duong District, Tuyen Quang Province; The Central region consists of the Co Tu people in Nam Giang District, Quang Nam Province and the Van Kieu people in Huong Hoa District, Quang Tri Province; and South of Vietnam was the Cho Ro, Kinh people in Vinh Cuu District, Dong Nai Province; Co Ho, Kinh people in Don Duong, Lac Duong District, Lam Dong Province; M'Nong, Tay and Kinh people in Dak Song District, Dak Nong Province (Figure 1). Consult with plant colorists and experts.

The name of the plant species used by people to create food coloring was determined by collecting plant specimens in the field. Experienced local people were selected to guide the plants they use. We collected samples, pictures, and recorded information on that basis, then compared them with specialized documents, standard samples were kept at research institutes and botanical museums to determine the species name. In addition, data about the parts used, life form, color, origin, and purpose of use were also collected. Samples were collected and processed according to the Handbook of biodiversity research (Thin 1997).

Data analysis

Comparative morphological and expert methods were applied to treat and identify plant specimens. After being collected and processed, the specimens were compared and contrasted with the standard specimens kept at the Vietnam National University of Forestry-Dong Nai Campus, Vietnam National University of Forestry, Hue University of Agriculture and Forest, and Southern Institute of Ecology. We used specialized documents for species identification for specimens that do not have a reference sample to search. The references included An Illustrated Flora of Vietnam, volumes 1-3 (Ho 1999); Timber Resources in Vietnam (Hop 2002); 1900 Useful Plant Species of Vietnam (Ly 1993); Vietnamese medicinal plants and Herbs (Loi 2001); Dictionary Medicinal of Vietnam (Chi 2012). The scientific name of the plants was determined and updated by Plants of the World Online (2022) and World Flora Online (2022). The Angiosperm plant species are arranged according to the taxonomy of the APG IV (APG IV 2016). Family and scientific names of species in the list were ordered alphabetically. The life form was evaluated according to documents An Illustrated Flora of Vietnam, volumes 1-3 (Ho 1999). Color classification, intended use, and origin were according to the findings of data synthesis. The threatened species composition was determined based on the VNRB (2007) and IUCN Red List (2022).

RESULTS AND DISCUSSION

Results

Species component

A total of 110 taxa, 94 genera belonging to 54 families, were discovered for food coloring in Vietnam. All species of plants belong to Angiosperms, and most of them belong to Eudicots (over 80% at the taxonomic level) (Table 1, Table 4).

Among the identified plants, 28 species were used frequently by local people (25.45%), 35 species (31.82%) were used occasionally, and 47 species (42.73%) were rarely used. The species-rich families (40.91% of total species) were represented by Fabaceae (9 species, 8,65%), Rubiaceae (8 species, 7.27%), Amaranthaceae, Zingiberaceae, Poaceae, Rubiaceae, and Cucurbitaceae (5 species, 4.55%); Scrophulariaceae, Rosaceae (4 species, 3.64%). In addition, Marantaceae, Asparagaceae, Phyllanthaceae, Moraceae, Malvaceae, Acanthaceae (3 species, 2.73%); Asteraceae, Theaceae, Polygonaceae, Pedaliaceae, Lythraceae, Euphorbiaceae, Primulaceae (2 species, 1.82%); and 33 single species families determined.

Buddleja was the most species (4 species, 3.64%), followed by *Dracaena* (3 species, 2.73%). There were 11 genera with two species, i.e., *Alpinia, Curcuma, Phrynium, Camellia, Phyllanthus, Sesamum, Morus, Paederia, Momordica, Amaranthus*, and *Dicliptera*; and 81 species genera were counted.

There were 15 species identified as conservation values at different domestic and global levels. Fourteen species at Least Concern (LC) and four at Data Deficient (DD) were shown in the IUNC Red List (2022). In addition, two species were categorized as Vulnerable (VU) in the Ministry of Science and Technology (2007) (Table 2).

Diversity of life-form

There were five life forms found for food coloring. The shrubs were the most (32.73%), followed by wood (30.91%), vines (17.27%), herbaceous (14.55%), and the lowest was bamboo (4.55%). Shrubs accounted for the highest percentage, but 13/36 species were domesticated and grown in home gardens and upland fields; 4/36 were cultivated and in the wild; 21/36 were recorded in the forest (Figure 2). While the wood plants were primarily native to natural forests, only a few species were cultivated by local people.

Table 1. Plant distribution for food coloring in the eudicots and monocots

Phylum	Family		Genera		Species	
	N	(%)	N	(%)	N	(%)
Angiosperms	54	100	94	100	110	100
Eudicots	44	81.48	77	81.91	88	80.00
Monocots	10	18.52	17	18.09	22	20.00

Notes: N: Number; (%): Percentage

Table 2. Species composition with conservation value

Family name	Botanical name	Local name	IUCN (2022)	VNRB (2007)
Altingiaceae	*Liquidambar formosana* Hance	Sau sau	LC	
Anacardiaceae	*Rhus chinensis* Mill.	Muối	LC	
Buseraceae	*Canarium pimela* K.D.Koenig	Trám đen		VU
Combretaceae	*Barringtonia asiatica* (L.) Kurz	Bàng vuông	LC	VU
Fabaceae	*Biancaea sappan* (L.) Tod.	Tô mộc	LC	
Lamiaceae	*Gmelina arborea* Roxb. ex Sm.	Lõi thọ	LC	
Lythraceae	*Lawsonia inermis* L.	Móng tay	LC	
Magnoliaceae	*Magnolia mediocris* (Dandy) Figlar	Giổi xanh	LC	
Myrtaceae	*Rhodomyrtus tomentosa* (Aiton) Hassk.	Hồng sim	LC	
Phyllanthaceae	*Breynia androgyna* (L.) Chakrab. & N.P.Balakr.	Bồ ngót	LC	
Phyllanthaceae	*Phyllanthus emblica* L.	Me rừng	LC	
Phyllanthaceae	*Phyllanthus reticulatus* Poir.	Phèn đen	LC	
Rosaceae	*Prunus salicina* Lindl.	Mận	LC	
Scrophulariaceae	*Buddleja officinalis* Maxim.	Mật mông	LC	
Theaceae	*Camellia oleifera* C.Abel	Sở	LC	

Note: VNRB (2007): Vietnam Red Data Book (2007); EN: Endangered; VU: Vulnerable; LC: Least Concern

Diversity of parts used

Thirteen parts used were discovered with the capability to color food. Leaves were the most (40 species, 36.36%), followed by fruit (28 species, 25.45%), wood (18 species, 16.36%), flower (14 species, 12.73%), and root the least (1 species, 0.91%) (Table 3). Although leaves accounted for the highest percentage, they did not influence the growth and evolution of trees, significantly not impacting the structure and stability of forest resources.

The number of species for one part used for food coloring predominates (94 species, 90.38%), followed by two parts used (15 species, 14.42%), and *Tamarindus indica* for three parts used (0.96%) (Figure 3).

Diversity of colors

We found ten colors from plant species. The most were red (31 species, 28.18%), yellow (24 species, 21.82%), green (22 species, 20.00%), black (18 species, 16.36%), and the most petite grey (1 species, 0.91%) (Table 5).

Three species (2.73%) were *M. philippensis*, *T. indica*, and *R. alceifolius* gave the greatest number of colors (3 colors), seven species (6.36%) showed two colors, most species gave one color (100 species, 90.91%).

Diversity of plant origin for food coloring

Of the overall number of species recorded as food coloring, 44 species (40.00%) were domesticated and cultivated in home gardens and upland fields; 43 species (39.09%) were naturalized, and 23 species (20.91%) were in the wild and cultivated (Figure 4).

Table 3. Diversity of part-used

Part-used	No. of species	Percentage (%)
Root	1	0.91
Sap	2	1.82
Young shoots	2	1.82
Rhizomes	3	2.73
Tuber	5	4.55
Seed	7	6.36
Bark	8	7.27
Flower	14	12.73
Wood	18	16.36
Fruit	28	25.45
Leaves	40	36.36

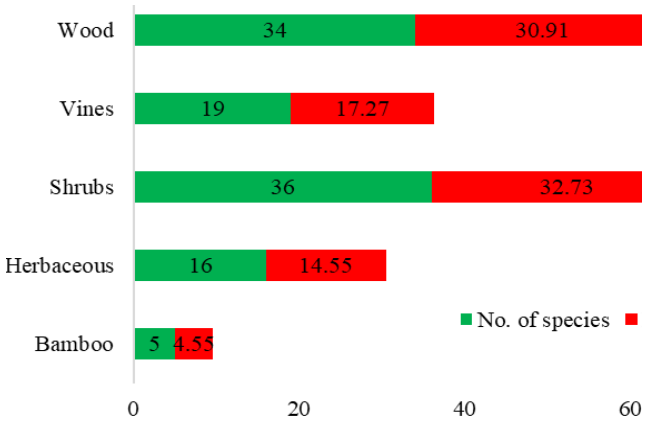

Figure 2. Diversity of life-form

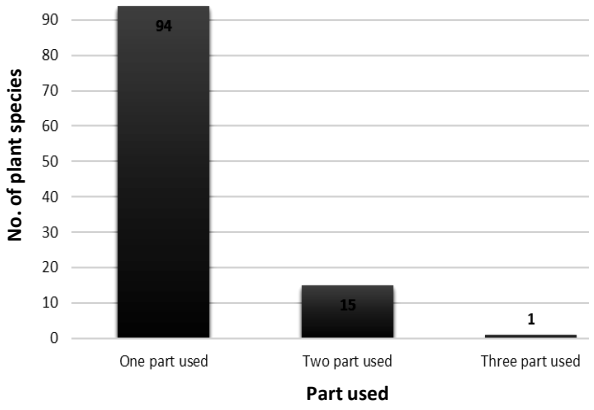

Figure 3. Number of plant species for food coloring by part used

Table 4. The composition of colorant plants for food coloring in Vietnam

Family	Botanical name	Local name	Parts-used	Color	Life form	Origin	Frequency	Food
Eudicots								
Acanthaceae	*Dicliptera chinensis* (L.) Juss.	*Lá diễn*	Leaves	Red	Shrubs	(1)	3*	Rice
Acanthaceae	*Dicliptera tinctoria* (Nees) Kostel.	*Cẩm*	Leaves	Purple	Shrubs	(2)	3*	Rice, drink
Acanthaceae	*Strobilanthes cusia* (Nees) Kuntze	*Chàm mèo*	Leaves	Blue	Shrubs	(1)	2*	Rice
Altingiaceae	*Liquidambar formosana* Hance	*Sau sau*	Leaves	Black	Wood	(1)	2*	Rice
Amaranthaceae	*Amaranthus caudatus* L.	*Dền tía*	Wood, leaves	Red	Shrubs	(2)	3*	Rice, cake, soup
Amaranthaceae	*Amaranthus cruentus* L.	*Dền đỏ*	Wood, leaves	Red	Shrubs	(2)	3*	Cake, rice, soup
Amaranthaceae	*Beta vulgaris* L.	*Dền*	Tuber	Pink	Shrubs	(2)	*	Cake
Amaranthaceae	*Iresine diffusa* f. *herbstii* (Hook.) Pedersen	*Nhung hoa*	Leaves	Red	Shrubs	(2)	3*	Rice
Amaranthaceae	*Spinacia oleracea* L.	*Rau chân vịt*	Leaves	Green	Shrubs	(2)	2*	Cake
Anacardiaceae	*Rhus chinensis* Mill.	*Muối*	Bark	Black	Shrubs	(1)	2*	Rice
Apiaceae	*Daucus carota* L.	*Cà rốt*	Tuber	Orange	Herbaceous	(2)	3*	Cake, rice, drink
Asteraceae	*Artemisia vulgaris* L.	*Ngải cứu*	Leaves, young shoots	Green	Shrubs	(2)	*	Cake
Asteraceae	*Pseudognaphalium affine* (D.Don) Anderb.	*Rau khúc*	Leaves	Green	Herbaceous	(2), (1)	3*	Cake, drink
Basellaceae	*Basella alba* L.	*Mồng tơi*	Fruit	Purple	Vines	(2)	*	Rice
Bignoniaceae	*Oroxylum indicum* (L.) Kurz	*Núc nác*	Wood	Black	Wood	(1)	3*	Cake
Buseraceae	*Canarium pimela* K.D.Koenig	*Trám đen*	Fruit	Black	Wood	(1)	2*	Rice
Brassicaceae	*Brassica oleracea* L.	*Bắp cải tím*	Leaves	Purple	Herbaceous	(2)	3*	Rice
Caricaceae	*Carica papaya* L.	*Đu đủ*	Fruit	Green	Wood	(2)	*	Rice
Combretaceae	*Barringtonia asiatica* (L.) Kurz	*Bàng vuông*	Leaves	Green	Wood	(2), (1)	2*	Cake
Convolvulaceae	*Ipomoea batatas* (L.) Lam.	*Khoai lang tím*	Rhizomes	Purple	Vines	(2)	2*	Rice, cake, drink
Cucurbitaceae	*Citrullus lanatus (*Thunb.) Matsum. & Nakai	*Dưa hấu*	Fruit	Red	Vines	(2)	3*	Rice, drink
Cucurbitaceae	*Cucumis melo* L.	*Mướp*	Leaves	Green	Vines	(2)	*	Rice
Cucurbitaceae	*Cucurbita moschata* Duchesne	*Bí đỏ*	Fruit	Yellow	Vines	(2)	2*	Rice, cake
Cucurbitaceae	*Momordica cochinchinensis* (Lour.) Spreng.	*Gấc*	Fruit	Red	Vines	(2)	3*	Rice, cake, drink
Cucurbitaceae	*Momordica sp.*	*Gấc vàng*	Seed	Yellow	Vines	(2), (1)	*	Rice
Datiscaceae	*Datisca cannabina* L.	*Đa tích*	Wood, leaves	Yellow	Shrubs	(2)	*	Rice
Ebenaceae	*Diospyros kaki* L.f.	*Hồng*	Fruit, leaves	Brown, yellow	Wood	(2)	*	Rice
Elaeagnaceae	*Elaeagnus latifolia* L.	*Nhót*	Leaves	Black	Vines	(2)	*	Rice
Ericaceae	*Vaccinium corymbosum* L.	*Việt quất*	Fruit	Purple	Shrubs	(2)	*	Rice, cake, drink
Euphorbiaceae	*Aporosa octandra var. octandra*	*Thẩu tấu*	Wood	Red	Wood	(1)	*	Rice
Euphorbiaceae	*Mallotus philippensis* (Lam.) Müll.Arg.	*Cánh kiến*	Fruit, flower	Yellow, orange, red	Wood	(1)	*	Rice
Fabaceae	*Biancaea sappan* (L.) Tod.	*Tô mộc*	Wood	Pink, brown, red	Wood	(2), (1)	3*	Rice
Fabaceae	*Caesalpinia crista* L.	*Móc mèo*	Flower	Yellow	Shrubs	(1)	*	Rice
Fabaceae	*Cassia fistula* L.	*Muồng hoàng yến*	Bark	Red	Wood	(2)	*	Rice
Fabaceae	*Clitoria ternatea* L.	*Đậu biếc*	Leaves, flower	Purple	Vines	(2), (1)	3*	Rice, cake, drink
Fabaceae	*Millettia sp.*	*Thàn mát*	Wood	Red	Wood	(1)	*	Drink
Fabaceae	*Saraca dives* Pierre	*Vàng anh*	Flower	Red	Wood	(2), (1)	*	Rice, cake
Fabaceae	*Spatholobus suberectus* Dunn	*Huyết đằng*	Wood, bark	Red	Vines	(1)	2*	Drink
Fabaceae	*Tamarindus indica* L.	*Me chua*	Leaves, fruit, seed	Yellow, brown	Wood	(1)	*	Drink

Fabaceae	*Vigna unguiculata subsp. unguiculata*	*Đậu đen*	Seed	Black	Vines	(2)	2*	Rice, cake, drink
Hypericaceae	*Cratoxylum neriifolium* Kurz	*Thành ngạnh lá hẹp*	Bark	Brown	Wood	(1)	*	Cake, Rice
Lamiaceae	*Gmelina arborea* Roxb. ex Sm.	*Lõi thọ*	Flower	Yellow	Wood	(1)	*	Rice
Lardizabalaceae	*Sargentodoxa cuneata* (Oliv.) Rehder & E.H.Wilson	*Huyết rồng*	Wood, leaves	Red	Vines	(1)	2*	Drink
Lauraceae	*Neolitsea cassia* (L.) Kosterm.	*Quế*	Bark	Brown	Wood	(2), (1)	*	Cake, Rice
Lythraceae	*Lawsonia inermis* L.	*Móng tay*	Wood, leaves	Red	Shrubs	(2)	*	Rice
Lythraceae	*Punica granatum* L.	*Lựu*	Fruit	Red	Wood	(2)	2*	Cake, rice, drink
Magnoliaceae	*Magnolia mediocris* (Dandy) Figlar	*Giổi xanh*	Leaves	Green	Wood	(2), (1)	*	Rice
Malvaceae	*Hibiscus sabdariffa* L.	*Bụp giấm*	Flower, fruit	Red	Shrubs	(2), (1)	3*	Drink, cake
Malvaceae	*Hibiscus sp.*	*Bụp*	Flower	Red	Shrubs	(1)	*	Drink
Malvaceae	*Theobroma cacao* L.	*Ca cao*	Fruit, seed	Brown	Wood	(2)	2*	Cake, rice, drink
Menispermaceae	*Fibraurea tinctoria* Lour.	*Hoàng đằng*	Wood	Yellow	Vines	(1)	2*	Wine
Moraceae	*Ficus simplicissima* Lour.	*Vú bò*	Leaves	Green	Shrubs	(1)	*	Rice
Moraceae	*Morus alba* L.	*Dâu tằm*	Fruit	Red	Wood	(2)	2*	Drink
Moraceae	*Morus cathayana* Hemsl.	*Dâu bầu*	Fruit	Red	Wood	(2), (1)	*	Drink
Myristicaceae	*Knema sp.*	*Máu chó*	Resin, resin	Red	Wood	(1)	*	Drink
Myrtaceae	*Rhodomyrtus tomentosa* (Aiton) Hassk.	*Hồng sim*	Fruit	Pink	Shrubs	(1)	3*	Wine
Pedaliaceae	*Sesamum indicum* L.	*Vừng trắng*	Wood	Black	Shrubs	(2)	*	Cake
Pedaliaceae	*Sesamum radiatum* Thonn. ex Hornem.	*Vừng đen*	Seed	Black	Shrubs	(2)	2*	Cake
Phyllanthaceae	*Breynia androgyna* (L.) Chakrab. & N.P.Balakr.	*Bồ ngót*	Leaves	Green	Shrubs	(2), (1)	3*	Rice, cake, drink
Phyllanthaceae	*Phyllanthus emblica* L.	*Me rừng*	Fruit	Black	Wood	(1)	*	Cake, Rice
Phyllanthaceae	*Phyllanthus reticulatus* Poir.	*Phèn đen*	Fruit	Black, purple	Shrubs	(1)	*	Rice, cake
Polygonaceae	*Fagopyrum esculentum* Moench	*Mạch ba góc*	Leaves	Yellow	Shrubs	(1)	*	Rice
Polygonaceae	*Reynoutria japonica* Houtt.	*Cốt khí*	Tuber	Yellow	Herbaceous	(2), (1)	2*	Sticky
Primulaceae	*Ardisia tinctoria* Pit.	*Cơm nguội nhuộm*	Fruit	Black	Shrubs	(1)	*	Cake, Rice
Primulaceae	*Embelia parviflora* Wall. ex A.DC.	*Chua ngút*	Wood	Red	Shrubs	(1)	*	Rice
Rosaceae	*Fragaria* × *ananassa* (Duchesne ex Weston) Duchesne ex Rozier	*Dâu tây*	Fruit	Red	Herbaceous	(2)	*	Drink, cake
Rosaceae	*Prunus salicina* Lindl.	*Mận*	Fruit	Red	Wood	(2)	2*	Drink
Rosaceae	*Rosa* sp.	*Hoa hồng*	Flower	Pink, red	Shrubs	(2)	*	Cake
Rosaceae	*Rubus alceifolius* Poir.	*Mâm sôi*	Young shoots	Yellow, green, grey	Shrubs	(1)	3*	Cake, drink
Rubiaceae	*Coffea arabica* L.	*Cà phê*	Fruit, seed	Black	Wood	(2), (1)	2*	Cake, rice, drink
Rubiaceae	*Dioecrescis erythroclada* (Kurz) Tirveng.	*Dành dành*	Sap	Black	Shrubs	(1)	*	Rice, cake
Rubiaceae	*Gardenia jasminoides* J.Ellis	*Dành dành*	Fruit	Yellow	Wood	(2)	3*	Rice, cake, drink
Rubiaceae	*Gynochthodes umbellata* (L.) Razafim. & B.Bremer	*Nhàu mặt quỷ*	Bark	Yellow, red	Vines	(1)	*	Rice, cake
Rubiaceae	*Morinda tomentosa* B.Heyne ex Roth	*Nhàu nhuộm*	Bark	Red, yellow	Wood	(1)	*	Rice
Rubiaceae	*Luculia gratissima* (Wall.) Sweet	*Gạc nai*	Wood	Yellow	Wood	(1)	*	Drink
Rubiaceae	*Paederia foetida* L.	*Mơ dây*	Leaves	Green	Vines	(2)	2*	Rice
Rubiaceae	*Paederia lanuginosa* Wall.	*Mơ tam thể*	Leaves	Black	Vines	(2), (1)	2*	Rice
Rutaceae	*Citrus* × *aurantium* L.	*Cam*	Fruit	Orange	Wood	(2)	*	Cake, drink
Schisandraceae	*Illicium verum* Hook.f.	*Hồi*	Fruit	Black	Wood	(1)	*	Cake, Rice
Scrophulariaceae	*Buddleja davidii* Franch.	*Búp lệ da*	Flower	Yellow	Shrubs	(1)	3*	Rice
Scrophulariaceae	*Buddleja macrostachya* Benth.	*Búp lệ chùm to*	Flower	Yellow	Shrubs	(1)	2*	Rice
Scrophulariaceae	*Buddleja officinalis* Maxim.	*Mật mông hoa*	Flower	Yellow	Shrubs	(1)	3*	Rice
Scrophulariaceae	*Buddleja paniculata* Wall.	*Búp lệ chùm tụ tán*	Flower	Yellow	Shrubs	(1)	2*	Rice

