

Diversity, distribution, and nest characteristics of stingless bees (Hymenoptera: Meliponini) in Baluran National Park, East Java, Indonesia

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Abstract. Rachmawati RD, Agus A, Umami N, Agussalim, Purwanton H. 2022. Diversity, distribution, and nest characteristics of stingless bees (Hymenoptera: Meliponini) in Baluran National Park, East Java, Indonesia. *Biodiversitas* 23: 3890-3901. Baluran National Park is a conservation area located in Situbondo, East Java. It has become a natural habitat for many animals, including stingless bees. Stingless bees, known as klanceng in Java, are eusocial insects that produce honey and play an important role as pollinators. This study aimed to explore the diversity of stingless bees in BNP, East Java. Two species of stingless bees were found in nine sites with various types of habitats: teak forest, secondary forest, evergreen, savanna, mangrove, and settlements. Based on their morphological and morphometric characteristics, the bees were identified as *Lepidotrigona terminata* and *Tetragonula laeviceps*. The results of species identification were confirmed by molecular analysis using the 16S rRNA gene from mitochondrial deoxyribonucleic acid. Furthermore, we described the distinction of nest entrance appearances of stingless bees from their natural nest and beehive in meliponiculture. The stingless bee *Tetragonula laeviceps* is considered an adaptive stingless bee because it is distributed in all types of habitats in this study. These data would add information to the biology and distribution of stingless bee species in Indonesia, especially in Java Island, and provide bases for their utilization in the future.

Keywords: Baluran National Park, distribution, diversity, insects, stingless bees

INTRODUCTION

Stingless bees (Hymenoptera: Apidae: Meliponini) are known as klanceng in Javanese. They are eusocial insects that live in colonies and consist of a queen with several worker bees and drones (Hamid et al. 2016). Stingless bees utilize nectar, pollen, and resin from several different plant species as sources of nesting and food supply (Leonhardt et al. 2009). Stingless bees play many important roles in ecology, economy, pharmacy, and socio-culture. Bees act as pollinators for important tropical plants and crops (Engel et al. 2018). Furthermore, bees produce honey, pollen, and propolis, which are of economic value (Abd Jalil et al. 2017). The propolis of stingless bees contains therapeutic compounds with antioxidant, antimicrobial, and anti-inflammatory properties, which are useful in medicine (Abd Jalil et al. 2017; Lavinias et al. 2019).

In Indonesia, 46 species of stingless bees belong to 10 genera, namely, *Austroplebeia*, *Geniotrigona*, *Heterotrigona*, *Homotrigona*, *Lepidotrigona*, *Lisotrigona*, *Papuatrigona*, *Pariotrigona*, *Tetragonula*, and *Wallacetrigona* (Kahono et al. 2018). These bees have been collected mainly from Sumatra and Kalimantan (Kahono et al. 2018); still, certain bees from Indonesia, especially in Java, have not been recorded. The stingless bees that had been recorded in Java Island include *Lepidotrigona nitidiventris*, *L. terminata*, *Heterotrigona itama*, *Tetrigona*

apicalis, *T. fuscobalteata*, *T. drescheri*, *T. laeviceps*, *Tetragonula laeviceps*, *T. iridipennis*, *T. sapiens*, *T. sarawakensis*, *T. cf. biroi*, and *T. drescheri* (Efin et al. 2019; Trianto and Purwanto 2020a,b; Purwanto and Trianto 2021). *Heterotrigona itama* and *T. laeviceps* are common and widely kept stingless bee species in Indonesia. Both species of bees are highly demanded by stingless beekeepers because they are productive and easy to maintain. These stingless bees are easily found in the forests of Kalimantan. Therefore, some beekeepers in Java import them from Kalimantan for their meliponiculture stock. However, the introduction of stingless bees into new habitats can have a negative impact on the native Java stingless bee. Based on a study in Brazil, introduced bees are potential hosts for transmissible diseases or parasites to local stingless bees (Nunes-Silva et al. 2016). The introduction of bees can also harm the native ecosystem. This practice will result in the competition for floral resources or nest sites with native pollinators, cointroduction of natural enemies, pollination of exotic weeds, and interference with native plant pollination (Goulson 2003). Accordingly, the introduction of stingless bees from Kalimantan or another island to Java is suspected to have similar impacts. Thus, scholars should focus on the study of stingless bees that are indigenous to Java as a prudent alternative for stocking the meliponiculture industry in Java.