Smilacaceae	*Smilax glabra* Roxb.	*Thổ phục linh*	Wood	Red	Vines	(1)	*	Drink
Theaceae	*Camellia oleifera* C.Abel	*Sở*	Bark	Brown	Wood	(2), (1)	*	Cake
Theaceae	*Camellia sinensis* (L.) Kuntze	*Trà xanh*	Leaves	Green	Wood	(2), (1)	3*	Cake, drink
Urticaceae	*Boehmeria nivea* (L.) Gaudich.	*Lá gai*	Leaves	Black	Shrubs	(2), (1)	3*	Cake
Vitaceae	*Vitis vinifera* L.	*Nho*	Fruit	Purple	Vines	(2)	*	Drink
Monocots								
Arecaceae	*Cocos nucifera* L.	*Dừa*	Fruit	Brown	Wood	(2)	*	Soup, Rice
Asparagaceae	*Dracaena angustifolia* (Medik.) Roxb.	*Bồng bồng lá nhỏ*	Leaves	Green	Shrubs	(1)	3*	Cake, drink
Asparagaceae	*Dracaena cambodiana* Pierre ex Gagnep.	*Huyết giác*	Wood	Red	Shrubs	(1)	*	Cake, drink
Asparagaceae	*Dracaena cochinchinensis* (Lour.) S.C.Chen	*Bồng bồng nam bộ*	Leaves	Green	Shrubs	(2), (1)	3*	Cake, drink
Cactaceae	*Selenicereus costaricensis* (F.A.C.Weber) S.Arias & N.Korotkova ex Hammel	*Thanh long*	Fruit	Red	Wood	(2)	3*	Rice, cake, drink
Dioscoreaceae	*Dioscorea alata* L.	*Khoai mỡ*	Tuber	Purple	Vines	(1)	*	Rice
Iridaceae	*Eleutherine bulbosa* (Mill.) Urb.	*Sâm đại hành*	Roots, tuber	Red	Herbaceous	(1)	2*	Drink
Liliaceae	*Lilium longiflorum* Thunb.	*Huệ tây*	Flower	Yellow	Herbaceous	(2)	*	Rice, drink
Marantaceae	*Phrynium imbricatum* Roxb.	*Lá dong*	Leaves	Green	Herbaceous	(2)	2*	Rice, cake
Marantaceae	*Phrynium pubinerve* Blume	*Lá dong*	Leaves	Green	Herbaceous	(2)	2*	Rice, cake
Marantaceae	*Stachyphrynium placentarium* (Lour.) Clausager & Borchs.	*Lá dong*	Leaves	Blue	Herbaceous	(2)	3*	Rice, cake
Pandanaceae	*Pandanus amaryllifolius* Roxb. ex Lindl.	*Dứa thơm*	Leaves	Green	Herbaceous	(2)	3*	Rice, cake, drink
Poaceae	*Bambusa bambos* (L.) Voss	*Tre*	Leaves, wood	Green, black	Bamboo	(2), (1)	2*	Rice, cake, drink
Poaceae	*Oryza sativa* L.	*Lúa nếp*	Seed	Black	Bamboo	(2)	2*	Rice, cake
Poaceae	*Saccharum officinarum* L.	*Mía*	Wood	Brown	Bamboo	(2)	*	Soup, rice, cake, drink
Poaceae	*Thysanolaena latifolia* (Roxb. ex Hornem.) Honda	*Chít*	Leaves	Yellow	Bamboo	(2), (1)	2*	Rice, cake
Poaceae	*Thyrsostachys siamensis* Gamble	*Tầm vông*	Leaves	Green	Bamboo	(1)	2*	Rice
Zingiberaceae	*Alpinia gagnepainii* K.Schum.	*Riềng*	Leaves	Green	Herbaceous	(1)	2*	Rice
Zingiberaceae	*Alpinia officinarum* Hance	*Riềng*	Leaves	Green	Herbaceous	(2), (1)	2*	Rice
Zingiberaceae	*Curcuma longa* L.	*Nghệ*	Rhizomes	Yellow	Herbaceous	(2), (1)	3*	Rice, soup, cake, drink
Zingiberaceae	*Curcuma aeruginosa* Roxb.	*Nghệ đen*	Rhizomes	Yellow	Herbaceous	(2), (1)	*	Rice
Zingiberaceae	*Zingiber officinale* Roscoe	*Gừng*	Leaves	Green	Herbaceous	(2)	2*	Rice

Note: (1): Naturalized; (2): Cultivated; 3*: Frequently used species; 2*: Occasionally used species; *: rarely used

Diversity of foods group using colorant plants

Colorant plants for food coloring in Vietnam were used for six food groups: rice, cake, drink, soup, wine, and sticky. The colors created were commonly employed for cooking sticky rice (75 species, 68.18%) in the traditional festivals of ethnic groups such as Kinh, Tay, Nung, H'Mong, Thai, and Co Tu (Table 6). The three primary colors are blue, yellow, and red. In comparison, several others, such as purple, pink, orange, and blue, are less frequent.

For example, on the cake (52 species, 47.27%), local people used formulated cakes during Lunar New Year, Pure Brightness Festival, and Mid-Autumn Festival. Popular products in this category include Chung cake, Troi cake, Day cake, Khuc cake, Duc cake, and Tet cake. Drink (41 species, 37.27%) was used for daily drinking. At the same time, soup (5 species, 4.55%) was cooked for people's local meals.

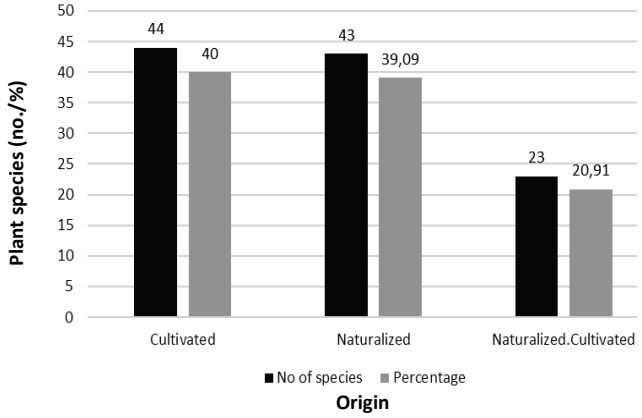

Figure 4. Diversity of plant origin for food coloring

Table 5. Diversity of colors

Colors	No. of species	Percentage (%)
Grey	1	0.91
Blue	2	1.82
Orange	3	2.73
Pink	4	3.64
Brown	9	8.18
Purple	9	8.18
Black	18	16.36
Green	22	20.00
Yellow	24	21.82
Red	31	28.18

Table 6. Diversity of foods using colorant plants

Food	No. of species	Percentage (%)
Sticky	1	0.91
Wine	2	1.82
Soup	5	4.55
Drink	41	37.27
Cake	52	47.27
Rice	75	68.18

Discussion

The present study showed that the species composition of food coloring plants was quite diverse, with 110 species and subspecies of 94 genera belonging to 54 families identified. Several previous studies on this topic were conducted in Vietnam and initially recorded native plant species used by ethnic communities for food coloring. However, these studies only found several case studies concentrated in several specific ecological regions, not systematic and not guaranteed to represent different ecological regions in Vietnam. For example, a study on natural plant colorants widely used in Vietnam's traditional food culture conducted in the Northwest region has recorded 49 species of vascular plants belonging to 30 families (Ung et al. 2018) less than this current study. Another study conducted in the North of Vietnam showed that 43 species of vascular plants belonging to 24 families had been identified with the ability to provide color for food coloring (Luu-Dam et al. 2016). Thus, this study added 61 species of food-coloring plants compared to Ung et al. (2018); and 67 species compared with Luu-Dam et al. (2016). This finding shows that the number of species capable of creating food coloring in the studies is different, possibly due to the difference in scope, research subjects, and sampling methods. Several studies in the Asian region showed that up to 106 plant species of 46 families in the Northwestern Himalayas and 46 species of plants from the Northern Western Ghats were collected for food coloring. Meanwhile, 25 Himalayan plant species have been identified as potential sources of coloring materials in high demand in food processing (Das and Kalita 2016). The ethnobotanical study of the Dong ethnic group in China showed that, in total, seven species were used to color food items, and three species were used to color foods and clothing (Liu et al. 2014).

This study found that, out of 28 species frequently used by people, 15 species were used by local people living in rural and urban areas, namely: *A. caudatus, A. cruentus, D. carota, P. affine, B. oleracea, C. lanatus, M. cochinchinensis, C. ternatea, H. sabdariffa, C. sinensis, B. nivea, S. costaricensis, P. amaryllifolius, D. chinensis, D. tinctoria*. The remaining 13 species were used by ethnic minorities living in mountainous areas where economic conditions were difficult. People living in rural and urban areas mainly use plants grown in home gardens or can be easily purchased from markets and supermarkets. Meanwhile, due to a difficult life living near the mountains and forests, where many native tree species grow naturally, searching for food-coloring plants through the ancestors' experience is also possible. Therefore, people living in rural and urban areas often use plants for food coloring more frequently than in mountainous areas. Because most people living in rural and urban areas are Kinh ethnic groups, they have a better life than people in mountainous areas. Moreover, finding these plants is also easier. People in the mountainous areas are mainly minority ethnics; they only use food coloring plants during traditional holidays and Lunar New Year, Qingming New Year, and Mid-Autumn Festival.

Plants for coloring are still used today by people in this study area, which shows their important role in food coloring. The most recorded use of dye plants is for food coloring, specifically glutinous rice, followed by cakes, beverages, and common soups. Pigments derived from dye plants can produce different colors (Liu et al. 2014). The present study noted that red predominates, followed by yellow, dark blue, black, purple, and brown. These pigments are obtained from different parts of plants, i.e., roots, leaves, flowers, stems, bark, and tubers. In addition to their coloring function, they are also used for medicinal, decorative, preservation, edible, and timber (Liu et al. 2014).

The color type also differs depending on the plant species and part of the plants. Therefore, the featured products of this study were five-colored sticky rice, three-color sticky rice, and gac sticky rice, for example: (1) five-color sticky rice, this product is: an attractive specialty of the northwest mountains because there were up to five colors: red, white, yellow, green, and black (Table 7), each color embodied a vibrancy, a color of its own; this is also the color of the girl's dress in this region. All combine to devise a color palette with high aesthetics. There is also other five-color sticky rice because the composition of the color-producing plants of each ethnic group is different, for example: the Kinh peoples use the red color from the fruit of *M. cochinchinensis*; purple color from the leaves of *D. tinctoria*; green color from the leaves of *A. officinarum*; and yellow from the bulbs of *C. longa*, and white from the seeds of *O. sativa* (Figure 6); (2) three-color sticky rice, this product is composed by three primary colors, i.e., yellow of *G. jasminoides*, red of *M. cochinchinensis*, and green of *P. amaryllifolius* combined to make a harmonious color, and aroma (Table 8). Quan et al. (2016) recorded five varieties of *P. bivalvis*, a synonym of *D. tinctoria*, a source of raw materials for dyeing food and medicine, in which the plant can produce three color varieties, i.e., purple, red, and yellow-orange.

Table 7. Species composition and color for making five-color sticky rice in Vietnam

Species	Color	Parts used
Curcuma longa L.	Yellow	Tuber
Bambusa bambos (L.) Voss	Back	Stem
Rubus alceifolius Poir.	Red	Fruit
Pandanus amaryllifolius Roxb. ex Lindl.	Blue	Leaves
Oryza sativa L.	White	Seed

Table 8. Species composition and color for making three-color sticky rice in Vietnam

Species	Color	Parts used
Gardenia jasminoides J.Ellis	Yellow	Fruit
Momordica cochinchinensis (Lour.) Spreng.	Red	Stem
Pandanus amaryllifolius Roxb. ex Lindl.	Green	Leaves
Oryza sativa L.	White	Seed

Yellow sticky rice is also called Bo phon sticky rice by the people, it is a speciality of the Northwestern people of Vietnam with its natural bright yellow color and delicious taste. The ingredients to make this dish are glutinous rice, and the yellow color is taken from the flowers of *Buddleja officinalis*. Yellow color is obtained from the flower by placing the whole bunch of flowers in boiling water until the desired color is achieved. Then soak glutinous rice in that yellow water and cook. Flowers can be dried, preserved for 1-2 years, and after use, can be reused 2 to 3 times. Subsequent uses, the yellow color of this species of flower is brighter and more natural (Figure 5).

Figure 5. Yellower sticky rice is called Bo phon by the Tay and Dao ethnic groups in Northwestern Vietnam, it is made from the flowers of *Buddleja officinalis*. (Image source: Nong Dinh Don and Mai Tay Bac)

Figure 6. Five-color sticky rice of the King peoples (Image source: Thuy Huong)

This study also found that coloring plants have health care and disease treatment functions besides creating attraction for food consumers; for example, leaves of *D. tinctoria* have diuretic effects and treat kidney stones; urinary incontinence; Flowers of *B. officinalis* Maxim. Cook and drink water to treat eye pain, red eyes or branches, leaves, and root bark as medicine for rheumatism. Several studies in China are similar to this present study. *Persicaria tinctoria* Spach has the effect of clearing heat, detoxifying, cooling blood, curing mumps, reducing swelling, and relieving pain; and itching (Chinese Pharmacopoeia Commission 2010). *S. cusia* can prevent and treat the influenza virus (Liu 1998) and clear heat, reduce toxicity, cool blood, relieve sore throat, and improve immunity by killing pathogenic microorganisms (Chinese Pharmacopoeia Commission 2010). *B. officinalis* is a beverage with medicinal effects recorded in the Chinese Pharmacopoeia (Chinese Pharmacopoeia Commission 2005) that can be used as a substitute for tea in Yunnan province with its cool and refreshing taste (Tang and Zheng 1991). It is used to treat dry eyes, blurred vision, and cataracts. It has also been shown to have anti-inflammatory and blood-sugar-lowering activities, potentially boosting immunity (Li and Sun 1996).

According to the cultural traditions of the ethnic groups in the study areas, indigenous knowledge about the coloring plants of the communities is passed down from generations of grandfathers and fathers to children and grandchildren. However, this method nowadays faces many challenges. The culture of community activities, even between generations in the family, is the traditional way to pass on the father's experience to future generations, which is also difficult to do. Furthermore, because of the change in all aspects of life, today's young generations are being driven away by social development; most young people are looking for work outside of their community, so this knowledge will not be passed on to the younger generation. Moreover, forest resource degradation is also an important reason; some species of color-producing plants could be easily collected in the past, but now it is not easy to access; even moving 5-10 km is searchable.

On the other hand, the use of plants for food coloring of natural origin often occurs within households, is small, fragmented, has low yields, and can only happen during festivals. These are major challenges for conserving and promoting indigenous knowledge of ethnic communities in the study area. Therefore, future studies should be conducted in other communities and ecoregions. These studies could systematize and document this unique knowledge. Some other necessary studies to be carried out to maintain and preserve this knowledge are to direct in-depth studies on the chemical composition of natural colorants, nutritional composition, uses, and method of extracting colorants as a basis for industrial-scale production for the food and beverage industries. These future studies will help to provide guidelines for community-based production and ultimately preserve indigenous knowledge about using plants for food coloring. Therefore, restoring traditional festivals, including culinary culture, is one of the solutions that have been implemented in some regions. Promoting products to tourists by providing traditional products to tourist centers, restaurants, and hotels in tourist areas is the trend to restore and preserve public knowledge on using plants for coloring in Vietnam.

In conclusion, based on indigenous knowledge, this study illustrates the richness of plants for food coloring in Vietnam. Accordingly, 110 taxa of 94 genera belonging to 54 families were recorded. Twenty-eight species were used regularly in classical cultural festivals of the indigenous people. This study also confirmed 15 species with domestic and global conservation values. Fabaceae and *Buddleja* were the species-rich families and genera. There were five life forms, i.e., shrubs, wood, vines, herbaceous, and bamboo. Eleven part-used plants were used for coloring by local people, with leaves used with the most frequency. These plant species produced ten colors: red, yellow, green, black, purple, brown, pink, orange, blue, and grey. Local people observe these species in the wild, then tame and cultivate them in home gardens or fields, remaining wild or cultivated. Therefore, by indigenous information from previous generations, local people have used dye plants to color cakes, rice, drink, soup, wine, or sticky. Faced with the challenges of the decline of color plants and the loss of indigenous knowledge of communities, we recommend the need for the conservation and development of plant colorants and the unique knowledge of communities on the use of food coloring plants through the domestication of native plants and the restoration of festivals' traditional cuisine to preserve them for future generations.

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Embryo and larvae development of Nilem Fish, *Osteochilus vittatus* reared in batik liquid waste

GISELA HARSIWI NUGRAHESTHI, GRATIANA EKANINGSIH WIJAYANTI, AULIDYA NURUL HABIBAH♥
Faculty of Biology, Universitas Jenderal Soedirman. Jl. Dr. Soeparno No. 63, North Purwokerto, Banyumas 53112, Central Java, Indonesia.
Tel.: +62-281-638794, ♥email: aulidya.nurul.habibah@unsoed.ac.id

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Abstract. *Nugrahesthi GH, Wijayanti GE, Habibah AN. 2023. Embryo and larvae development of Nilem Fish,* Osteochilus vittatus *reared in batik liquid waste. Nusantara Bioscience 15: 105-112.* The embryonic and larval stages are critical phases of an organism's development. For aquatic organisms, development is affected by environmental factors such as liquid waste. A batik liquid waste is a waste product of the textile industry usually streamed directly into the aquatic environment. This study aimed to observe the effects of exposure to batik liquid waste effects on developing Nilem (*Osteochilus vittatus* Valenciennes, 1842) fish embryos and larvae. The research was conducted using a completely randomized design. One hundred two-celled embryos were kept in batik liquid waste with dilution concentrations of 0% (the control), 5%, 10%, 15%, and 20% of water until the age of 4 days after hatching with five replications. The time of embryo evaluation was 60th minutes, 120th minutes, and 180th minutes after fertilization; the larval stage evaluation time was the 24th, 48th, 72nd, and 96th hour after fertilization; five embryos were evaluated for each replication. The results showed that embryo exposure to batik liquid waste affected the height of the blastoderm embryo, accumulation of waste in the chorion of the embryo, deceleration of embryonic development, increased larval abnormalities, decreased the survival rate of larvae, and acceleration of yolk absorption of fish larvae. Embryos were successfully hatched and produced larvae only in the control and 5% batik liquid waste medium. Batik liquid waste interfered with *O. vittatus* embryo development and generated mortality above 5%.

Keywords: Batik waste, blastoderm, development, embryogenesis, Nilem Fish

INTRODUCTION

Batik was recognized as one of the cultural heritage of Indonesia by the United Nations, Educational, Scientific and Cultural Organization (UNESCO). Therefore, it enhanced batik production and increased economic growth (Ariyanti et al. 2021). Small and medium entrepreneurs carry out batik production; they conduct traditional techniques for producing batik (Muslimah et al. 2020). This technique generated wastewater released before treatment into the river or environment (Muslimah et al. 2020).

The wastewater problem hinders increasing batik industry production (Indrayani et al. 2020; Oedjijono et al. 2021). Batik liquid waste contains dyes and organic compounds that contaminate water bodies. Some techniques are required to separate and degrade the organic compounds from the dyes, requiring a continuous process (Ariyanti et al. 2021). Wirosoedarmo et al. (2019) stated that wastewater produced by the batik industry contains dark-colored organic waste, elevated temperature, pH, Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD), and Total Suspended Solid (TSS) content. The wastewater is due to chemical and coloring substances used during the batik production and coloration process. Batik production comprises techniques: canting, hand-drawn batik, block, printing, *tritik*, and tie-dye. Canting is widely used in the small batik industry; this is a traditional technique for making batik. There are six stages for making batik through the traditional technique: material preparation, waxing, dyeing, dye fixation, wax disposal, and product drying. These processes run as follows: the cloth was submerged into the desired color and toward the sun drying. Dye use during the preparation process was done if only necessary. The following step was dipping in starch solution; this process aimed to shed the glossy effect. Rinsing and sun drying are the next steps. Next, the waxing process uses a block design, dipping into the wax, then applying it to the cloth. The dyeing process applies the desired color of batik; The cloth is submerged into a sodium silicate solution for about 3-4 hours, then submerged in water. The following steps are rinsing and drying, also wax disposal. This process takes about 1-1.5 hours for submerging, boiling, and rinsing; 18 hours for submerging before the batik product's drying process. Washing and rinsing are carried out repeatedly to remove the oil, excessive wax, and dyes (Daud et al. 2022). These batik-making processes mostly utilize clean and not-so-clean water; the water utilization quantity for batik production is related to the wastewater (Handayani et al. 2021). The water used in batik production created an environmental problem (Widianarko et al. 2021). Batik production is part of the textile industry that is the major contributor to environmental water contamination. Therefore, water treatment before releasing wastewater from the textile industry, including batik production, is necessary. However, batik industries' wastewater is usually disposed of without treatment. This batik liquid water consists of several toxic components of heavy metals such as chromium, ferrum (Fe), lead (Pb),

silicon (Si), calcium (Ca), magnesium (Mg), cuprum (Cu), and zinc (Zn) (Daud et al. 2022).

Heavy metals were toxic to the fish embryo; for example, they caused subtraction of the eye size of the embryo (Zhang et al. 2015). Therefore, heavy metal is a major pollutant for freshwater fish species. Furthermore, several reports on heavy metal affected many fish species length and body weight (Häder et al. 2021), such as chromium, copper, and cadmium, affected the Pejjerey fish's embryo (*Odonthestes bonariensis* Valenciennes, 1835) (Miranda and Somoza 2022). Also, chromium significantly affected the development of the *Danio rerio* Hamilton, 1822 (zebrafish) embryo; it caused several abnormalities such as yolk sac edema, faint pigmentation, impairing skeleton, pericardial edema, lordosis, hatching overdue, tail shortening, notable subtraction of development of the embryonic eye, movements of the embryo, and heartbeats (Nisha et al. 2020). Furthermore, Xu et al. (2021) investigated chromium's effect on zebrafish larvae; it proved that chromium had a neurotoxicity effect on zebrafish embryos. In addition, Krzykwa et al. (2018) stated that neurological and cardiovascular systems are more reliable for measurable endpoints of toxicity tests.

Like heavy metals, dyes, especially synthetic dyes, were harmful to aquatic animals like fish. Several eco-toxicological effects from dyes for fish, such as alteration of enzyme activity, morphology, size, histopathological significances, and diminishing of carbohydrates, lipids, and protein in tissue, resulted in mortality (Al-Tohamy et al. 2022). Several dyes used for batik making usually depend on fabric material. For example, indigo and naphthol dyes were used for cotton and linen (Dailin et al. 2022). Indigo dye was insoluble in water, and naphthol contained more hazardous chemicals; this was harmful to fish (Dailin et al. 2022). Nasrin et al. (2022) examined Basic Red 18 dye to tilapia. Therefore, this dye was toxic for the treated fish; it showed causing abnormalities in several organs: gills, heart, stomach, intestine, and liver of *Tilapia mossambica* Peters, 1852.

Moreover, fish usually were used to asses water status (Braunbeck et al. 2014). The embryo phase is the critical phase of the organism. This phase is one of the most important stages for increasing the production in fish aquaculture (Olaniyi and Omitogun 2013). Moreover, Dahlke et al. (2020) stated that the embryo and larvae stages were the critical phase in determining their developmental stage further. Therefore, the embryo was a convenient model for testing some toxic material, and this stage was sensitive and suitable for the toxicology test (Wlodkowic and Campana 2021).

Furthermore, batik liquid waste damage the chorion of Nilem Fish embryo (Habibah et al. 2022). As the embryo protector, the chorion was totally damaged in the incubation of 20% batik liquid waste for 24 hours. Embryos of Nilem Fish were incubated in several batik liquid waste concentrations: 5%, 10%, 15%, 20%, and 0% as the control. Observation of the chorion showed a breakdown of the chorion in 20% batik liquid concentration. However, research about the effects of batik liquid waste on embryo and larva development of Nilem Fish were not conducted yet. Therefore, this research aimed to determine the effect of batik liquid waste exposure on embryos and larvae development of Nilem Fish (*Osteochilus vittatus* Valenciennes, 1842).

MATERIALS AND METHODS

Material

This study used Nilem Fish (*O. vittatus*), 10% Neutral Buffered Formalin (NBF) as a fixative solution, 0,9% NaCl solution for diluting fish milt, cavity slides, and eyepiece micrometer. The study used a Completely Randomized Design (CRD) model with five batik liquid-waste medium concentrations for embryo rearing medium. These five concentrations were 0% (control), 5%, 10%, 15%, and 20%. The evaluation was conducted at the embryonic development stage of 60^{th}, 120^{th}, and 180^{th} minutes after fertilization and the larval stage of 24^{th}, 48^{th}, 72^{nd}, and 96^{th} hours after fertilization. The treatment was replicated five times. Therefore, a total of 25 experimental units were maintained during the research, and 110 embryos per unit.

Procedures

Dilution of batik liquid waste

Batik liquid waste was obtained from the waste collection tank after the dyeing process of the batik industry in Sokaraja Sub-district, Banyumas District, Central Java, Indonesia. Batik liquid waste was diluted to fulfill percentage levels of 5%, 10%, 15%, and 20%. The dilution of batik waste was done by adding water obtained from the campus environment of the Faculty of Biology, Universitas Jenderal Soedirman, Banyumas District, Central Java, Indonesia. Wastewater from each percentage level was put into a basin with the addition of water until it reached ± 1,000 mL volume and was equipped with aeration.

Assisted spawning of Nilem Fish

Mature females and males of Nilem Fish were induced using GnRH analog (Ovaprim) at a dose of 0.5 mL/kg for females and 0.3 mL/kg for males (Simanjuntak and Wijayanti 2005). After 8 hours of induction, induced fish were then stripped. Eggs collected were accommodated in a container, a small concave basin with size 15 cm in diameter until assisted fertilization takes 5-10 minutes. Milt of male fish was taken using a syringe without a needle and then diluted with 0.9% NaCl solution with a ratio of 1:9. Assisted fertilization was done by putting milt into a container containing eggs and slowly adding water to activate spermatozoa and gently shaking the container. After the eggs reached the 2-cell stage, the eggs were put into each basin of the experimental unit equipped with aeration.

Sampling of embryo and larvae

Samples of 25 embryos were obtained from each treatment. In addition, five embryos were collected from each basin of 5 replication for each treatment. Embryo samples were taken at incubation times of 60^{th} minutes, 120^{th} minutes, and 180^{th} minutes. A sampling of embryos that had developed into larvae was continued at the 24^{th}, 48^{th}, 72^{nd}, and 96^{th} hours. All samples obtained were immediately fixed in a 10% NBF fixative solution.

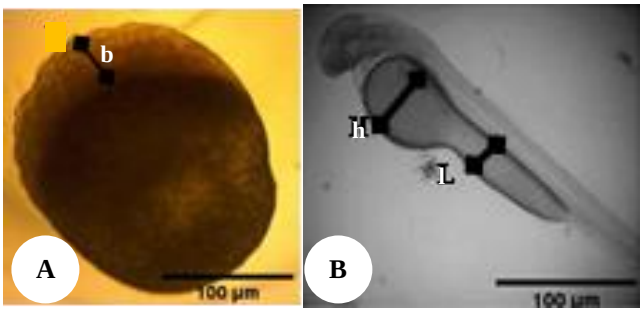

Figure 1. A. Embryo of Nilem Fish (*O. vittatus*) morula stage. Description: b. Height of blastoderm. B. Nilem larvae age 96 hours (Windari 2013). Description: l. Elongated yolk sac, h. Shortened yolk sac

Embryo and larvae observation

Embryos and larvae were observed under a light microscope at an objective magnification of 4 X. The blastoderm height was measured using an eyepiece micrometer previously calibrated. Measurement of blastoderm height is represented in the Figure 1A.

The proportion of an abnormal sample was observed and quantified. Abnormal embryos were categorized if there was the disintegration of the vitelline membrane, the blastomere size was not uniform, or a blastomere detached from the blastoderm and was found in the perivitelline space (Hallare et al. 2005). Abnormal larvae were categorized if there were one or more forms of abnormality, such as pericardial edema, bent or curved tail vertebrae, and bent or curved pectoral fins (Mukti 2005). The cumulative time required in the development process to reach a certain stage was calculated and recorded. Then the cumulative development time of treatment embryos was compared with the cumulative development time of the control embryos. Finally, the percentage of larval survival rate was calculated from the number of larvae that lived at the end of each evaluation time compared to the total larvae of initial rearing, which was 400 embryos.

The yolk absorption rate was calculated based on the volume of the yolk sac using an eyepiece micrometer with the following formula:

$$LPKT = \frac{V_0 - V_t}{T}$$

Where:
LPKT : Yolk sac absorption rate
V0 : Early yolk sac volume
Vt : End yolk sac volume
T : Time (hour)

V=0,1667 π 〖LH〗 ^2

V : Egg yolk volume
L : Elongated yolk sac diameter
H : Shortened yolk sac diameter

The measurement of the length of the yolk sac diameter is represented in the Figure 1B.

Data analysis

Height of embryo blastoderm, abnormal embryo, and larvae, cumulative development time, larval survival rate, and yolk absorption rate were analyzed using ANOVA with SPSS software version 16.0. The data were first tested for homogeneity using Levene and normality using Kolmogorov-Smirnov before being analyzed using ANOVA. Then, when data were normally distributed and homogenous, it was analyzed using ANOVA or Independent Sample T-Test (if only two data groups were being tested) with a significance of P<0.05. Finally, post hoc analysis was carried out to determine the time of embryo maintenance with the highest level of damage. The post hoc used was the Tukey HSD for data with ANOVA significance value <5%.

RESULTS AND DISCUSSION

Height of embryo blastoderm

Blastoderm formation indicates embryo development after fertilization (Figure 2). The average blastoderm height at the 60th and 180th minutes evaluation time showed subtle differences (p= 0.08 and p=0.232, respectively). Therefore, it can be interpreted that the batik liquid waste treatment has no significant effect (p>0.05) on the height of the embryo's blastoderm at 60th and 180th minutes after fertilization. Meanwhile, the results for 120th minutes evaluation time showed a significance value (p = 0.002), which means that the treatment of batik liquid waste has an effect (p<0.05) on the height size of the embryo's blastoderm. Therefore, Tukey HSD further tests were carried out to know the details of the concentration of batik liquid waste, which affects the size of the blastoderm height at the 120th minutes evaluation time. Based on Tukey's test, 20% batik liquid waste significantly affected the mean height of the blastoderm embryo, which was 271.72±19.68 µm.

Embryo abnormality-2

Batik liquid waste was not affected (p>0.05) on the level of abnormality of Nilem Fish embryos at all three evaluation times (60th minutes, 120th minutes, 180th minutes) (Figure 2). Abnormalities of the control and treatment embryos tend to show a constant abnormality rate. This was possible because the chorion function, which acts as a protector, could still protect the embryo in the range of immersion time of 60th minutes, 120th minutes, and 180th minutes. The chorion that surrounds the embryo functions as a means of respiration and excretion of the embryo. In addition, the chorion also acts as the first line of defense against foreign substances entering the embryos. Research on the development of zebrafish embryos by Henn and Braunbeck (2011) proved the role of the chorion as a protector. It revealed that embryos without chorion incubated in pronase increased mortality, which was not found in these control embryos. Furthermore, the number of coagulated embryos identified physical damage to dechorionized embryos.

Larvae abnormality

Embryos reared in batik liquid waste at concentrations of 10%, 15%, and 20% did not continue until the hatching

stage, so the treatment did not obtain larvae (Figure 3). The Independent Samples T-Test (between the control larvae and those exposed to 5% batik liquid waste) showed a significant value of ($p<0.001$) (Figure 4), which means that batik liquid waste had a highly affected ($p<0.01$) on the level of larval abnormalities resulted hatching failure at 24 hours post-fertilization (hpf). Larval abnormalities included pericardial edema, spinal curvature, and bent pectoral fins (Figure 5).

Larvae survival rate

Batik liquid waste affected the survival rate of Nilem Fish. The concentration of 5% batik liquid waste decreased the survival rate of Nilem and along with the evaluation time (Figure 6).

Larval yolk absorption rate

Larval yolk absorption was observed between control and treatment group of 5% batik liquid waste medium. The absorption of yolk was presented in Figure 7.

Time cumulative development

The time required for each stage to complete the stages of development is called the time cumulative development (Table 1).

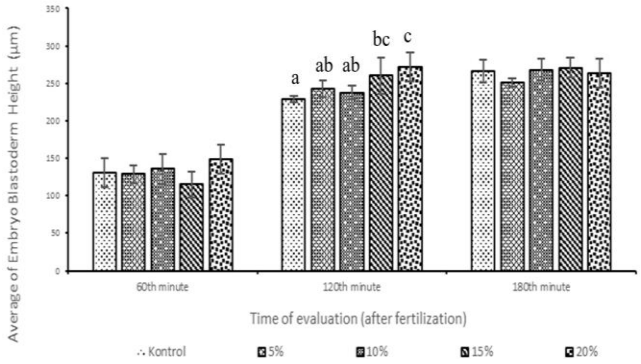

Figure 2. Average and the standard deviation of embryo blastoderm height of Nilem Fish (*Osteochilus vittatus*) in batik liquid waste medium and control at 3 evaluation times; 60th minute, 120th minute, and 180th minute after fertilization. Note: different letters were significant

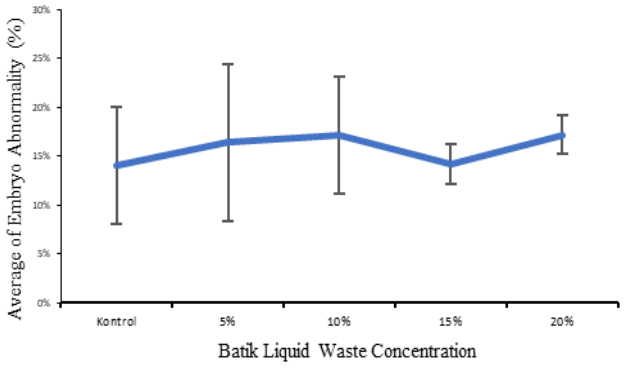

Figure 3. Average and standard of deviation of embryo abnormality of Nilem Fish (*O. vittatus)* control and in batik liquid waste medium

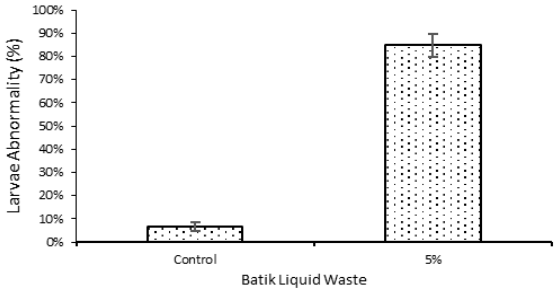

Figure 4. Abnormality of Nilem Fish larvae (*O. vittatus*) control and in 5% batik liquid waste medium

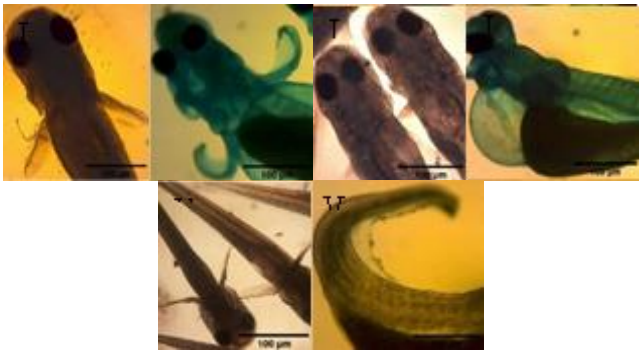

Figure 5. Morphology of pectoral fins, heart, and vertebrae of Nilem (*O. vittatus*) larvae 0% (Control) and 5% in batik liquid waste medium: I. Morphology of the control group larvae of pectoral fins, II. The morphology of the pectoral fins in the 5% batik

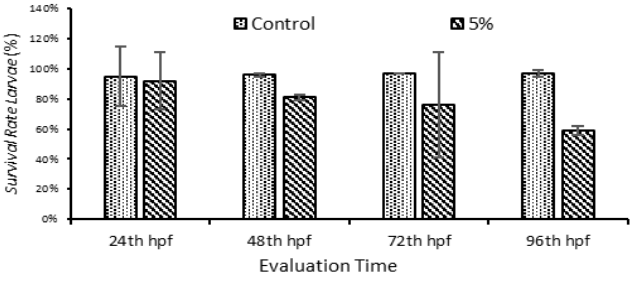

Figure 6. Larvae survival rate and standard of deviation of Nilem Fish (*O. vittatus*) in 5% batik liquid waste medium

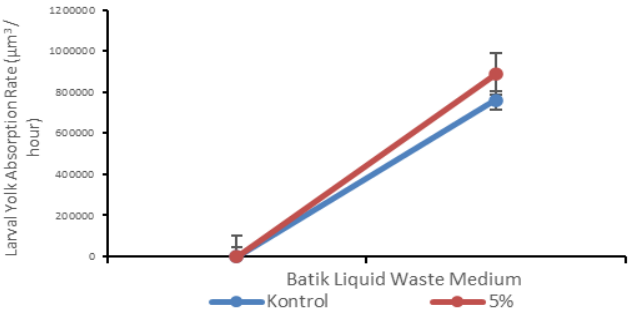

Figure 7. Absorption rate of yolk larvae Nilem Fish (*O. vittatus*) the control and in 5% batik liquid waste medium

Table 1. Cumulative developmental time (minutes) of Nilem Fish embryo (*O. vittatus*) control and in batik liquid waste for each embryonic stage

Developmental stage	Batik liquid waste concentration	Cumulative time of embryo development (minutes)				
		0% (Control)	5%	10%	15%	20%
2 cell–Morula		59±3[b]	40.33±3,51[a]	105.67±2,51[c]	113.67±2,08[d]	57.33±2,08[b]
Morula–Blastula		14.66±1.15[a]	30.33±4.50[b]	49±1.00[d]	42.66±3.51[cd]	40.33±1.52[c]
Blastula–Gastrula		66.66±3.78[b]	63±3.00[ab]	63.33±0.57[ab]	66±2.00[ab]	57±5.56[a]
Gastrula–Hatching		1390.3±2.51	1997.7±3.78	-	-	-

Discussion

The development of the embryos of the research subjects was affected by toxicants when the embryos entered the two-cell stage or 30th minutes after fertilization. The development of blastoderm embryos in the control and reared in batik liquid waste at 60th minutes tended to be normal due to the short exposure to batik liquid waste. Embryo development up to the 60th minute was included in the early stages of embryo development so that maternal factors largely regulated development. Furthermore, O'Farrell (2015) suggested that the development of eggs outside the mother's body was supported by maternal factors deposited into the oocyte cytoplasm during the oogenesis phase so that the oocyte has been prepared by the mother to start the cell division cycle.

Embryos in batik liquid waste at the time of evaluation of 120th minutes tend to show faster development than the controls; the higher average of height of the blastoderm proved it. The highest blastoderm size indicates the embryo is at the peak stage of the blastula. At 120th minutes, the control embryos were in the late morula stage until the early blastula, while the embryo in exposure to batik liquid waste currently entered the blastula stage. Toxicants accelerate blastula development in embryos exposed to batik liquid waste; it is assumed this stage possesses high sensitivity to toxicants. Häder and Erzinger (2018) stated that conditions of water temperature, water pH, and form of toxicants affect the level of toxicity and sensitivity of organisms to toxicants. O'Farrell (2015) suggested that embryonic development during the late blastula stage to early gastrula is a transitional cycle at the end of development involving maternal gene products toward zygotic transcriptional activation. This embryonic development is characterized by the appearance of cell shape and morphogenetic changes in a relatively short period. Changes in internal factors due to the activation of embryonic genes occur, especially in development at the 120th minute to the 180th minute. The development of the control embryos at the 180th minute reached the late gastrula stage. In contrast, the embryos reared in the waste showed an average development up to the late blastula or early gastrula stage. Furthermore, the development of embryos exposed to batik waste would be continued because of the possibility of embryos initiating adaptation mechanisms to tolerate toxicity due to the presence of the chorion as a protective blastomere (Braunbeck et al. 2005).

In this study, developmental deterioration was experienced by embryos in exposure to batik liquid waste treatment. The maintenance of treatment embryos at 180th minutes showed development to the late blastula or early gastrula stage, while the control embryos reached the late gastrula stage. Von Hellfeld et al. (2020) reported the developmental retardation of late blastula to early gastrula in zebrafish (*D. rerio*) embryos exposed to toxic mercury. Delayed embryo hatching evidenced the developmental delay in this research subjects in 5% batik liquid waste-treated embryos with a delay of about 60th minutes longer when compared to the control embryos. Meanwhile, embryos in batik liquid waste treatment above 5% did not hatch. Slow developments due to exposure to toxicants, as suggested by Küçükoğlu et al. (2013), on zebrafish (*D. rerio*) embryos on exposure to zinc chloride began to hatch on the 7th day after fertilization, compared to the control embryos hatched on the 4th day after fertilization. Moreover, Nisha et al. (2020) found that toxic material (chromium) in water caused abnormalities in zebrafish embryos, such as eye and tail development. Like zebrafish embryos, chromium that might be involved in the batik liquid waste becomes toxic to Nilem Fish embryos. The mechanism of heavy metal could cause abnormalities in fish embryos through oxidative stress, and free radicals evocation was an indication.

The accumulation of batik liquid waste in the chorion was characterized by color alteration of the chorion layer. The higher the concentration of batik liquid waste, the darker the color. The role of the chorion as a respiratory and excretory pathway was supported by a pore structure evenly distributed throughout the chorion layer. The small pore size has the potential as a route for toxicants to enter the embryo. Toxicants that successfully penetrated the chorion layer were able to interfere with the development of the embryo's blastomere. Habibah et al. (2022) investigated the chorion and oolemma of Nilem Fish embryos reared in batik liquid waste. It showed that a concentration of 20% batik liquid waste affected supreme damage of chorion and oolemma compared to other concentrations, 0%, 5%, 10%, and 15%.

The cumulative time required for embryos in batik liquid waste medium to reach certain stages of development is shown in Table 1. Batik liquid waste at a concentration of 10% to 20% interferes with embryonic development time, especially at the morula to blastula stages of development. The 10% and 15% concentrations of batik liquid waste tended to slow the development, while the 20% concentration tended to accelerate the development to the early gastrula stage. The formation of morula-stage blastoderm by 10% and 15% batik liquid waste embryos in long duration is due to the toxicant content that has

successfully penetrated the chorion, as reported by Feitosa et al. (2021) that accumulation of toxicants around the chorion causes a decrease in oxygen levels so that it affects embryonic development. The difference in development time can be caused by the quantity of the toxicant used as a medium for embryo maintenance. The high toxicant content of batik liquid waste, especially at a dilution concentration of 20%, may accelerate embryo development. However, the development will be stopped at a certain stage due to toxicants that damage the blastoderm. In line with the observations, embryos reared in waste concentrations above 5% stopped at the late gastrula stage, so they did not develop into larvae. The difference in hatching duration of 5% batik liquid waste embryos was about ±60 minutes compared to the control embryos. Batik liquid waste with high polarity and a small molecular weight is estimated to interfere with the normal development of embryos and cause embryos to experience hatching delays (Cao et al. 2009).

The anomaly observed in the test could be attributed to the inhibition of DNA synthesis, which causes protein and enzyme activity disruption due to excessive levels of toxicants. Research by Scholz et al. (2008) reported that the activity of certain transporter proteins, such as the ATP binding cassette-transporter, a transporter that can prevent the entry of certain molecules into cells, can be affected by environmental chemicals. Inhibition of the transporter activity can potentially increase the entry of toxicants through the chorion so that the number of toxicants in contact with the embryo increases. In addition, toxicant interactions at the molecular level could cause gene transcription or protein expression changes, which can disrupt embryonic metabolism. Researchers Wiegand et al. (1999) suggested that embryos exposed to toxicants will increase excretory metabolism by controlling stress due to exposure to foreign substances. One of the metabolisms observed is the increase in glutathione as a detoxifying enzyme. Increased activity of glutathione allows detoxification to be carried out through the physiological degradation of toxicants. However, the activity of detoxifying enzymes cannot tolerate high concentrations of toxicants.