Stingless bee species can be identified from the morphological and morphometric characteristics of the worker bees. However, two different species of stingless bees may show similar characteristics and vice versa. The same species may have differences in their appearance, thus allowing errors in the identification. The species distinction between morphologically similar species of stingless bee can be studied thoroughly through the molecular analysis of its DNA, such as the 16S rRNA gene (Thummajitsakul et al. 2013). Furthermore, the sequence of 16S rRNA gene data has been widely used to determine the phylogeny of bees in the order Hymenoptera (Costa et al. 2003). Costa et al. (2003) showed that 16S rRNA markers could reveal the phylogenetic relationships for large intercontinental *Meliponini* groups (Africa, Australia, and Neotropical).

Baluran National Park (BNP) is a conservation area located in Situbondo, East Java. The area has a size of about 25,000 hectares at an altitude between 0 and 247 m above sea level and features a dry climate type (Istomo and Hartarto 2019). BNP has a variety of flora and fauna, including insects (Rohman et al. 2020). The diversity of stingless bees in BNP, East Java, has never been reported. Therefore, this study was conducted to determine the diversity of stingless bees in BNP based on their morphological, morphometric, and nest entrance characteristics. To support the morphological

characteristic, we also conducted a molecular study using the 16S rRNA gene.

MATERIALS AND METHODS

Study area

This research was conducted from July 2021 until December 2021 in Baluran NP, Situbondo, East Java, Indonesia ($7^{\circ}49'52.3''\text{S}$, $114^{\circ}23'15.2''\text{E}$). Nine sampling sites, namely: Bekol, Bama, Balanan, Labuhan Merak, Watu Numpuk, Bitakol, Perengan, and Batangan, represented the division of the surveillance area (resort) (Figure 1). The sampling sites were selected by purposive sampling method, and they represented the diversity of habitat types (teak forest, secondary forest, evergreen, savanna, mangrove, and settlements).

Procedures

Sampling and preservation of samples

The sampling of stingless bees was carried out by directly capturing bees from the nest entrance or at spots where individual bees were spotted. The stingless bees were captured using a sweeping net with a diameter of 380 mm and a $0.9 \times 0.3 \text{ mm}^2$ polyester mesh. Then, the specimens were placed in 90% ethanol and labeled with a description of the specimen code, location, and sampling time.