Changes in the rearing environment in the form of toxicants resulted in abnormal larval development. Changes in the shape of the vertebrae can characterize the form of larval abnormalities. Researcher Küçükoğlu et al. (2013) informed that one of the mechanisms that cause vertebral damage is disruption during early vertebral development caused by inhibition of collagen synthesis. Toxic substances contained in batik liquid waste inhibited collagen synthesis during the development of vertebrae of Nilem Fish larvae during the study.

The survival rate of the control group larvae tends to be stable; there was no change, while 5% batik liquid waste larvae have decreased linearly (Figure 6). The respiratory disorders due to toxicants in the waste were assumed to cause the decreased survival rate of larvae reared in 5% batik liquid waste from time to time. In addition to the mouth, exposure to toxicants can enter through the gill surface, which would then be transported to the entire surface area of the fish's body (De Oliveira et al. 2016). The linear decreased curve was in line with the increase in larval yolk consumption. Yolk as an energy source was used by larvae until they reached the age of day 4 or 96 hours after fertilization. Research by Wulandari et al. (2020) reported that yolk shrinkage in 24 hours post-fertilization (hpf) larvae tended to be faster than yolk shrinkage in 36 hpf to 96 hpf larvae. Based on observing the 5% batik liquid waste larval survival rate reduction curve, the external feed can be given earlier at 50 hpf to replace the energy source previously provided by the yolk.

The larval yolk absorption rate showed a higher value of 5% batik liquid waste than the control (Figure 7). Rearing in batik liquid waste caused stress on Nilem Fish larvae. Under stressful conditions, larvae require more energy, so their metabolic rate is faster than the control larvae. A higher metabolic rate caused higher energy consumption. When yolk reserves were nearly depleted, the metabolic needs were fulfilled from tissue-level catabolism for energy production. Therefore, the external feed can be given earlier, shortly after the opening of the larvae's mouth, to replace the energy source used (Mariska et al. 2013). The increase in the absorption rate of 5% batik liquid waste larvae was due to the larval effort to maintain homeostasis to stay alive by hydrolysis of the yolk into the larval body tissue. Research by Wiegand et al. (1999) informed that the enzymatic process in embryos requires additional energy used for growth, but in embryos exposed to toxicants, more energy was needed for detoxification. Research by Long et al. (2011) reported that the detoxification of aquatic organisms was mediated by the ATP Binding-Protein Cassette (ABC) transporter. Gills, as organs of excretion and detoxification, were the main link between animals and their environment. Exposure to toxicants causes an increase in the expression of ABC transporter genes in the gills to limit the absorption of environmental toxicants.

Based on the results of the research that has been done, it can be concluded that the exposure of batik liquid waste to embryos in the third observation evaluation time (60^{th} minutes, 120^{th} minutes, and 180^{th} minutes) affected the height of the blastoderm of the embryo, deteriorate the development of the embryo, and accumulated batik liquid waste in the chorion characterized by changing the color of the embryo's chorion. Furthermore, exposure to batik liquid waste on larvae aged 24 to 96 hours after fertilization also increased larval abnormalities, decreased larval survival rates, and accelerated the absorption rate of larvae yolks.

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Anti-nephrotoxic activity of aqueous extract of polyherbal mixture against renal toxicity induced by paracetamol in Wistar albino rats

MICHAEL SUNDAY ABU[1,♥], RUKAIYAT LAWAL MASHI[2], JAMILA YAHAYA LAWAL[2], OYEBISI SUNDAY SAMUEL[1]

[1]Department of Biochemistry, Faculty of Pure and Applied Sciences, Federal University Wukari. Ibi Road, 670102, PMB 1020, Taraba State, Nigeria. Tel.: +234-7064945026, ♥email: abulex77@yahoo.com

[2]Department of Biological Science, Federal College of Education Katsina. Dutsin-ma Road, Katsina, Nigeria

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Abstract. *Abu MS, Mashi RL, Lawal JY, Samuel OS. 2023. Anti-nephrotoxic activity of aqueous extract of polyherbal mixture against renal toxicity induced by paracetamol in Wistar albino rats. Nusantara Bioscience 15: 112-117.* Medicinal plant materials as sources of therapeutic compounds continue to play an important role in maintaining human health for ages. This study investigated the effect of polyherbal extract (*Carica papaya* L., *Allium sativum* L., *Curcuma longa* L., and *Azadirachta indica* A.Juss.) on urea, creatinine, sodium ion, potassium ion, and chloride ion concentrations, and body weight of Wistar rats intoxicated with paracetamol. Thirty Wistar rats were randomly distributed into six groups, with five in each group. Group 1 is the normal control group; Group 2 is the negative control group (paracetamol-induced but untreated); Group 3 received 140 $mgkg^{-1}$ of silymarin; Groups 4, 5, and 6 received 100, 300, and 500 $mgkg^{-1}$ body of the aqueous polyherbal extract respectively for seven days. Therefore, the blood samples were collected and evaluated for creatinine, urea, sodium, potassium, and chloride ions concentrations to measure kidney function. The results revealed that the aqueous extract of the polyherbal mixture significantly ($p<0.05$) ameliorated the kidney function test parameters that were analyzed by lowering their concentrations previously elevated by the paracetamol intoxication comparable with the normal control rats. Therefore, it can be concluded that the polyherbal mixture extract expressed an anti-nephrotoxic effect against renal toxicity induced by paracetamol in Wistar albino rats.

Keywords: Acetaminophen, aqueous extract, nephrotoxicity, paracetamol, polyherbal, renal toxicity

INTRODUCTION

The liver, gastric tract, and kidneys metabolize drugs in the body. Drug and metabolite excretion can take one of two routes: renal or extra-renal (Perazella 2019). In terms of kidney elimination, medications can be removed via either glomerular filtering or tubular secretion. Each excretion pathway exposes the tubules and the interstitium to potentially harmful chemicals. The proximal tubules are exposed by apical contact with chemicals released into the tubular lumen, tubular epithelial cell absorption, or apical efflux from the peritubular circulation (the basolateral regions of tubular cells) into the tubular lumen (Perazella 2019). The chemicals are secreted from the proximal tubule into the loop of Henle and then into the distal tubule via glomerular filtration and tubular secretory traffic. In addition, drugs may precipitate, crystallize, or create casts in the distal tubules, resulting in tubular blockage (Luque et al. 2017). Another pathway involves interstitial tubule inflammation, which causes interstitial nephritis (Chamarthi et al. 2018). Therefore, drug-induced nephrotoxicity occurs through one of the three routes mentioned (Zuk and Bonventre 2016). Finally, a dose-dependent mechanism is caused by apical contact with drugs or their metabolites, transport of drugs and metabolites from the apical surface, and secretion of drugs from the basolateral surface into the tubular lumen (Chawla et al. 2017), which causes interstitial nephritis (Qu et al. 2017).

Allium sativum L., also known as garlic, is a member of the Amaryllidaceae family. Its biological qualities have been known to humans for many centuries. Garlic is native to Central Asia and has long been used as a crop in the Mediterranean region and as a flavoring in continents such as Africa and Europe. India is the world's second-largest producer of garlic after China (Raj et al. 2022). Garlic's anti-inflammatory, antioxidant, antibacterial, antiviral, antifungal, and antimutagenic effects are well known (Tsai et al. 2012).

Papaya (*Carica papaya* L.) is a popular and commercially significant species in tropical nations (Nugroho et al. 2017). Traditionally, it has been used to cure various conditions such as gastrointestinal problems, diarrhea, skin illnesses, male contraception, and cold household remedies. Numerous studies have found that papaya has anticancer properties for colon, prostate, cervical, and breast malignancies. Extracts of the chosen plant's fruit, seeds, and leaves have also been demonstrated to have strong cytotoxic activity against cancer cell lines such as breast, liver, and cancer of hematopoietic cell lines (Singh et al. 2020).

Although neem (*Azadirachta indica* A.Juss.) is native to India, the Meliaceae family is widely distributed globally, and it can thrive in most tropical and subtropical nations, including Indonesia. In Indonesia, Neem is known as Imba, Nimba, or Mimba (Sitasiwi et al. 2018). According to

previous studies, neem leaf extract has numerous biological and pharmacological activities such as antipyretic, analgesic, antihepatotoxic (Ogbuewu et al. 2011), spermicide, anti-implantation (Asif 2013; Sarkar et al. 2021), antihyperglycemic, antiulcer, antifungal, antibacterial, anti-inflammatory, immunomodulatory, antimutagenic, anticancer, antimalarial, antiviral, antioxidant (Alzohairy 2016; Gupta et al. 2017), antifertility (Gbotolorun et al. 2008), and contraception (Kumar et al. 2016).

Curcuma longa L. (turmeric or curcuma) is a rhizomatous monocotyledonous annual herbaceous plant in the ginger family (Zingiberaceae), native to tropical and temperate areas such as India, China, and Southeast Asia. India is the world's major producer, user, and exporter of turmeric (Trujillo et al. 2013). Curcuma is derived from the Arabic term Kourkoum, which was the original name for saffron (Benzie and Wachtel-Galor 2011). Curcuminoids have been consumed as therapeutic infusions over the years worldwide. In Ayurvedic medicine, curcumin is a well-documented treatment for various respiratory ailments such as asthma, bronchial hyperactivity, and allergy, as well as for liver disorders, rheumatism, anorexia, runny nose, cough, sinusitis, and diabetic wounds (Araújo and Leon 2001).

This study investigated the effect of the aqueous extract of a herbal mixture (*C. papaya, A. sativum, C. longa,* and *A. indica*) on urea, creatinine, sodium ion, potassium ion, chloride ion concentrations, and body weight of Wistar rats intoxicated with paracetamol.

MATERIALS AND METHODS

Study area

This study was conducted at the Biochemistry Department, Federal University Wukari Nigeria, Taraba State, Nigeria, from October 2022-2023.

Plant collection and identification

Matured and healthy-looking *C. papaya*, *A. indica, C. longa,* and *A. sativum* were collected from Wukari, Taraba State. The plants were washed with tap water thoroughly to remove the dust and soil particles. The leaves were air-dried under the shade to prevent ultraviolet rays from inactivating the chemical constituents (Ncube et al. 2008; Das et al. 2010). The dried planets were pulverized into a fine powder by a mortar and pestle, stored, and labeled in dry containers until needed.

Extract preparation

One hundred and twenty-five (125) grams of each pulverized plant powder was mixed and soaked for 48 hours in 250 mL of distilled water with periodic stirring and mixing (Abubakar and Haque 2020). The solution was subsequently sieved through Whatman filter paper. The extract, after filtration, was evaporated and concentrated using a water bath at 99°C. The percentage yield was calculated as 15.7%. The extract was stored at 4°C until further analysis.

Experimental animals

The rats weighing 150-230 g were purchased from Wukari, Taraba State, Nigeria, and were allowed to be acclimatized in the Animal House of the Department of Biochemistry, Federal University Wukari, for two weeks before the commencement of the experiment. All the rats were allowed free access to water ad libitum and feed throughout the experiment.

Animal grouping

Thirty (30) Wistar albino rats were used for the study. The rats were distributed into six groups of five rats in each group. The rats received the following treatment schedule:

Group 1: Non-paracetamol-induced rats (normal control).

Group 2: Paracetamol-induced rats (negative control), 500 $mgkg^{-1}$ body weight paracetamol.

Group 3: Paracetamol-induced (500 $mgKg^{-1}$) Nephrotoxic rats treated with 140 $mgkg^{-1}$ of silymarin (standard control) (Hamidian et al. 2020).

Group 4: Paracetamol-induced (500 $mgKg^{-1}$) Nephrotoxic rats treated with 100 $mgkg^{-1}$ body weight of extract.

Group 5: Paracetamol-induced (500 $mgKg^{-1}$) Nephrotoxic rats treated with 300 $mgkg^{-1}$ body weight of extract.

Group 6: paracetamol-induced (500 $mgKg^{-1}$) Nephrotoxic rats treated with 500 $mgkg^{-1}$ body weight of extract.

Treatment and induction

Nephrotoxicity was induced by oral administration of paracetamol 500 $mgkg^{-1}$ body weight after dissolution of paracetamol in distilled water. The paracetamol administration was continued for ten days. After three days of paracetamol induction, treatments with the extract of the mixture of *C. papaya, A. indica, C. longa, A. sativum*, and standard drug (silymarin) was carried out concomitantly with the induction for seven days. At the end of the experimental period, the rats were fasted for twelve (12) hours, anesthetized using chloroform, and sacrificed. Consequently, blood was collected from the heart via cardiac puncture using sterile syringes and needles to analyze kidney function tests.

Estimation of serum sodium, potassium, and chloride ions

A flame photometer Model 143, equipped with an automatic diluter Model 144 (ratio of the dilution of 200:1) (Instrumentation Laboratory, Inc., Lexington, Mass., USA), was used. The flame photometer was calibrated with twice distilled water and a standard with a Na^+ concentration of 140 mequiv/L and a K^+ concentration of 5 mequiv/L (Instrumentation Laboratory, Inc., Lexington, Mass., USA). After each sample measurement, the instrument's stability was checked with the standard solution.

Determination of serum urea concentration

The serum urea concentration was assessed using the method described by Fawcett and Scout (1960).

Principle: Urease breaks down urea into ammonia and carbon dioxide. In an alkaline medium, ammonia reacts with hypochlorite and salicylate to form dicarboxy indophenol, a colored compound. The reaction is catalyzed

by sodium nitroprusside. The intensity of color produced is measured spectrophotometrically at 578 nm.

$$\text{Urea} + H_2O \xrightarrow[\text{Urease}]{} 2NH_3 + CO_3$$

$$NH_3 + \text{Hypochlorite} + \text{salicylate} \rightarrow \text{dicarboxyindophenols (blue compound)} + CO_3$$

Procedure: Reagent (1 mL) containing sodium nitroprusside and urease was added into three clean test tubes labeled as a test sample, standard, and reagent blank containing 0.01 mL sample, 0.01 mL standard reagent, and 0.01 mL distilled water, respectively. The content in each test tube was mixed and incubated at room temperature (25-30°C) for 10 minutes. The absorbance of the test sample and standard were read against the reagent blank at 578 nm.

Calculation: The serum urea concentration was calculated using the formula below:

$$\text{Urea Conc. (mg/dl)} = \frac{\text{Absorbance of Test}}{\text{Absorbance of Standard}} \; x \; \text{Concentrationof Standard}$$

BUN Concentration (mg/dL) = 0.467 x Urea Concentration (mg/dL)

Determination of serum creatinine concentration

The colorimetric method was used to determine serum creatinine concentration, according to Bertels et al. (1973).

Principle: Creatinine in the serum reacts with alkaline picrate to form a colored complex. The rate of formation of the colored complex is directly proportional to creatinine concentration. This reaction rate (intensity of orange color produced) is measured colorimetrically at 510 nm and compared with the standards.

$$\underset{\text{(Yellow)}}{\text{Creatinine} + \text{Picric acid}} \xrightarrow[\text{(Alkaline medium)}]{\text{NaOH}} \underset{\text{(Orange)}}{\text{Creatinine picrate (510 nm)}}$$

Procedure: Working reagent (1 mL) containing picric acid and sodium hydroxide was added into two clean test tubes labeled sample test and standard, containing 0.1 mL of the test sample and 0.1 mL of standard solution. The content in each test tube was mixed, and after 20 seconds, the absorbance of the standard (ST1) and test sample (TS1) was read at 510 nm. Exactly 80 seconds later, absorbance for (ST2) and (TS2) of the standard and sample were read at 510 nm against distilled water (blank).

Calculation: The Concentration of creatinine in serum (mg/dL) was calculated using the formula below:

$$\text{Creatinine Conc. (mg/dl)} = \frac{TS2 - TS1}{ST2 - ST1} \; x \; \text{Concentration of Standard}$$

Where:
ST : Standard
TS : Test sample

Statistical analysis

The biochemical results were subjected to statistical analysis using One-Way Analysis of Variance (ANOVA) and further duncan multiple comparisons using Statistical Package for Social Science (SPSS) version 21. The means were compared for significance at $p<0.05$, and the group results were presented as mean ± standard deviation.

RESULTS AND DISCUSSION

Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) on electrolytes levels of experimental rats

The study revealed a significant ($p<0.05$) increase in the concentration of sodium ions in Group 2 compared to Group 1. In contrast, Groups 3, 4, and 5 showed a significant ($p>0.05$) decrease in sodium ion levels compared to Groups 1 and 2. However, Group 6 did not display significant differences compared with Group 1 but decreased significantly ($p<0.05$) compared to Group 2. This implies that the paracetamol's adverse effects on the kidney were experimentally reversed, as shown in Table 1.

Also, Groups 2 and 3 showed a significant ($p<0.05$) rise in potassium ion concentration compared to Groups 1, 4, 5, and 6. Although, numerically, Group 3 has a lower potassium ion value than Group 2. Meanwhile, Groups 1, 4, 5, and 6 were all observed to be significantly ($p<0.05$) lower in potassium concentration when compared to Group 2.

Moreover, a significant ($p<0.05$) increase in the chlorine level in Group 2 compared to Groups 1, 3, 4, 5, and 6 was observed. The experiment demonstrated a significant ($p< 0.05$) reduction in chlorine concentration in Groups 3, 4, 5, and 6, comparable with Group 1 due to the extracts' administration.

Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on urea level of experimental rats

There was a significant ($p<0.05$) rise in the concentration of urea in Group 2 as compared with the other groups. The urea level in Groups 3, 4, 5, and 6 was ($p<0.05$) significantly ($p<0.05$) lowered after the treatments with both the silymarin and the polyherbal extract when compared with Group 2. However, the values in these groups were significantly higher compared to Group 1, which served as the normal control in the experiment. Repairing the paracetamol damage increases the urea's excretion rate and reduces its accumulation in the system, as evident in Figure 1.

Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on creatinine level of experimental rats

The creatinine concentration demonstrated a significant ($p<0.05$) increase in Groups 2, 3, and 4 compared to Groups 1, 5, and 6. However, upon treatments, Groups 5 and 6 significantly ($p<0.05$) reduced compared to Groups 1 and 2. The increased creatinine concentration indicated renal obstruction in Groups 2, 3, and 4, while the evidential reduction in Groups 1, 5, and 6 was attributed to kidney clearance, as shown in Figure 2.

Table 1. Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on serum electrolytes of albino rats

Groups	Na^+ (mmol/L)	K^+ (mmol/L)	Cl^- (mmol/L)
Group 1	175.77± 13.11^b	8.70 ± 2.83^c	118.37 ± 3.90^b
Group 2	250.06 ± 1.43^a	17.89 ± 1.15^a	139.82 ± 27.61^a
Group 3	145.03 ± 63.62^c	15.90 ± 6.51^a	121.16 ± 2.05^b
Group 4	102.65 ± 7.67^d	6.55 ± 0.92^c	113.35 ± 030^c
Group 5	140.58 ± 91.19^c	11.95 ± 5.16^b	118.69 ± 0.33^b
Group 6	188.52 ± 13.44^b	7.65 ± 2.05^c	109.93 ± 12.05^c

No = 5; the result is represented as mean ± standard deviation. Results within a column with the same superscript indicate no level of significance (p>0.05), while result within a column with different superscripts indicates a level of significance (p<0.05). Group 1: Non-paracetamol-induced rats (Normal control), Group 2: Paracetamol-induced rats (negative control), 500 mgkg^{-1}body weight paracetamol; Group 3, Paracetamol-induced (500 mgKg^{-1}) Nephrotoxic rats treated with 140 mgkg^{-1} of silymarin (standard control) (Hamidian et al. 2020), Group 4: Paracetamol-induced (500 mgKg^{-1}) Nephrotoxic rats treated with 100 mgkg^{-1} body weight of extract, Group 5: Paracetamol-induced (500 mgKg^{-1}) Nephrotoxic rats treated with 300 mgkg^{-1} body weight of extract, Group 6: paracetamol-induced (500 mgKg^{-1}) Nephrotoxic rats treated with 500 mgkg^{-1} body weight of extract.

Table 2. Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on body weight of albino rats

Groups	Body weight		
	Week 0 (g)	Week 1 (g)	Week 2 (g)
Group 1	142.68±15.72^b	170.00±11.78^a	178.00±14.79^a
Group 2	162.34±17.51^a	158.60±13.46^a	163.20±12.58^a
Group 3	136.26±5.93^b	144.00±3.63^a	150.00±15.14^a
Group 4	158.80±14.72^b	174.60±14.92^a	172.60±14.32^a
Group 5	140.66±5.45^b	148.40±4.8^{ab}	156.60±12.66^a
Group 6	137.40±13.19^b	152.00±15.4^a	158.00±16.88^a

No = 5; the result is represented as mean ± standard deviation. Results within a row with the same superscript indicate no level of significance (p>0.05) difference, while different superscripts indicate a level of significance (p< 0.05)

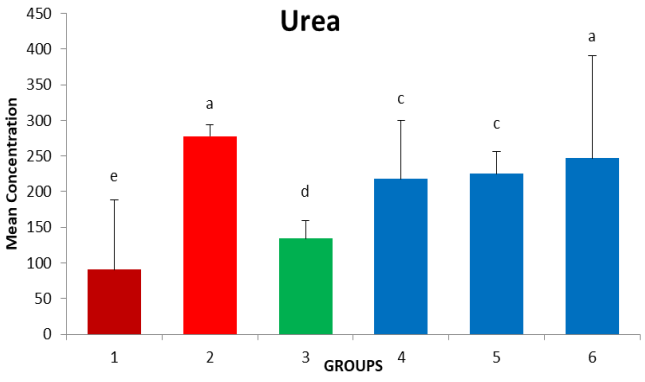

Figure 1. Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on urea of albino rats. No = 5; the result is represented as mean ± standard deviation. Results with the same superscript indicate no level of significance (p>0.05) difference, while different superscripts indicate a level of significance (p< 0.05)

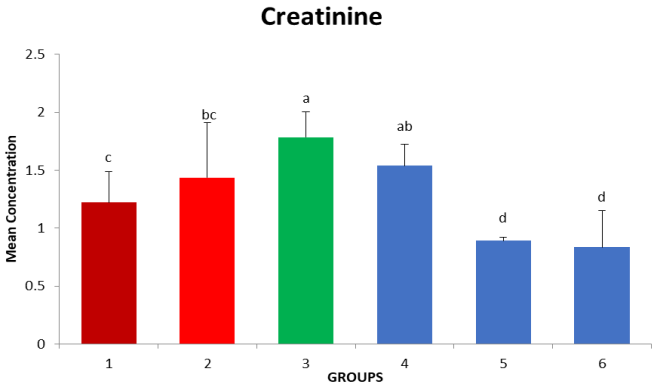

Figure 2. Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on creatinine of albino rats. No = 5; the result is represented as mean ± standard deviation. Results with the same superscript indicate no level of significance (p>0.05) difference, while different superscripts indicate a level of significance (p< 0.05)

Effect of polyherbal (*Carica papaya, Allium sativum, Curcuma longa,* and *Azadirachta indica*) extract on body weight of experimental rats

The average body weight of Group 2 rats in week 0, showed no significant (p<0.05) difference compared to the week 2 body weight. However, Groups 1, 3, 4, 5, and 6 in week 0 displayed a significant (p<0.05) lower body weight compared to week 2. The weight gain experienced in the extract-treated groups was attributed to kidney damage restoration, as shown in Table 2.

Discussion

In this research, upon induction of the rats with 500 mgkg^{-1} of paracetamol, the levels of the various kidney function parameters determined were significantly (p<0.05) increased. Meanwhile, administration of the different concentrations of the polyherbal extract (*C. papaya, A. sativum, C. longa,* and *A. indica*) and silymarin considerably reduced the levels of these parameters in the intoxicated rats, which were comparable with the normal control rats.

One of the most essential electrolytes in extracellular fluid is sodium, an osmotically active cation. It is in charge of controlling extracellular fluid volume as well as regulating cell membrane potential. The bulk of sodium reabsorption occurs in the kidney's proximal tubule (Shrimanker et al. 2022). Sodium transport occurs via sodium-chloride symporters activated by the hormone aldosterone; potassium functions primarily as an intracellular ion. The sodium-potassium adenosine triphosphatase pump is the major regulator of sodium and potassium homeostasis, pumping sodium out in exchange for potassium, which goes into the cells (Udensi and Tchounwou 2017). The glomerulus in the kidneys is where potassium is filtered. Chloride is an anion that is mostly present in extracellular fluid. The kidneys are the primary regulators of serum chloride levels. Most chloride filtered by the glomerulus is reabsorbed by both the proximal and

distal tubules (mostly the proximal tubule) via active and passive transport (Gattineni and Baum 2015).

Usually, nephrology defects caused by xenobiotics, such as CCl_4 and DEN toxicity, may truncate renal functions and result in irregular distribution of these ions in the ECF (Ganie et al. 2011), as it was the case of this research where paracetamol was able to cause similar alteration. However, the treatments with 100, 300, and 500 $mgkg^1$ of the polyherbal extract significantly ($p<0.05$) lowered the levels of sodium, potassium, and chloride ions of the paracetamol-intoxicated rats compared with the normal control and the standard drug groups after seven days of daily oral administration. This result can be deduced that administering the evaluated polyherbal extract can ameliorate the elevated level of serum electrolytes orchestrated by paracetamol toxicity, probably due to some bioactive components such as polyphenol, glycoside, and flavonoid in the different plants. The normalization of electrolytes in this study could liken to the findings of Abu et al. (2022), that used an n-butanol fraction of *F. glumosa* to reverse the distorted levels of sodium, chloride, and potassium ions in albino rats.

Amino acid deamination occurs in the liver, which is also the site of the urea cycle, where ammonia is converted into urea and excreted through urine (David et al. 2014). The urea excretory function of the kidney is often obstructed by injuries arising from toxic substances, as experienced with paracetamol in the present report. Whereas creatinine is a breakdown waste product formed in the muscle by creatinine phosphate metabolism, its retention in the blood is evidence of kidney impairment. However, it was revealed that the administration of 100, 300, and 500 $mgkg^{-1}$ of the polyherbal extract reduced urea and creatinine levels significantly ($p<0.05$) compared to the untreated Group 2. However, the reductions were not sufficiently comparable with the Group 1 and Group 3 rats. A similar finding was observed by the administration of *A. marmelos* (Kore et al. 2011), *T. terrestris* (Abdel-Kader et al. 2016), and *B. diffusa* (Olaleye et al. 2010) in rats have also been reported. This report has established that the aqueous extract of the polyherbal mixture examined can ameliorate the high urea and creatinine levels in the paracetamol-intoxicated rats by exploring the wound healing and antioxidant capacities of the varying phytoconstituents of the plants. On the other hand, the increased serum creatinine and urea level in the paracetamol-induced rats decreased the glomerular filtration rate in rats (Jesurun and Lavakumar 2016). The mechanism behind elevated serum urea and creatinine might be that the paracetamol increases calcium ions' entry into the mesangial cells, leading to a reduced glomerular filtration rate (Stojiljković et al. 2008). However, urea can also increase other ailments such as upper gastrointestinal bleeding, dehydration, catabolic process, and a high-protein diet (Gounden et al. 2023). Consequently, the near-normal reversal of the elevated creatinine and urea in this report might result from the mechanical reversal or obstruction of the paracetamol nephrotoxicity pathway in the experimental rats by the polyherbal extract.

Furthermore, Kpela et al. (2013) also found that the rat fed with the neem leaf extract at 500 $mgkg^{-1}$ body weight dose for 14 days caused a decrease in urea and creatinine concentrations in the rat induced by cisplatin for kidney damage. Based on the result obtained, induction of paracetamol without treatment showed no significant ($p<0.05$) increase in the body weight in Group 2 in week 0 compared to week 2, indicating a weight gain restriction. However, the administration of the polyherbal extract in the other groups showed a significant ($p>0.05$) increase in body weight between weeks 0 and 2, which may be an indication of restoration of normalcy from the nephrotoxic effects of the paracetamol. These findings also support the previous studies (Ali et al. 2005). The insignificant impart in the body weight in Group 2 could be attributed to the decrease in the animal's oral food intake due to acidosis and anorexia caused by acute kidney injury (Basile et al. 2012). Reduced body weight may have also been due to impaired water reabsorption by damaged renal tubules and consequent dehydration (Ali et al. 2005).

In conclusion, based on the result obtained, aqueous extract of polyherbal extract (*C. papaya, A. sativum, C. longa*, and *A. indica*) ameliorated the lost body weight. In addition, it reversed kidney functions of nephrotoxic albino rats, as evidenced by urea, creatinine, and electrolyte results.

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Butterfly (Lepidoptera: Papilionoidae) diversity and structure community in Lumajang, East Java, Indonesia

NAJMATUL MILLAH♥, AMIN SETYO LEKSONO♥♥, BAGYO YANUWIADI

Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Brawijaya. Jl. Veteran, Malang 65145, East Java, Indonesia.
Tel.: +62-341-575841, ♥email: najmatulm@gmail.com, ♥♥amin28@ub.ac.id

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Abstract. *Millah N, Leksono AS, Yanuwiadi B. 2023. Butterfly (Lepidoptera: Papilionoidae) diversity and structure community in Lumajang, East Java, Indonesia. Nusantara Bioscience 15: 118-128.* Lumajang is one of the district in East Java, Indonesia with an area of around 1,790.90 km^2 with various ecosystems. Unfortunately, several ecosystems in Lumajang have been degraded, affecting biodiversity, especially butterflies. The aim of this study was to analyze the structure of butterfly communities in Lumajang and analyze the species as bioindicators. The study was conducted in 5 habitats: Village, Agriculture, City Park, Community Forest, and Conservation Forest. The butterfly sampling method in this study was an Active Visual Survey using the Transect Pollard Walk; environmental factors were also measured during the study (temperature, humidity, light intensity, wind velocity, and altitude). The butterfly diversity and the measurement of environmental factors were analyzed for correlation using the Principal Component Analysis (PCA). The results of the study consisted of 124 species. Several were endemic species (*Cyrestis lutea* (Zincken, 1831), *Ypthima nigricans* (Snellen, 1892), *Parantica albata* (Zincken, 1831), *Mycalesis sudra* (Felder, 1867), and *Pachliopta adamas* (Zincken, 1831)), then protected species (*Troides helena* (Linnaeus, 1758), *Troides cuneifera* (Oberthür, 1879), and *Troides amphrysus* (Cramer, 1779)). The Shannon-Wienner diversity index values from highest to lowest are Community Forest (3.52), Conservation Forest (3.32), City Park (2.98), Village (2.79), and Agriculture (2.29). The results can be used as basic data and are expected to support butterfly conservation strategies in Indonesia, especially in Lumajang, East Java, Indonesia.

Keywords: Butterfly, community structure, diversity, Lepidoptera, Lumajang

INTRODUCTION

Butterflies are insects that have many roles in functioning ecological systems. Butterflies also play an important role in a plant's reproductive success because of their role as pollinator agents (Pe'er and Settele 2013). Butterflies and vegetation have a tight correlation because butterflies require vegetation for larval feeding so butterflies can be used as an indicator of the presence of specific plant species (Miller III et al. 2011). Butterflies also serve as bioindicator agents because of their sensitivity to environmental changes (Forister et al. 2010; Ren et al. 2022). Changes in environmental conditions can be observed in the structure of the butterfly community changes, both in terms of abundance and its correlation with the environment (Zografou et al. 2014).

The number of individuals and insect populations is decreasing yearly, and some species are even threatened with extinction globally; one of the endangered insects are thea butterfly (Cardoso et al. 2020; Warren et al. 2021). Declining butterfly populations can disrupt ecosystem services, disrupting phylogenetic diversity, functional and ecological networks (Cardoso et al. 2020). The decline in butterfly populations is caused by anthropogenic factors such as pollution (industrial pollution and pesticides), invasive species, climate change, exploitation, and declining hostplant species (Zografou et al. 2014; Cardoso et al. 2020). One of the biggest threats to butterflies is habitat degradation (Basset et al. 2015). Degradation and fragmentation of habitats can affect butterfly populations because habitat conditions can affect an area's biodiversity (Koneri et al. 2020). Degraded habitats will result in the loss of areas where butterflies seek to feed and reproduce, while fragmented habitats will increase isolated species (Haddad et al. 2015).

Butterfly studies are important because they have many roles in functioning ecological systems, as mentioned before, at the population and ecosystem levels. Furthermore, butterflies can be easily observed and directly identified by their wing patterns; if they cannot be identified directly, they can be caught, tagged, or photographed and released (Ren et al. 2022). The study of butterfly community structure generally describes the diversity of butterflies present in a habitat in terms of abundance and how the butterfly correlates with other organisms (Adey and Loveland 2007). Studying the diversity and structure of the butterfly community worldwide is very popular, especially in Indonesia. Indonesia has a high butterfly population, with over 2,000 butterflies from 17,280 species worldwide (Peggie and Harmonis 2014; Shahroni et al. 2022). Several studies of butterfly diversity in Indonesia, especially in Java, have been carried out in various habitats, such as forest areas (Widhiono 2015) and urban areas (Leksono et al. 2016; Azizah et al. 2021). In addition, approximately 640 species of butterflies on the island of Java have been recorded, and 46 are endemic species (Peggie and Harmonis 2014; Shahroni et al. 2022).

Studies on similar topics in Lumajang are still very limited, especially on the communities structure and the diversity of butterflies. Lumajang is one of the districts located in eastern Java. Lumajang has an area of about 1,790.90 km^2 with various ecosystems, such as agricultural areas, forest areas, bushes, residential areas, and other areas, such as lakes, rivers, waterfalls, and wells. Based on topography, Lumajang has a land altitude ranging from 0 to > 2,000 meters above sea level (masl), so it also has coastal and mountain areas (RPJMD of Lumajang District, 2018-2023). Based on this, Lumajang has a high diversity of ecosystems that can affect the high species diversity, including butterflies. Some ecosystems have changed due to land conversion, thus threatening the diversity and existence of butterflies. Data on the diversity of butterflies in Lumajang is limited, so monitoring the butterfly population changes is difficult. Thus, the study aims to analyze the structure of butterfly communities in Lumajang, East Java, as basic data useful for butterflies conservation strategies and to find species with a high sensitivity to environmental changes that have the potential as bioindicator agents for environmental quality.

MATERIALS AND METHODS

Study site

The study was conducted at the end of the dry season, from July to September 2022, in Lumajang, East Java, Indonesia (Figures 1 and 2). The study was conducted in five locations: Village, Agriculture, City Park, Conservation Forest, and Community Forest (Table 1). The selection of these different study locations is based on the land use of each location so that it can represent several types of ecosystems in Lumajang. There are three transect lines in each location, and each transect has a length of 600 m. Therefore, distances between one transect line and another vary according to the conditions of each location, ranging from 300 to 600 m.

Sampling procedurs

The butterfly sampling was conducted using the active visual survey method with the Pollard Walk Transect. The Pollard Walk Transect is visualized within the view of 5 m side-by-side and 5 m forward when walking through the transect line by direct observation or with cameras and insect nets (Montgomery et al. 2021). The samplings were conducted from 8:30 AM to 3:30 PM and repeated five times on different days (Ren et al. 2022). Butterfly observations included the identification of species and counting the number of individuals. Identification of butterfly species was based on observing the morphological characters. Furthermore, the butterfly species identification was by the butterfly application Kupunesia and several identification books by Smith and Vane-Wright (2008), Wilson (2008), Rositawati (2017), Baskoro et al. (2018), Fitri (2021), and Iqbal et al. (2021).

Environmental factors were measured by air temperature and humidity measurements using a thermo-hygro-meter, light intensity using a lux meter, wind velocity using an anemometer, and altitude using the Global Positioning System (GPS) at the beginning and the end of each transect line.

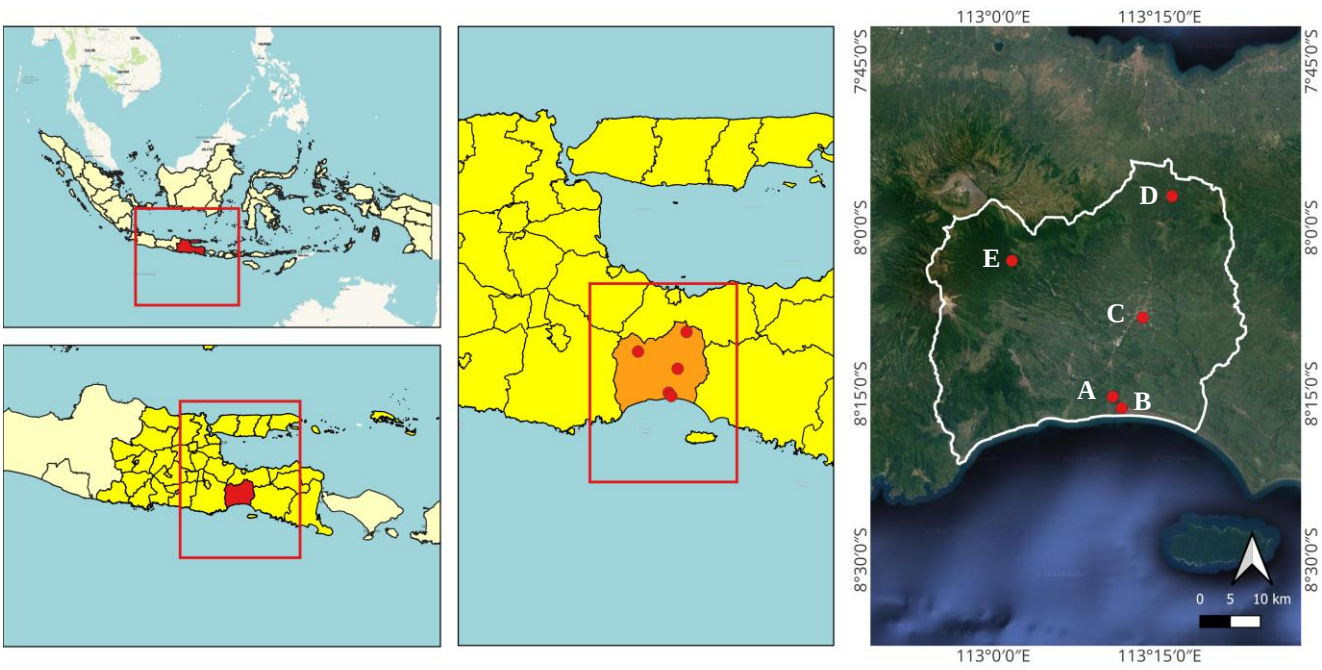

Figure 1. Map of study site in Lumajang, East Java, Indonesia. Note: A. Village, B. Agriculture, C. City Park, D. Community Forest, E. Conservation Forest

Table 1. Description of the conditions of the study site habitat

Habitat	Coordinate	Altitude	Condition
Village	8°15'15.67"S 113°10'46.54"E	28-40 m asl.	Artificial ecosystems, high human activities, controlled vegetation growth, trees, shrubs, and herbs exist. The vegetation found in this area is *Mangifera indica* L. (Anacardiaceae), *Carica papaya* L. (Caricaceae), *Citrus* spp. (Rutaceae), and *Acalypha siamensis* Oliv. ex Gage (Euphorbiaceae).
Agriculture	8°16'16.48"S 113°11'35.10"E	4-12 m asl.	Artificial ecosystem, controlled vegetation, use of fertilizers and pesticides, and high human activity. The vegetation found in this area are *Pennisetum purpureum* Schumach. (Poaceae), *Digitaria ciliaris* (Retz.) Koeler (Poaceae), *Ipom*ea spp. (Convolvulaceae), and *Oryza sativa* L. (Poaceae).
City Park	8°08'06.72"S 113°13'28.13"E	55-58 m asl.	Artificial ecosystems, plants selected based on certain functions (pollution and water absorption) and high human activity. The vegetation found in this area are Arecaceae, *Pterocarpus indicus* Willd. (Fabaceae), *Eupatorium capillifolium* (Lam.) Small (Asteraceae), *Pseuderanthemum reticulatum* (hort. ex Hook.fil.) Radlk. (Acanthaceae), and *Cupressus Papuanus* (Cupressaceae).
Community Forest	7°57'09.47"S 113°16'07.47"E	167-280 m asl.	In the secondary forest that local people use, some plants grow naturally and are cultivated; there are human activities (traveling, grazing, and fishing). The vegetation found in this area are *Pennisetum purpureum* Schumach. (Poaceae), *Mimosa pudica* L. (Fabaceae), *Tridax procumbens* L. (Asteraceae), *Hibiscus tiliaceus* L. (Malvaceae), *Ipomea* sp. (Convolvulaceae), *Antigonon leptopus* Hook. & Arn. (Polygonaceae), and Arecaceae.
Conservation Forest	8°02'58.93"S 113°01'40.34"E	1103-1420 m asl.	In the Primary Forest and conservation area, plants grow naturally. They are very diverse, dominated by trees, so the canopy tends to be more closed when compared to other locations, with minimal human activity. The found in this area are Malvaceae, *Swietenia mahagoni* (L.) Jacq. (Meliaceae), *Ficus* spp. (Moraceae), *Toona sureni* (Blume) Merr. (Meliaceae), *Albizia chinensis* (Osbeck) Merr. (Fabaceae), and Urticaceae.

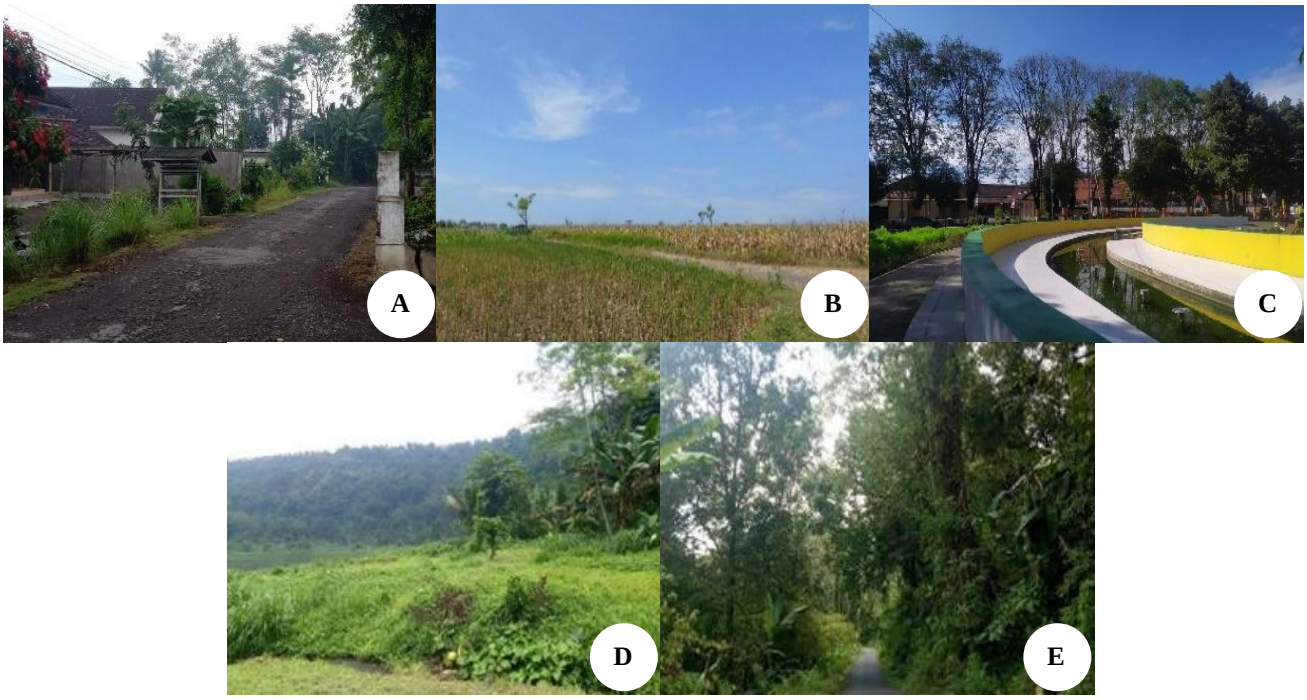

Figure 2. Study site in Lumajang, East Java, Indonesia. A. Village, B. Agriculture, C. City Park, D. Community Forest, E. Conservation Forest

Data analysis

Butterfly data on species and individual numbers are analyzed for diversity values regarding species abundance (n) and taxa richness (S). The diversity index used is the Shannon-Wienner diversity index (H'=-Σpi ln pi) to describe the diversity of butterflies. The Eveness (E=H'/lnS) to determine the pattern of species distribution (Magurran 2004; Ismail et al. 2020). The results of the diversity values are analyzed using the One-Way ANOVA test that aims to analyze different butterfly diversity compositions at each study site. Furthermore, Indval analysis is carried out to measure the percentage of

association for all species found in all habitats through butterflies' consistency and relative abundance. Indval analysis is useful to identify species that could be indicator species in each habitat (Bugno-Pogoda and Durak 2021). All analyses were calculated with the PAST 4.09 software (Hammer et al. 2001).

Individual analysis was also carried out to measure the percentage of associations for all species found in all habitats through their relative consistency and abundance; this analysis is useful for identifying species that could be indicators in a habitat (Bugno-Pogoda and Durak 2021). Individual analysis was calculated using PAST 4.09 (Hammer et al. 2001). Data from measuring environmental factors were analyzed using the Kruskal Wallis test using R studio software (Hollander et al. 1973). The relationship between environmental factors and butterfly diversity correlation analysis using a biplot Principal Component Analysis (PCA) by PAST 4.09 software (Hammer et al. 2001).

RESULTS AND DISCUSSION

Community structure of butterfly species

The study of butterflies in Lumajang in several habitats found 2,958 individuals from 124 species and six families (Table 2). The family consists of Hesperiidae, Lycaenidae, Nympalidae, Papilionidae, Pieridae, and Riodinidae. The family that has the highest number of species and individuals is Nymphalidae, which has 63 species (51.22%) and 1,420 individuals (47.97%), and the smallest were from the Riodinidae family, only 1 species (0.81%) and 1 individual (0.03%) (Table 2). The study recorded several endemic and protected species. The endemic species are *Cyrestis lutea* (Zincken, 1831), *Mycalesis sudra* (Felder, 1867), *Ypthima nigricans* (Snellen, 1892), and *Pachliopta adamas* (Zincken, 1831). Meanwhile, three protected species were recorded from the genus of *Troides*, consisting of *T. helena* (Linnaeus, 1758), *T. cuneifera* (Oberthür, 1879), and *T. amphrysus* (Cramer, 1779) (Table 2 and Figure 6).

Diversity of butterfly species

Diversity analysis showed that the Community forests have the highest species richness and abundance (1,372 individuals dan 72 species). While the lowest number of species is in Agricultural areas (30 species), and the lowest abundance is in City park. (280 individuals). The results of the analysis of the Shannon-Wiener diversity index and Evennes index were the highest in Community forests (H'=3.52 and E=0.47), and the lowest value was an agricultural area (H'=2.12 and E = 0.30) (table 3). The results of the one-way ANOVA analysis showed that between the five study sites, there were significant differences in terms of species richness (F= 23.15, p= 0.00005), abundance (F=11.41, p=0.001), Shannon-Wiener diversity index (F = 17.18, p= 0.0002), and Evennes index (F=5.82, p=0.01) (Figure 3).

Indicator species analysis (Indval)

The Indval analysis shows the species can be used as an indicator species if the Indval value is close to 100% and has a significance value of $p<0.05$. The analysis results in Table 2 showed that Agriculture has six species that can be used as indicators. These species have Indval values above 50% and significance values $p<0.05$; the species consist of *Danaus chrysippus* (Linnaeus, 1758), *Hypolimnas missippus* (Linnaeus, 1764), *Phalanta phalantha* (Drury, 1773), *Graphium agamemnon* (Linnaeus, 1758), *Appias lyncida* (Cramer, 1779), and *Eurema sari* (Horsfield, 1829). In the Village area, six indicator species were found, such as *Borbo cinnara* (Wallace, 1866), *Zizula hylax* (Fabricius, 1775), *Euploea core* (Cramer, 1780), *Papilio memnon* (Linnaeus, 1758), *Papilio polytes* (Linnaeus, 1758), and *Appias olferna* (Swinhoe, 1890). In the City Park, two species could be indicator species, including *Tanaecia pelea* (Fabricius, 1787) and *Delias periboea* (Godart, 1819). The Community Forest have the highest indicator species among other locations, 22 species have a significance value $p<0,05$, and 12 have a perfect indval value (100%). The species consist of *Chersonnesia rahria* (Westwood, 1857), *Hebomoia glaucippe* (Linnaeus, 1758), *Ideopsis juventa* (Cramer, 1777), *Mycalesis horsfieldii* (Moore, 1892), *Mycalesis janardana* (Moore, 1857), *Orsotriaena medus* (Fabricius, 1775), *Pachliopta adamas* (Zinken, 1831), and *Ypthima horsfieldii* (Moore, 1884). There are 21 species as indicators in Conservation forests, and 11 have an indval value of 100%. These species include *Heliophorus epicles* (Godart, 1823), *Ideopsis gaura* (Horsfield, 1829), *Notocrypta curvifascia* (C. Felder & R.Felder, 1862), *Papilio helenus* (Linnaeus, 1758), *Symbrenthia anna* (Semper, 1888), *Symbrenthia hypselis* (Godart, 1823), *Tanaecia japis* (Godart, 1823), *Troides cuneifera* (Snellen, 1889), *Udara akasa* (Horsfield, 1828), *Vagrans egista* (Cramer, 1780), and *Ypthima nigricans* (Snellen, 1892).

Environmental factors

The result of measuring the environmental quality of all habitats varies. For example, the Kruskal Wallis test showed that temperature (X2= 8,607, p= 0,072) and humidity (X2= 7,067, p= 0,132) had no significant differences between habitats. Meanwhile, the wind speed (X2= 13,987, p= 0,017), the intensity of the signal (X2= 11,433, p= 0.022), and the altitude (X2= 13,5, p= 0.009) between habitats have significant differences (Table 4; Figure 4).

Correlation between butterfly species diversity and environmental factors

The PCA analysis used five variables of environmental parameters and two results of an analysis of butterfly diversity in five habitats. The total variance in the results of the PCA analysis is 77.5%. PC 1 explains 52.8% of the variance, and PC 2 explains 24.7%. The results of the PCA analysis showed that the City park and Agricultural had high temperature, light intensity, and wind velocity characteristics. In contrast, low humidity resulted in low diversity and evenness of butterflies. Conversely, the Community forest was characterized by high diversity and evenness of butterfly populations with moderate temperature and humidity (Figure 4).

Table 2. List species each family and Indval value

Species	Agriculture		Village		City Park		Community Forest		Conservation Forest	
	Indval (%)	*p*	Indval (%)	*p*	Indval (%)	*p*	Indval (%)	*p*	Indval (%)	*p*
Hesperiidae										
Ancistroides sp.	0	1	0	1	0	1	33.33	0.2026	0	1
Borbo sp.	33.33	0.206	0	1	0	1	0	1	0	1
Borobo cinnara	0	1	66.67	0.029	0	1	0	1	0	1
Notocrypta curvifascia	0	1	0	1	0	1	0	1	100	0.0019
Oriens gola	25.64	0.176	35.9	0.084	0	1	2.564	0.731	0	1
Pelopidas sp.	0	1	0	1	8.333	0.520	50	0.053	0	1
Pirdana sp.	0	1	33.33	0.201	0	1	0	1	0	1
Potanthus omaha	40	0.082	0	1	0	1	26.67	0.1553	0	1
Potanthus sp.	0	1	0	1	0	1	0	1	33.33	0.198
Pseudocoladenia dan	0	1	0	1	0	1	50	0.0389	25	0.1534
Suastus gremius	0	1	0	1	33.33	0.203	0	1	0	1
Tagiades ultra	0	1	0	1	0	1	0	1	33.33	0.1975
Telicota augias	0	1	0	1	0	1	33.33	0.1982	0	1
Lycaenidae										
Acytolepis Puspa	0	1	0	1	0	1	33.33	0.2026	0	1
Arhopala centaurus	0	1	6.667	0.741	26.67	0.241	6.667	0.737	6.667	0.7406
Castalius rosimon	0	1	0	1	0	1	33.33	0.1986	0	1
Heliophorus epicles	0	1	0	1	0	1	0	1	100	0.0019
Jamides sp.	0	1	0	1	0	1	50	0.0191	33.33	0.1111
Lampides sp.	0	1	2.778	0.639	0	1	0	1	91.67	0.0035
Leptotes plinius	0	1	12.82	0.272	0	1	61.54	0.0092	0	1
Loxura atymnus	0	1	0	1	0	1	0	1	33.33	0.1948
Luthrodes pandava	4.255	0.489	8.511	0.401	0	1	80.85	0.0103	0	1
Miletus sp.	0	1	0	1	0	1	33.33	0.1982	0	1
Nacaduba sp.	0	1	0	1	16.67	0.377	0	1	16.67	0.3686
Prosotas dubiosa	0	1	0	1	0	1	0	1	33.33	0.1975
Ramelana jangala	0	1	0	1	33.33	0.203	0	1	0	1
Udara akasa	0	1	0	1	0	1	0	1	100	0.0019
Udara sp.	0	1	0	1	0	1	0	1	33.33	0.1948
Zizina otis	0	1	1.333	0.737	7.556	0.421	73.33	0.0087	0	1
Zizula hylax	0.2331	0.880	49.65	0.027	8.159	0.540	25.17	0.1628	0	1
Nymphalidae										
Acraea issoria	0	1	0	1	0	1	0	1	33.33	0.1948
Acraea tepsicore	44.44	0.087	0	1	11.11	0.512	0	1	0	1
Amanthusia pidippus	0	1	33.33	0.201	0	1	0	1	0	1
Ariadne ariadne	0	1	33.33	0.200	0	1	0	1	0	1
Chersonesia rahria	0	1	0	1	0	1	100	0.0022	0	1
Cupha erymanthis	0	1	33.33	0.206	0	1	0	1	0	1
*Cyrestis lutea**	0	1	0	1	0	1	0	1	66.67	0.0284
Danaus chrysippus	77.97	0.002	2.26	0.671	0.565	0.833	11.3	0.3663	0	1
Dichorragia nesimachus	0	1	0	1	0	1	0	1	33.33	0.1975
Discophora celinde	0	1	33.33	0.201	0	1	0	1	0	1
Dolescalia bisaltidae	11.11	0.577	18.52	0.370	18.52	0.377	27.78	0.1464	0	1
Elimnias hypermnestera	1.55	0.931	16.28	0.382	26.36	0.173	41.86	0.0399	0	1
Euploea climena	0	1	0	1	0	1	7.692	0.444	88.46	0.0035
Euploea core	0	1	62.5	0.013	0	1	25	0.179	0	1
Euploea corrina	0	1	0	1	100	0.002	0	1	0	1
Euploea eleusina	0	1	33.33	0.206	0	1	0	1	0	1
Euploea eunice	0	1	0	1	0	1	0	1	66.67	0.0284
Euploea mulciber	26.32	0.163	3.509	0.816	0	1	63.16	0.0094	0	1
Euthalia aconthea	0	1	100	0.002	0	1	0	1	0	1
Euthalia malaccana	0	1	0	1	0	1	0	1	33.33	0.1975
Euthalia sp.	0	1	0	1	0	1	33.33	0.2026	0	1
Faunis canens	0	1	0	1	0	1	0	1	66.67	0.0266
Hypolimnas anomala	0	1	0	1	0	1	33.33	0.2026	0	1
Hypolimnas bolina	3.922	0.828	32.35	0.099	44.12	0.037	7.843	0.6675	0	1
Hypolimnas missippus	66.67	0.032	0	1	0	1	0	1	0	1
Ideopsis gaura	0	1	0	1	0	1	0	1	100	0.0019
Ideopsis juventa	0	1	0	1	0	1	100	0.0022	0	1
Ideopsis vulgaris	0	1	0	1	0	1	66.67	0.0291	0	1
Junonia almana	9.677	0.303	1.075	0.789	0	1	58.06	0.0417	0	1
Junonia atlites	6.061	0.545	1.515	0.775	6.061	0.556	45.45	0.05	0	1

Junonia erigone	0	1	0	1	0	1	33.33	0.1986	0	1
Junonia hedonia	0	1	0.5602	0.611	0	1	98.32	0.0022	0	1
Junonia iphita	0	1	0	1	0	1	99.36	0.0022	0.2123	0.6366
Junonia orithya	40	0.078	0	1	0	1	13.33	0.3716	0	1
Lete confusa	0	1	0	1	0	1	0	1	33.33	0.1948
Lethe minerva	0	1	0	1	0	1	33.33	0.199	0	1
Melanitis leda	11.11	0.507	11.11	0.520	0	1	11.11	0.524	0	1
Mycalesis horsfieldi	0	1	0	1	0	1	100	0.002	0	1
Mycalesis janardana	0	1	0	1	0	1	100	0.002	0	1
Mycalesis perseus	0	1	2.083	0.641	0	1	93.75	0.002	0	1
*Mycalesis sudra**	0	1	0	1	0	1	52.94	0.019	47.06	0.029
Neptis hylas	0	1	11.11	0.513	10.58	0.535	63.49	0.002	6.349	0.747
Neptis vikasi	0	1	0	1	0	1	63.16	0.029	1.754	0.517
Orsotriaena medus	0	1	0	1	0	1	100	0.002	0	1
Paedhima columella	38.71	0.069	6.452	0.683	35.48	0.075	5.376	0.7	0	1
Phalanta phalantha	66.67	0.028	0	1	0	1	0	1	0	1
Pantoporia hordonia	0	1	0	1	0	1	66.67	0.029	0	1
Parantica albata	0	1	0	1	0	1	0	1	33.33	0.198
Polyura hebe	0	1	33.33	0.2007	0	1	0	1	0	1
Symbrenthia anna	0	1	0	1	0	1	0	1	100	0.002
Symbrenthia hypselis	0	1	0	1	0	1	0	1	100	0.002
Symbrenthia lilaea	0	1	0	1	0	1	0	1	33.33	0.198
Tanaecia japis	0	1	0	1	0	1	0	1	100	0.002
Tanaecia palguna	0	1	0	1	0	1	0	1	33.33	0.198
Tanaecia pelea	0	1	0	1	66.67	0.030	0	1	0	1
Tanaecia sp.	0	1	0	1	0	1	33.33	0.199	0	1
Vagrans egista	0	1	0	1	0	1	0	1	100	0.002
Yoma sabina	0	1	0	1	0	1	66.67	0.029	0	1
Ypthima baldus	0	1	0	1	0	1	89.83	0.006	6.78	0.396
Ypthima horsfieldii	0	1	0	1	0	1	100	0.002	0	1
*Ypthima nigricans**	0	1	0	1	0	1	0	1	100	0.002
Ypthima pandocus	0	1	0	1	0	1	52.94	0.023	47.06	0.028
Ypthima philomela	0	1	9.524	0.393	0	1	71.43	0.008	0	1
Papilionidae										
Atrophaneura priapus	0	1	0	1	0	1	0	1	66.67	0.027
Graphium agamemnon	64.91	0.002	8.772	0.611	21.05	0.301	1.462	0.940	0.292	0.981
Graphium doson	0	1	0	1	44.44	0.086	11.11	0.514	0	1
Graphium sarpedon	0	1	0	1	18.18	0.220	3.03	0.737	42.42	0.041
*Pacliopta adamas**	0	1	0	1	0	1	100	0.002	0	1
Papilio demoleus	33.33	0.075	14.81	0.468	25.93	0.119	1.852	0.919	0	1
Papilio helenus	0	1	0	1	0	1	0	1	100	0.002
Papilio memnon	0.641	0.978	61.54	0.002	15.38	0.413	21.15	0.272	0	1
Papilio polytes	18.18	0.362	51.52	0.021	8.081	0.740	10.1	0.604	1.01	0.992
*Troides amphrysus***	0	1	0	1	0	1	0	1	33.33	0.198
*Troides cuneifera***	0	1	0	1	0	1	0	1	100	0.002
*Troides helena***	0	1	0	1	0	1	76.67	0.002	23.33	0.165
Pieridae										
Appias albina	33.33	0.206	0	1	0	1	0	1	0	1
Appias olferna	0.5848	0.870	83.33	0.002	3.509	0.647	6.433	0.566	0	1
Appias sp.	0	1	0	1	0	1	66.67	0.029	0	1
Appias lyncida	66.67	0.028	0	1	0	1	0	1	0	1
Catopsilia pomona	5.128	0.826	30.77	0.134	26.92	0.168	23.08	0.229	0	1
Catopsilia scyla	0	1	4.762	0.548	9.524	0.348	38.1	0.071	0	1
Cepora iudith	0	1	0	1	0	1	5.556	0.379	27.78	0.195
Delias belisama	0	1	0	1	3.03	0.517	27.27	0.203	3.03	0.520
Delias hyparete	8.333	0.429	12.5	0.277	45.83	0.121	0	1	0	1
Delias periboea	0	1	0	1	97.22	0.002	0	1	0.926	0.606
Eurema blanda	0	1	0	1	0	1	7.619	0.366	77.14	0.005
Eurema hecabe	1.058	0.97	12.7	0.560	0.3527	0.991	44.44	0.048	40.21	0.064
Eurema sari	71.15	0.007	0	1	0	1	0.160	0.875	28.37	0.130
Eurema sp.	0	1	0	1	33.33	0.204	0	1	0	1
Eurema tilaha	0	1	0	1	0	1	44.44	0.085	22.22	0.136
Hebomoia glaucippe	0	1	0	1	0	1	100	0.002	0	1
Leptosia nina	0.4167	0.905	18.75	0.236	0.8333	0.889	78.12	0.004	0.208	0.977
Pithecops corvus	0	1	0	1	0	1	70.83	0.015	29.17	0.136
Riodinidae										
Zemeros fleygas	0	1	0	1	0	1	33.33	0.199	0	1

Note: The mark *: Endemic species, **: Protected species

Table 3. Butterfly diversity indices for each habitat

Diversity indices	Village	Agriculture	City Park	Community Forest	Conservation Forest
Taxa richness (S)	40	30	32	72	53
Abundance (n)	397	360	280	1372	559
Shannon-Wiener (H')	2.79	2.12	2.98	3.51	3.32
Evenness (E)	0.41	0.30	0.62	0.47	0.52

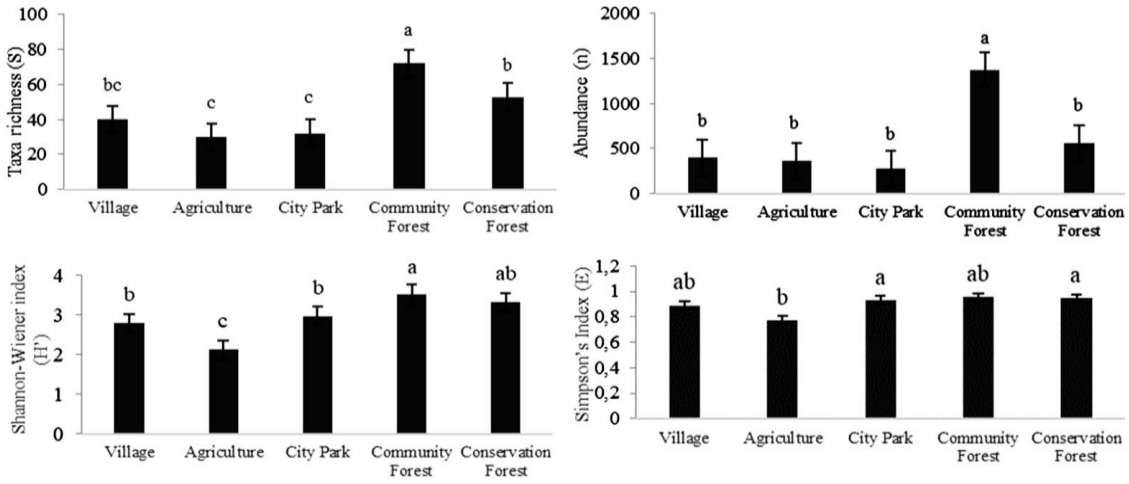

Figure 3. Butterfly diversity indices across habitats. Note: The same letter in the same diversity indices is significantly different according to Tukey's test at the 95% confidence level

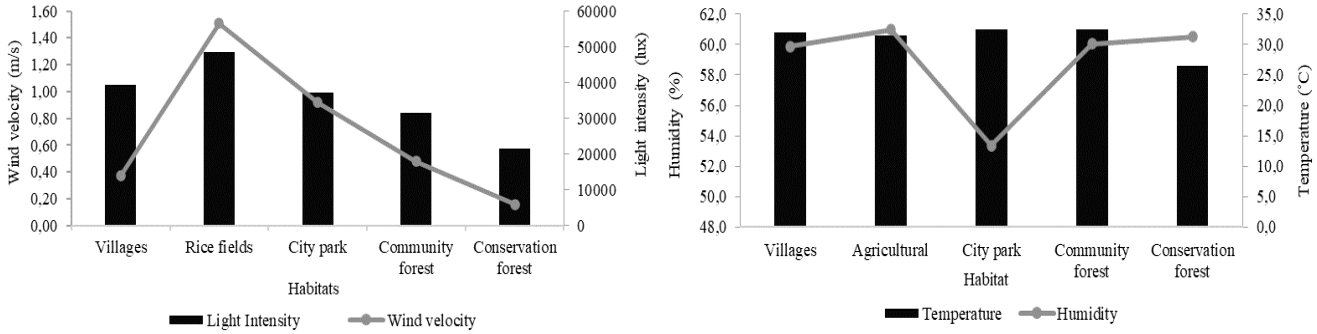

Figure 4. Environmental factor values

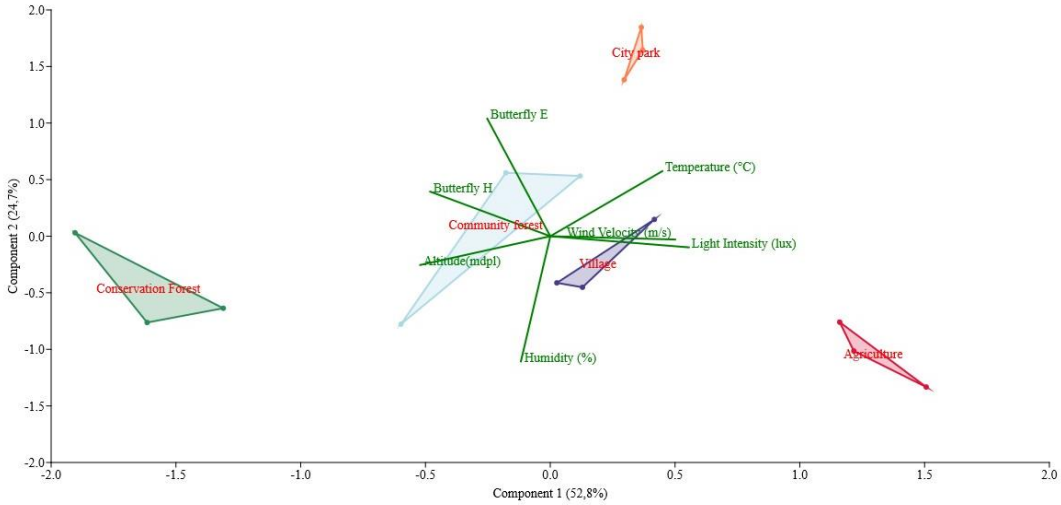

Figure 5. PCA biplot of the correlation between spesies diversity and environmental factors. H: Diversity index, E: Evenness Index

Table 4. The calculation of environmental factors with the Kruskal Wallis test

	T	H	WV	LI	ALT
chi-square	8.607	7.067	13.987	11.433	13.5
df	4	4	4	4	4
p-value	0.072	0.132	0.017	0.022	0.009

Note: Environmental factors. T: Temperature, H: Humidity, WV: Wind Velocity, LI: Light Intensity, ALT: Altitude

Discussion

The number of species of butterflies found in Lumajang is higher compared to similar studies in other areas of Java, Indonesia, such as Ledokombo Hillocks Jember (34 species) (Mubarok et al. 2023), Mount Muria Kudus, Central Java (40 species) (Sukma et al. 2021), Selorejo Waterfall Area, Ponorogo, East Java (26 species) (Zulaikha and Susanto 2021), Sumber Clangap and Waduk Selorejo, East Java (22 species) (Ashari et al. 2019), Baluran National Park, East Java (63 species) (Leo et al. 2016), green spaces in Malang and Pasuruhan, East Java (67 species) (Leksono et al. 2016), Mount Slamet, Central Java (99 species) (Widhiono 2015), Pasirlangu Village, Puncak Cisarua, Bandung, West Java (45 species) (Septianella et al. 2015). Suaka Elang (Raptory Santuary) Mount Halimun Salak National Park in West Java (40 species) (Murwitaningsih and Dharma 2014). The difference in the number of species of butterflies obtained is due to the difference in the study site, period (season) of study, and other environmental factors. The differences in the location of the study range from the area, altitude, type of habitat, and vegetation at each location. Periods or seasons of every study area also differ; this study was carried out in the rainy season near the raining-times, while other studies were those carried out when the rainy season shifts (Mubarok et al. 2023); seasonal differences affect the butterfly microhabitat (Koneri et al. 2020). Another environmental factor that influences the number of species of butterflies is the environmental disturbance caused by anthropogenic factors; the study conducted by Basri and Zakaria (2021) mentioned that environmental disruption could affect the number of species of butterflies significantly.

The butterfly species composition in each family in Lumajang is also different; in this study, the Nymphalidae family has the highest number of species and individuals among other families. Another study found that the Nymphalidae family is more dominant in urban areas (Azizah et al. 2021) and other forest areas (Widhiono 2015; Rusaman 2016). That is because the Nymphalidae family is a family type in which many species are polyphagous, so they can survive even if the main host plant is unavailable. In addition, the imago population of the Nymphalidae family is not only dependent on the availability of nectar from flowering plants (its source of nutrition) but also be found in rotting plants, fruits, and animals' urine. Host plants of the Nymphalidae family dominate the site of the study, consisting of *M. indica* (Anacardiaceae), *P. indicus* (Fabaceae), *E. capillifolium* (Asteraceae), *Ficus* spp. (Moraceae), and Malvaceae (Table 1). This result is consistent with other studies that mention the host plants of the family Nymphalidae are Fabaceae, Annonaceae, Asteraceae, Verbenaceae, Moraceae, Rubiaceae, Malvceae, and Anacardiaceae (Koneri et al. 2022). In addition, the Nymphalidae family has a broad distribution and the highest number of species than other families (Koneri et al. 2022). Meanwhile, the Riodinidae family found only one species and one individual, *Zemeros flegyas* (Cramer, 1780), in the Community Forest area. That is due to the narrow distribution of species in the family Riodinidae. The family is most common in the Neotropical region, which accounts for about 95% of the total species found only in the region (Siewert et al. 2014). About 40 species are found in Indonesia (Rusman et al. 2016).

Three species of the *Troides* genus found are protected species; these species consist of *T. helena, T. cuneifera,* and *T. amphrysus* (Peggie 2011). These species are protected by law Regulation of the Ministry of Environment and Forests of the Republic of Indonesia No. P.20/MENLHK/SETJEN/KUM.1/6/2018. In addition to being protected by law, three of these species listed in Appendix II have status in Cites and are Least Concern in IUCN (Böhm et al. 2018). Meanwhile, *T. helena* is Moderately Depleted (MD) in the Green Status IUCN (Sultana and Chowdhury 2021). In addition, several endemic species were found in this study, consisting of *C. lutea, M. sudra, Y. nigricans,* and *Y. horsfieldii* are endemic species of Java-Bali and *Parantiaca albata* (Zincken, 1831) and *P. adamas* are endemic species of Java. In addition, *P. adamas* previously belonged to *P. aristolochiae*, and then separation was proposed (Page and Tradeway 1995; Peggie et al. 2021).

The evenness, diversity, abundance, and taxa richness of butterflies in each habitat in Lumajang is different (Figure 3). The differences are because butterflies' activity, growth, reproduction, and even distribution depend on environmental factors (Jacquier et al. 2020; Koneri et al. 2022). The Community Forest was the habitat with the most butterfly species than other study sites. The Community Forest is a secondary forest with complex vegetation because it utilizes land by combining forestry and agriculture. Vegetations found in this habitat include *P. purpureum* (Poaceae), *M. pudica* (Fabaceae), *T. procumbens* (Asteraceae), and Arecaceae. Butterflies use these plants as larval host plants. The Community Forest also found flowering plants such as *H. tiliaceus* (Malvaceae), *Ipomea* sp. (Convolvulaceae), and *A. leptopus* (Polygonaceae), which make good habitats for butterflies to find nectar. Although the variation and heterogeneity, Conservation Forest have the second-highest number of species after Community Forest 30 species (24% from all study sites) are found there. The dominant families of vegetation in Conservation Forest are Malvaceae, Meliaceae, Moraceae, Fabaceae, and Urticaceae. This vegetation becomes a host plant for the butterfly species found. For example, plants from the Moraceae family are hosts of *Euploea* spp., and plants from the Fabaceae family are hosts of *Eurema* spp. (Rahman et al. 1985; Iqbal et al. 2021).

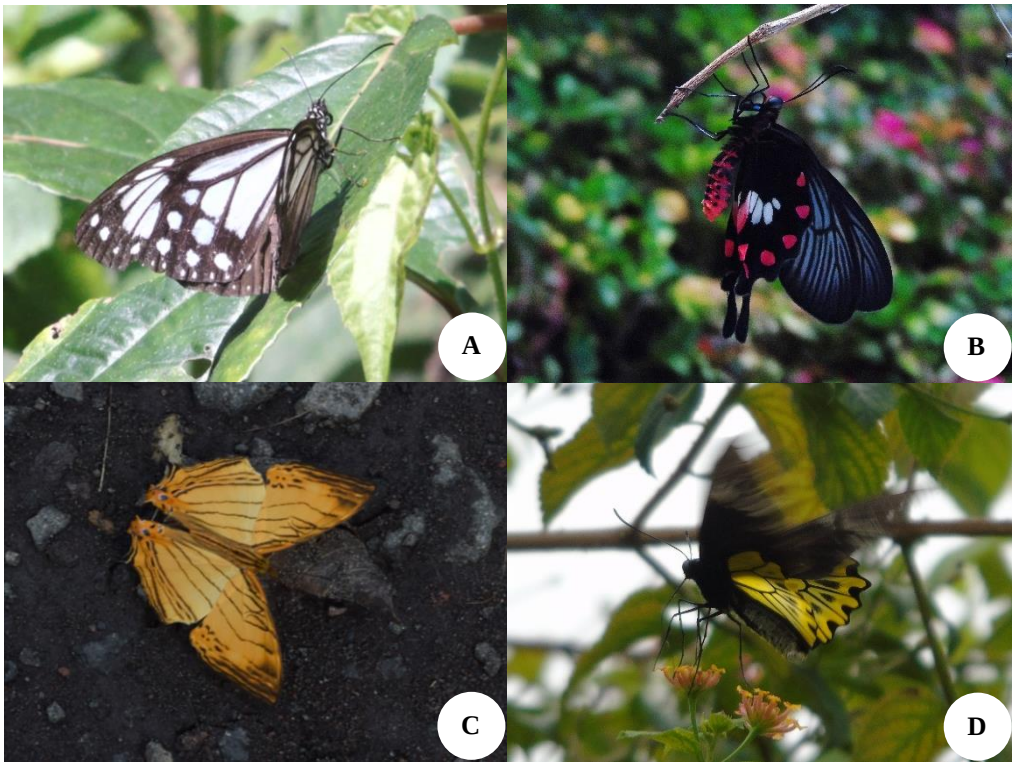

Figure 6. Photos of endemic and protected species. A. *Parantica albata*, B. *Pachliopta adamas*, C. *Cyrestis lutea*, D. *Troides cuneifera*

Furthermore, the presence of butterflies is closely related to the presence of vegetation. Different species of butterflies have adapted to feed on specific plant types, and the presence of these plants is necessary for their survival and reproduction. Therefore, vegetation diversity greatly affects butterfly diversity (Muto-Fujita et al. 2017; Okamura et al. 2019). Some studies show that more butterflies are found in habitats with complex structures and vegetation diversity (Han et al. 2021; Koneri et al. 2022).

In addition, the existence of butterflies also depends on environmental factors. The measurement of environmental factors in each habitat aims to determine the influence and relationship between environmental factors and butterfly diversity. The results of the PCA analysis show that environmental factors greatly influence the diversity and evenness of butterfly species. For example, biplot on PCA analysis shows that temperature is averse to altitude and humidity, which shows that temperature negatively correlates with altitude and humidity. In addition, the index of diversity and evenness of butterflies, as opposed to light intensity and wind velocity, shows a negative correlation (Figure 4). Light intensity that is too low or too high can also affect the presence of butterflies (Liao et al. 2017). Based on these findings, this study's results indicate that butterflies prefer habitats with optimal temperature and humidity, such as Community Forest. Furthermore, butterflies are poikilotherm organisms, so environmental temperature greatly influences their body temperature (Muhelni and Anwar 2020; Comay et al. 2021). Temperature can also affect butterfly activity; for example, high temperatures can cause the volume of secretions in flowering plants to decrease, resulting in a lack of food intake (Ramesh et al. 2012; Koneri et al. 2022).

Each butterfly species has a different tolerance level, either to host plants or to their environmental conditions. Based on indval analysis, some species in Conservation Forest inhabit forest areas. These species include *Troides* spp., *H. epicles*, *Symbrenthia spp*. and *Euthalia malaccana* (Fruhstorfer, 1899), and *Dichorragia nesimachus* (Boisduval, 1836) (Saha and Das 2012; Mehra et al. 2018; Bhowmik and Chowdhury 2021; Peggie et al. 2021). The result showed that butterflies could be bioindicators either in terms of the diversity of environmental characteristics (abundance and vegetation diversity) or sensitivity to environmental conditions (temperature, humidity, light intensity, wind speed, as well as the presence of pollutants) (Ghazanfar et al. 2016; Comay et al. 2021). This is consistent with some studies showing that several species tolerant to all conditions can be found in all habitat types. On the contrary, some found only in forest habitats tend to have lower tolerance (Forister et al. 2010; Ren et al. 2022).

This study concludes that butterflies' existence depends on their habitat conditions. This study shows a significant difference in the diversity of butterflies at each site. The Community Forest has the highest value of diversity among the other habitats (72 species and 1,372 individuals). This is because this habitat has complex vegetation and optimal environmental conditions for butterfly existence. Meanwhile, Conservation Forest have the second highest diversity rating after Community Forest, and 30 species (24% of the 124 species found) are inhabited only in this habitat. The results also show that some species that can be

considered indicator species in Conservation Forest habitats are native species of forest inhabitants. These species include *Troides* spp., *H. epicles*, *Symbrenthi*a spp., and *E. malaccana*. The study also recorded protected species (*T. Helena*, *T. cuneifera*, and *T. amphrysus*) and endemic species (*C. lutea*, *M. sudra*, *Y. nigricans*, *Y. horsfieldii*, *P. albata*, and *P. adamas*). Research on similar things in Lumajang is still limited, so the results of this research can be used as basic data to support the conservation efforts of butterflies in Indonesia, especially in Lumajang, East Java, Indonesia.

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Abundance and diversity of terrestrial free-living nematodes in potato agroecosystem

MUTALA'LIAH♥, ABDUL MANAN, LAFI NA'IMATUL BAYYINAH
Department of Agrotechnology, Faculty of Agriculture, Universitas Jenderal Soedirman. Jl. Dr. Soeparno No. 63, North Purwokerto, Banyumas 53122, Central Java, Indonesia. Tel.: +62-281-638791, ♥email: mutalaliah@unsoed.ac.id

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Abstract. *Mutala'liah, Manan A, Bayyinah LN. 2023. Abundance and diversity of terrestrial free-living nematodes in potato agroecosystem. Nusantara Bioscience 15: 129-136.* The presence of terrestrial free-living nematodes in agroecosystems is frequently overlooked. However, these microorganisms in agroecosystems soil are beneficial for supporting plant growth. These microorganisms could recycle the nitrogen in soil, decomposition and mineralization of organic matter, and suppress the densities of plant parasitic nematodes through their life strategy. Terrestrial free-living nematodes are classified according to trophic groups such as bacterivores, fungivores, predators, and omnivores. Reports on the abundance and diversity of terrestrial free-living nematode populations in agroecosystems, especially on potato plantations, were limited. They usually focused on the plant parasitic nematode's attack on potatoes. Therefore, this study aimed to examine the diversity and abundance of terrestrial free-living nematodes in potato agroecosystems. The current study was conducted in two potato fields in Pratin Village, Serang, Purbalingga, Central Java, Indonesia. Soil samples were collected from two potato fields planted with different varieties (Granola and Atlantik) and extracted using the Whitehead-tray method. The variables observed were the diversity of genera and the abundance of terrestrial free-living nematode populations from each field. Population density data were analyzed by T-test, and diversity (H'), evenness (E), and dominance index (D) were also calculated. The results showed that the mean population densities of terrestrial free-living nematodes in the two fields were significantly higher at 918.40 individuals/ 100 g of soil in the Atlantik field than in the Granola field 76.53 individuals/ 100 g of soil. However, the diversity, evenness, and dominance index were not significantly different in both fields. Genera of nematode found in the Granola field were *Diplogaster*, *Dorylaimus*, *Tripyla*, and *Lotonchus*, while in the Atlantik field were *Rhabditis* and *Dorylaimus*.

Keywords: Beneficial nematodes, biodiversity, genera, potato field

INTRODUCTION

The agricultural ecosystem consists of some valuable and invaluable organisms regarding plant productivity. Terrestrial free-living nematodes are aquatic organism requiring adequate soil moisture to move in the soil (Yadav et al. 2018). Nematodes are classified as soil microorganisms in the Animalia Kingdom, which have several roles in the agricultural ecosystem, i.e., free living in the soil, plant-parasitic, and entomopathogenic nematode (Iqbal and Jones 2017). Free-living nematodes are the most abundant in the agricultural ecosystem, feeding on some organisms like algae, bacteria, and fungi (Iqbal and Jones 2017). Free-living nematodes play an important role in decomposition and nutrient recycling. In addition, these organisms are very important in protecting the soil's organic nature, assisting colonization of microbial substrates and nutrients mineralization in the soil, and feeding on other soil microbes like plant pathogens and soil insect pests (Iqbal and Jones 2017; Hailu and Hailu 2020). Terrestrial free-living nematodes are also considered to be used as soil health and quality indicators (Linsell et al. 2014). Soil nematodes are classified into five trophic groups and life strategies i.e., bacterial feeders, fungal feeders, plant feeders, omnivores, and predators (Kekelis et al. 2022).

Terrestrial free-living nematodes play essential roles in the decomposition and mineralization of organic matter and recycling nitrogen in the soil and soil food webs, and suppress the densities of plant parasitic nematodes (Rahman et al. 2014; Mendoza-de Gives 2022). The organic amendments in soil systems like green manure and botanical extracts, could significantly reduce the number of plant parasitic nematodes and increase the abundance of bacterial and fungal feeder nematodes (Kekelis et al. 2022). Organic fertilizer applied on the pumpkin (*Cucurbita moschata* (Duchesne) Duchesne ex Poir.) plantation reduces the number of plant parasitic nematodes and increases the non-parasitic nematodes (Atungwu et al. 2018). Applying organic substrates also affected the soil nematode community by increasing the density of bacterivore and fungivore nematodes (Darmola et al. 2013). Diplogasterid predators are abundant in decomposing organic manure soil (Askary and Abd-elgawad 2017). The most abundant population found in organic soil amendments was *Rhabditis* (bacterivore) and *Dorylaimida* (omnivore, excluding plant parasitic nematode and carnivore) (Rahman et al. 2014). Soil nematodes, especially bacterivorous, were the most potential bioindicator agents in soil ecosystem health (Chen et al. 2020; Lu et al. 2020). Terrestrial free-living nematodes could act as bioindicators of soil health due to their response to the soil environmental change like

farming, pollution, acidification, insecticide, liming, and fertilization (Sun et al. 2013; Pothula et al. 2019).

Terrestrial free-living nematodes are beneficial organisms that play a role in soil nutrient cycling (Yadav et al. 2018). In the ecosystem, soil nematodes function in decomposition, like bacterivorous nematodes regulate decomposer microflora composition, litter decay rates, and element cycles (Neher 2010). The second role is nutrient cycling, which directly affects nitrogen availability by excreting excess N in the form of ammonium, by the bacterivorous and fungivorous nematode. While indirectly regulating the population of microbivorous nematodes which is done by omnivorous and predatory nematodes (Neher 2010; Buchan et al. 2013;). Bacterivorous and predatory nematodes are performed directly and indirectly for nitrogen mineralization in conventional and integrated farming systems (Yadav et al. 2018). The third role is free-living nematode as disease suppression and biological control (Neher 2010). Omnivorous and predatory nematodes affect plant pests and disease suppression as these nematodes regulate the plant parasitic nematode populations (Bull et al. 2018). Diplogasterid predatory nematodes are suited for biocontrol of nematodes because they are easy to culture, have a short life cycle, have prey specificity, and resistance to adverse conditions (Khan and Kim 2007), while Mononchidae is unsuccessful for biocontrol due to the scarcity and long-life cycle (Neher 2010). In addition, nematodes belonging to genera of *Steinernema* and *Heterorhabditis* could act as biocontrol agents as entomopathogenic nematodes (Askary 2010).

The important role of free-living nematodes has not been explored in the agricultural ecosystem, while the most reported nematode was in plant feeder or plant parasitic nematodes, which caused a detrimental loss in plant productivity. Studies on terrestrial free-living nematode in Indonesia has been reported in the coffee plantation, which depicted several genera of free-living nematodes, i.e., *Dorylaimus, Rhabditis, Aphanolaimus, Aphelenchus, Acrobeles,* and *Mononchus* (Widowati et al. 2014; Tarno et al. 2021). However, there was no information on terrestrial free-living nematodes in potato plantations. Therefore, this study aimed to investigate the diversity and abundance of terrestrial free-living nematodes in potato agroecosystems. This research was necessary to assess the land sustainability for agroecosystems caused by implementing agronomic management practices. Evaluating the presence of terrestrial free-living nematodes could also be used as a fundamental term to manage further agroecosystems to minimize the risk of attacking of plant pests and diseases. Information on the abundance and diversity of terrestrial free-living nematodes could be used to determine the agroecosystem condition and as environmental monitoring.

MATERIALS AND METHODS

Study area and soil sampling

The research was conducted at Pratin Village, Serang Sub-district, Purbalingga District, Central Java, Indonesia, in two fields with different varieties (Granola and Atlantik). This location was one of the centers of potato plantations in Central Java. Samples were taken on November 2022 by randomly taking ten potato rhizosphere soil samples from each site. The number of samples taken was based on the land area. Mulyadi (2009) stated in the land area of less than 500 m^2, it needs eight to ten soil samples.

Procedures

Nematode extraction and isolation

The rhizosphere soil samples (100 g) were extracted and isolated using the modified Whitehead and Hemming tray method (Bell and Watson 2001) with the component arrangement (top-bottom): tissue paper, nylon gauze, suspension tray, and container tray. The rhizosphere soil samples were laid on the tissue paper and spread evenly. The container tray was filled with water until the soil sample on the top surface was submerged. The rhizosphere soil samples were incubated for 24 hours; subsequently, the water in each container tray was decanted and filtered by a 400 mesh (37 µm) sieve and put into the sample bottle for further observation. The rhizosphere soil samples from each variety were extracted three times.

Nematode population observations and identification

Nematode suspension was homogenized using a syringe and poured into the counting dish at about 5 mL. The observation was carried out by a stereo microscope for counting the nematode population and a binocular microscope (400x) for morphological observation. Nematode preservation was carried out using a non-permanent mount by dripping sterile water into the slide and placing the nematode into the slide; then, it was briefly burned using a Bunsen burner. Morphological identification was done by an online identification key accessed at https://nematode.unl.edu/key/nemakey.htm from the University of Nebraska-Lincoln (UNL) Nematology Lab. Morphological characters for nematode identification were stoma, esophagus, tail shape, and another specific morphological character for each genus following the identification key. Nematode populations were calculated by multiplying the mean number of individuals in each genus with the total volume of the sample.

Data analysis

Data of nematode population densities on each field were tested for normality assumption using the Shapiro-Wilk test ($p= 0.898$; $p >0.05$ for Granola field and $p= 0.931$; $p>0.05$ for Atlantik field), subsequently analyzed using T-test at 5% error level. In addition, nematode diversity was evaluated by Shannon-Wiener Index (H'), Evenness index (E), and Simpson's dominance index (D) (Tarno et al. 2021). The statistical software used for data analyses was IBM SPSS Statistics 25 and R Statistic version 4.2.1.

RESULTS AND DISCUSSION

Nematode diversity

Results on the nematode diversity based on morphological character refer to the online identification key by UNL Nematology Lab. encountered from potato plantation revealed that there were five genera, i.e., *Diplogaster*, *Dorylaimus*, *Tripyla*, *Lotonchus*, and *Rhabditis*. Nematode genera found in the Granola potato plantation were *Diplogaster*, *Dorylaimus*, *Tripyla*, and *Lotonchus*, while in the Atlantik plantation only two genera, i.e., *Dorylaimus* and *Rhabditis*. These five nematodes were categorized as non-parasitic nematodes.

Diplogaster (Figure 1)

Below are the steps followed using an online identification key (https://nematode.unl.edu/key/nemakey_pt2.htm) for *Diplogaster*:

1. Cephalic setae indistinct or absent
2. Stylet absent
39. Esophagus expanded at mid-region
49. Lip region without rib-like armature …… *Diplogaster*

Diplogaster was categorized as a microbial feeder and predator which fed on other nematodes, bacteria, fungi, and ciliates (Bajaj and Kanwar 2015; Hodda 2022). *Diplogaster* has a large and strong stoma with a strong claw-like movable dorsal tooth to grind the prey (Figure 1.A) (Mendoza-de Gives 2022). Male and female tail usually filiform (Figure 1.B) (Sudhaus and Rehfeld 1990; Kanzaki et al. 2014). The cuticle character of the *Diplogaster* on the cuticle lining between stoma and median bulb appears as a set of several longitudinal ridges, and the lumen of the corpus region was wider than isthmus and basal bulb. The life cycle of the *Diplogaster* genera is short and has high fecundity (Bajaj and Kanwar 2015). *Diplogaster* was the most effective nematode's biocontrol regarding its short life cycle at about 8-15 days, ease to culture, specified on prey, chemotaxis sense, and resistance to the harmful condition (Askary and Abd-elgawad 2017). *Diplogaster* is generally found in decomposing organic manure (Askary and Abd-elgawad 2017). Furthermore, *Diplogaster* was found in several rhizospheres like pear, orchards, rice, and soybean plantation (Shrestha and Bam 2015; Musarrat et al. 2016). Some studies (Fauzia et al 1998; Khan and Kim 2005) revealed that *Diplogaster* effectively controlled root-knot nematodes by suppressing the population densities and reducing the root gall formations on tomato plants (Askary and Abd-elgawad 2017).

Dorylaimus (Figure 2)

Below are the steps followed using an online identification key (https://nematode.unl.edu/nemakey.htm) for *Dorylaimus*:

1. Cephalic setae indistinct or absent
2. Stylet present
3. Stylet knobs or flanges absent
29. Valvate median esophageal bulb absent
30. Stomal walls not cuticularized
31. Esophagus with basal expansions
32. Posterior third of the esophagus swollen
36. Stylet axial, positioned centrally…… *Dorylaimus*

The morphological character of *Dorylaimus* has a long body length of about 2-9 mm. The cuticle of *Dorylaimus* was thick with longitudinal ridges. The lip region was moderately offset from the body contour (Figure 2.A). *Dorylaimus* has a strong odontostyle with 2-3 times as long as the lip region width. The female tail was elongated to filiform (Figure 2.B) (Vinciguerra et al. 2016). *Dorylaimus* has a piercing and sucking stylet to puncture their prey and remove their content; thus, it could disturb the prey's internal organs (Mendoza-de Gives 2022). *Dorylaimus* was reported as an omnivorous nematode in freshwater, wet moss, and soil habitats (Vinciguerra et al. 2016). Tarno et al. (2021) reported that *Dorylaimus* was a dominant genus found in coffee plantations in Indonesia. The genus was also reported in garlic plantations in Indonesia (Kusuma et al. 2020), wheat roots and soil in Australia, and orange roots in the USA (Alvarez-Ortega and Pena Santiago 2010). In addition, this genus was found in all types of soils, climates, and habitats. *Dorylaimus* has an efficient prey-searching ability, attraction and aggregation activities at the feeding site, and a wide range of predation on plant parasitic nematodes and other soil microorganisms (Askary and Abd-elgawad 2017).

Tripyla (Figure 3)

Below are the steps followed using an online identification key (https://nematode.unl.edu/key/nemakey_pt4.htm) for *Tripyla*:

1. Cephalic setae present
69. Post cephalic setae absent
70. Stylet absent
71. Teeth absent, minute or indistinct
72. Esophagus uniformly cylindrical
82. Stoma narrow, elongated, collapsed, or inconspicuous
83. Gonads paired
84. Amphid inconspicuous ……. *Tripyla*

Genus *Tripyla* has a smooth cuticle, head not offset, composed of three large, fairly rounded lips with three circlets of short blunt and inconspicuous amphid. The form of a stoma was like a simple tube, and the esophagus was almost cylindrical (Figure 3.A). The cell of the esophago-intestinal valve was well developed (UNL Nematology Lab 2023). The tail of *Tripyla* on this study was grouped in short-tails with short cephalic setae (Figure 3.B) (Zhao 2009). *Tripyla* was a predaceous nematode that fed on small microbes like bacteria, fungi, rotifers, and unicellular algae; which categorized as a microbial feeder by ingesting them that can be sucked from suspension and requires some processing in the mouth with its teeth to access the contents (Bilgrami and Gaugler 2004; Hodda 2022). This genus was grouped as chewers nematodes which fed on animals of a similar size to the nematode by taking prey into the mouth and then accessing the contents using the teeth (Majdi et al 2016; Hodda 2022).

Lotonchus (Figure 4)

Below are the steps followed using an online identification key (https://nematode.unl.edu/nemakey.htm) for *Lotonchus*:

1. Cephalic setae indistinct or absent
2. Stylet absent
38. Teeth present, prominent
39. Esophagus without mid-region expansion
40. Tail-pointed or tapering
41. Male tail without setae
42. Stoma without denticles
45. Tooth anteriorly directed
46. Tooth in the basal part of stoma…… *Lotonchus*

The main characters of this genus have body length 0.8-5.2 µm, have medium to large stoma with a dorsal tooth at the base of stoma (Figure 4.A), pharyngo-intestinal junction tuberculate, the female genital was amphidelphic or monodelphic, spicule on male was more or less arcuate, bifurcate lateral guiding pieces present, and the tail was predominantly conoid or filiform in both sexes (Figure 4.B) (Vu et al. 2021). *Lotonchus* was categorized as a Mononchid nematode which has a strong sclerotized on the stoma or buccal cavity with a large pointed dorsal tooth, small teeth or denticles (Askary and Abd-elgawad 2017; Mendoza-de Gives 2022). The morphological character of the *Lotonchus* group has a large buccal cavity, and the small tooth was located in the basal part of the stoma. The body was large and robust with a smooth cuticle and indistinct striations. The lip region was set off by slight expansion. Esophagus was long, narrowing to a nerve ring at about ¼ of its length and expanding gradually to its base. The tail was curved and elongated ventrally, and it had asymmetrical muscles around the vagina and slender spicules (Khan and Araki 2002). Mononchid nematode is a broad-spectrum predatory that feeds extensively, not exclusively on plant parasitic or other nematodes (Kim 2015).

Rhabditis (Figure 5)

Below are the steps followed using an online identification key (https://nematode.unl.edu/key/nemakey_pt2.htm) for *Rhabditis*:

1. Cephalic setae indistinct or absent
2. Stylet absent
38. Teeth absent, minute, or indistinct
50. Esophagus with basal expansions
51. Esophagus expanded at mid-region
55. Gonads paired
56. Stomal walls straight amalgamated
57. Moderately swollen metacorpus, stoma not excessively elongate …….. *Rhabditis*

The morphological character of the *Rhabditis* genus has an elongated stoma, tiny, open and cylindrical (Figure 5.A). The cuticle was slightly annulated. This free-living nematode has long tail in males and females (Figure 5.B) (Rakhsanpour et al. 2012). *Rhabditis* do not have stylets or teeth, the lips are flat, and the stoma type was funnel-like channels consisting of cheilostom, gymnostom, and stegostom. The metacorpus of this genus was enlarged. The tail tended to be tapered and blunt to the tip (Mirsam et al. 2020). *Rhabditis* is a bacterial feeder that occurs in a wide range of bacteria. Rhabditis was reported on the maize rhizosphere in South Sulawesi, Indonesia (Mirsam et al. 2020).

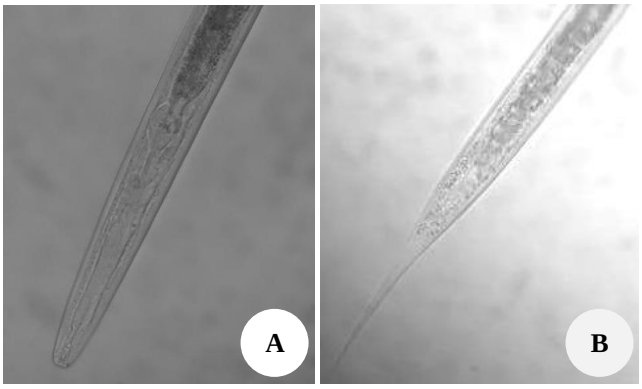

Figure 1. *Diplogaster*: A. Anterior; B. Posterior. Note: Microscope magnification 400x

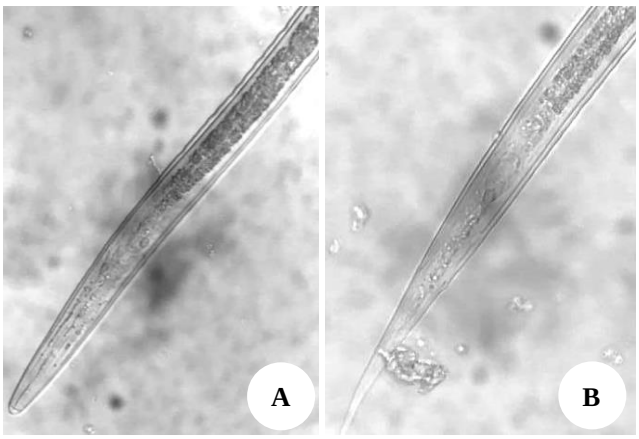

Figure 2. *Dorylaimus*: A. Anterior; B. Posterior. Note: microscope magnification 400x

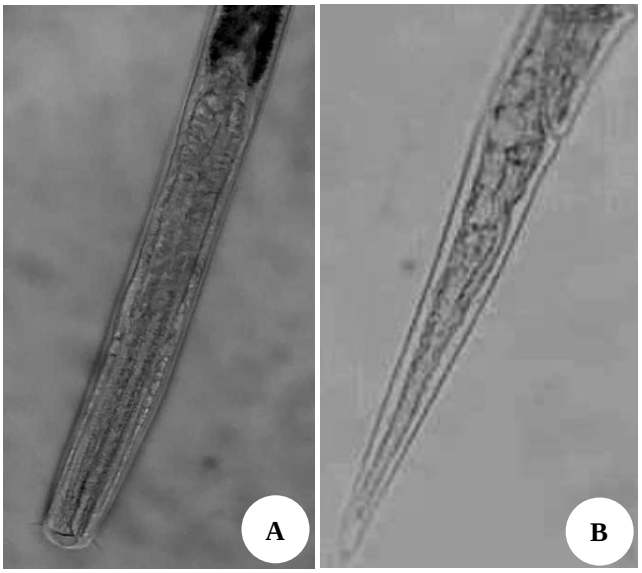

Figure 3. *Tripyla*: A. Anterior; B. Posterior. Note: microscope magnification 400x

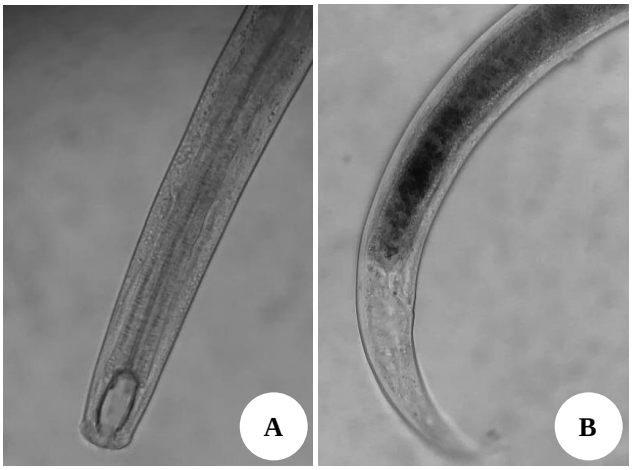

Figure 4. *Lotonchus*; A. Anterior; B. Posterior. Note: microscope magnification 400x

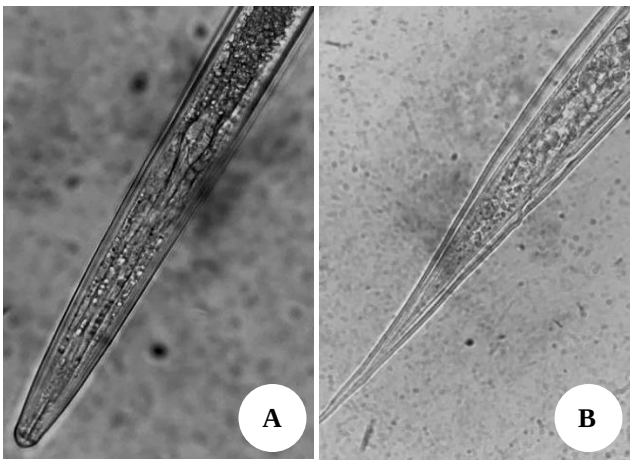

Figure 5. *Rhabditis*; A. Anterior; B. Posterior. Note: microscope magnification 400x

Nematode population

Results of this study depicted that the terrestrial free-living nematode population densities were significantly higher ($t = 4.89$; $P = 0.008$) in the Atlantik potato field (918.4 nematode/100 g of soil) than in the Granola potato field (76.53 nematode/100 g of soil) (Figure 6). Terrestrial free-living nematodes found in Atlantik potato field were *Rhabditis* (576.21 nematode/100 g of soil) and *Dorylaimus* (342.21 nematode/100 g of soil), while in Granola potato field were *Diplogaster* (33.21 nematode/100 g of soil), *Dorylaimus* (30.32 nematode/100 g of soil), *Tripyla* (5.77 nematode/100 g of soil), and *Lotonchus* (7.23 nematode/100 g of soil).

Rhabditis is a bacterial feeder with specified morphological character on stoma-like tubes or funnels (Mirsam et al. 2020). These terrestrial free-living nematodes in Rhabditida Family also interacted with other soil biota like arthropods and invertebrates. The species of *Rhabditis* associated with insects were *R. terricola* and *Mesorhabditis* sp. (Azizoglu et al. 2016). Slug and snail is also the common host for *Rhabditis;* thus, the slug and snail contribute to the spread of this genus (Sudhaus 2018). *Dorylaimus* was an omnivorous free-living nematode abundantly found in disturbed forest land use (Sagita et al. 2014) and post-coal mine reclamation land in Indonesia (Sofian et al. 2022). Genus *Dorylaimus* could act as a predator, decomposer, and bacterivore (Sofian et al. 2022). *Diplogaster* was a potential predator for plant parasitic nematodes, bacteria, and other soil microorganisms with a high predation rate (Khan and Kim 2005). The feeding habit of *Tripyla* was predatory nematodes which fed on protozoa, small nematodes, and rotifers. Its feeding habit as a predatory nematode was detected by ingested bodies of nematodes and a stylet inside the intestine of *Tripyla* (Asghari et al. 2017). Several species of *Tripyla* have been reported from Mexico and USA i.e. *Tripyla tropica, Tripyla alaecaudata,* and *Tripyla napaensis* (Cid del prado vera et al. 2012). Some species of *Tripyla* were newly found in Northern Iran and North China, i.e., *Tripyla paraffnis* and *Tripyla parafilicaudata* (Asghari et al. 2017) and *Tripyla aquatica* Brzeski & Winiszewska-Slipinska 1993 and *Tripyla setifera* Bütschli 1873, respectively (Liu et al. 2021). *Lotonchus* was a predatory associated with the rhizosphere of medicinal plants in India (Gupta and Mondal 2018). This genus is also found in the rhizosphere of tea plantation in organic, conventional, and semi-natural tea ecosystems, while there were not found in pollution-free tea ecosystems (Li et al. 2014). This predatory nematode is also found in the rhizosphere of the rice ecosystem in India with less population (IARI 2020). In addition, 77 species of *Lotonchus* have been reported, and a new species of *Lotonchus* was identified from the forest in Vietnam, namely *L. lotilabiatus* (Vu et al. 2021).

Regarding the life strategy of these five nematodes, it disclosed that the varieties were not the main factor influencing the abundance of their population in the soil. The nematode's abundance was due to their life strategy not being affected by each variety's root exudate release. Root exudate generally did not impact the activity and growth of free-living nematodes, whereas it was sensitive to the effect on plant parasitic nematodes as herbivorous nematodes (Sikder and Vestergard 2020). Root exudate is mainly affected by the plant parasitic nematode than free-living nematode due to the location of plant parasitic nematodes closer to the root than free-living nematode (Mathesius and Costa 2021).

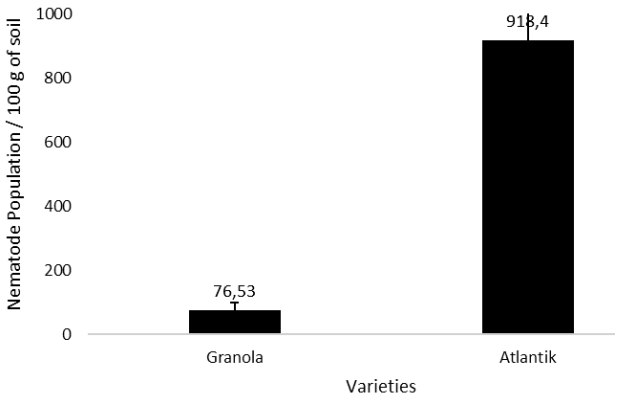

Figure 6. The terrestrial free-living nematode population density in different varieties of potato plantation

Table 1. Shannon-Weiner (H'), Evenness (E), and Simpson's Dominance Index from Nematode Population in Different Varieties of Potato Plantation

Varieties	H'	E	D
Granola	0.80	0.85	0.49
Atlantik	0.63	0.91	0.43

Note: Shannon-Weiner Diversity Index: < 1 = low diversity, 1-3 = moderate diversity, > 3 = high diversity; Evenness Index: 0.00 < E < 0.50 = low evenness, 0.50 < E < 0.75 = medium evenness, 0.75 < E < 1.00 = high evenness; Simpson's Dominance Index: 0 < D ≤ 0.5 = no dominance; 0.5 > D ≥ 1 = dominance

In addition, the densities of plant parasitic nematodes respond to the vegetation cover and photosynthetic activity. In contrast, soil properties influenced bacterivore nematodes more (van den Hoogen et al. 2019). In addition, the pesticide application affected the significance of population densities in two fields. The farmer stated that the Granola plantation was intensively sprayed with pesticides, while the Atlantik plantation did not apply pesticides. This condition significantly affected the terrestrial free-living nematode population densities in the soil. That was in line with Khanum et al. (2022), the agroecosystem atmosphere was massive in use of chemical properties like chemical fertilizers and pesticides, adversely affecting beneficial microorganisms and humans. That also affected soil fertility, nutrient disparity, and reduced water holding capacity (Khanum et al. 2022). Yang et al. (2020) stated that pesticides application in the field directly affects soil nematode abundance and diversity. Therefore, bacterivore, fungivore, omnivore, and predator nematodes reach the lowest population in the pesticides (organic phosphate or carbamate) application field (Koc et al. 2020).

The Shannon-Weiner diversity index in both fields was categorized as low diversity, i.e., 0.80 for Granola and 0.63 for Atlantik fields (Table 1). The index value in Granola field was higher than Atlantik field due to the more diverse genera found in Granola field: *Diplogaster*, *Dorylaimus*, *Tripyla*, and *Lotonchus*. In contrast, only two genera were found in Atlantik field, i.e., *Dorylaimus* and *Rhabditis*. The Evenness index value in Granola field was 0.85, and Atlantik field was 0.91 (Table 1), ranging from 0.75 < E < 1.00 and categorized as high genera evenness. The Simpson's Dominance index values were 0.49 for Granola field and 0.43 for Atlantik field (Table 1), indicating no dominance genera in both fields. The abundance and diversity of free-living nematodes are essential in the agroecosystem. The presence of non-parasitic nematodes in the agroecosystem was not only natural enemies that could control some herbivorous insect larvae and plant parasitic nematodes but also contributed to the decomposition process and provided inorganic nutrient availability. Therefore, the abundance and diversity of free-living nematodes could be considered a bioindicator for soil health (Sikder and Vestergard 2020). The ratio of free-living nematode and nonparasitic nematode on the field could be useful as an indicator of soil quality (Rahman et al. 2014). For example, the presence of *Rhabditis* and *Acrobeles* in the soil was positively correlated with the increasing soil nitrogen. Furthermore, the densities of plant parasitic nematodes were also suppressed in the abundant free-living bacterial-feeding nematodes and entomopathogenic nematodes (Khanum et al. 2022).

In conclusion, terrestrial free-living nematodes, categorized as non-parasitic nematodes found in potato plantations, belonged to five genera, i.e., *Diplogaster*, *Dorylaimus*, *Tripyla*, *Lotonchus*, and *Rhabditis*. The abundance of terrestrial free-living nematodes could be used as an indicator of soil health to support plant growth and represent sustainable agriculture. This study could be useful in agriculture regarding the abundance and densities of terrestrial-free living nematode that used as bioindicators and biocontrol. Information about this population could be used to make decisions in future cultivation practices.

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