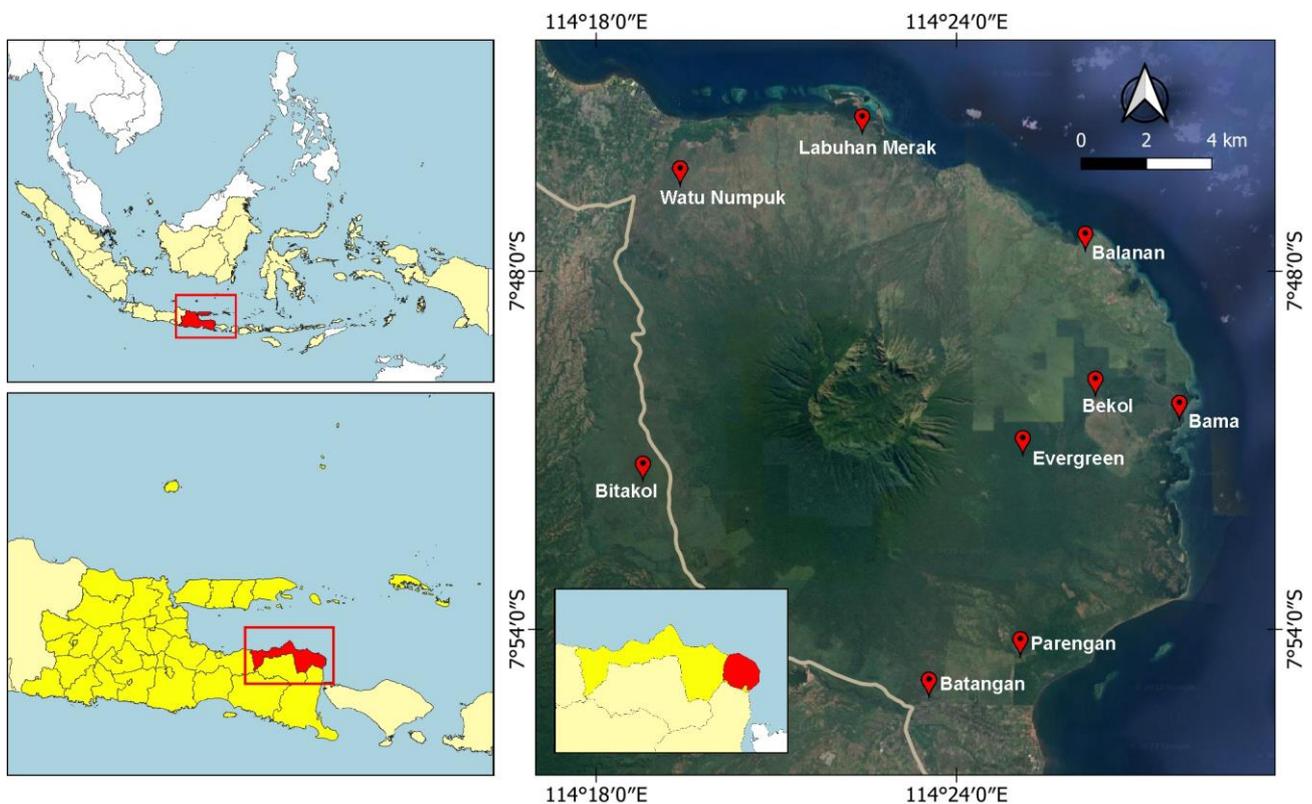


Figure 1. Sampling sites of stingless bees in Baluran National Park, East Java, Indonesia

Identification of specimen and morphometric measurement

The stingless bee specimens were identified in the Entomology Laboratory, Faculty of Biology, Gadjah Mada University. Specifically, the specimens were identified based on their morphological characteristics and morphometric measurements, as described by Sakagami et al. (1990) and Smith (2012). The morphological characteristics of the bees were observed and counted using a Supereyes™ digital microscope with Supereyes viewer® and Image Raster® software. Measurements were carried out on 20 specimens from four different colonies of the same species.

Nest entrance characterization

The images of the nest entrances of the stingless bee's nests were captured using a digital camera. Then, the nests were characterized based on the shape, ornaments, and color, diameter or width of the nest entrance, length or depth of the entrance tube, and height of the nest entrance from the ground surface. All measurements were conducted using the Image Raster® software.

Colony distribution in different types of habitats

The distributions of the stingless bees were estimated by the frequency of nests present at the sampling sites. Specifically, the calculations were performed using the following formula:

$$F = \frac{\text{the number of the nest of sp.i presence at the sampling point}}{\text{total number of sampling points}} \times 100\%$$

Molecular identification using 16S rRNA mitochondrial DNA (mtDNA)

The molecular identification of stingless bees was conducted through three stages, namely, extraction, amplification, and sequencing of DNA. One individual stingless bee species without a head and wings was extracted using Geneaid's gSYNCTM extraction kit, in accordance with the protocol. Polymerase chain reaction (PCR) amplification and DNA sequencing were carried out using the primer pairs of 16S rRNA (LR13107-F 5'-TGG CTG CAG TAT AAC TGA CTG TAC AAA GG-3') and (LR12647-R 5'-GAA ACC AAT CTG ACT TAC GTC GAT TTG A-3') (Thummajitsakul et al. 2013). PCR reactions were carried out using a GoTaq® Green master mix (Promega). The temperature profiles used during the PCR process for the 16S rRNA gene are as follows: predenaturation at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 30 s, and a final hold at 4°C (Thummajitsakul et al. 2013; Trianto and Purwanto 2020b; Purwanto and Trianto 2021). The amplified DNA (amplicons) were electrophoresed using 1% agarose gel and FluoroVue dye and run for ±45 min at 60 A and 60 volts. The DNA bands were visualized under an ultraviolet transilluminator, and the detection was documented using the gel documentation system (*gel-doc*). Then, DNA sequencing processes were carried out using the Sanger method (Trianto and Purwanto 2020b; Purwanto and Trianto 2021).

Data analysis

Table 1 lists the 35 body features of stingless bees that were measured in this study. The acquired morphometric data were analyzed using principal component analysis (PCA) with PAST4® software. The analysis was conducted to identify the grouping of characteristics to determine their diagnostic properties. The graphic forms of the scatter and loading plots were analyzed to determine the patterns of specimen groupings based on the role of each character in the grouping. The longer the arrow and the higher the graph on the loading plot graph, the greater the role of a characteristic in the grouping (Purwanto and Trianto 2021). Meanwhile, colony distribution data were displayed in the form of a bar graph to determine the difference in the number of colonies present in each type of habitat.

Bioinformatics analysis was performed by examining and editing DNA sequences using MEGA11® software. The sample forward- and reverse primer sequences were aligned with ClustalW. The DNA sequences were compared with the GenBank database using Nucleotide BLAST on the National Center for Biotechnology Information (NCBI) website (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). The BLAST results indicated the species of stingless bee that was most closely related to the sample. The phylogenetic reconstruction (phylogenetic tree) of sample DNA sequences and DNA sequences from the NCBI database was completed using the neighbor-joining method with a bootstrap value of 1000 using the Kimura 2-Parameter (K2P) model in MEGA11® (Tamura et al. 2021).

RESULTS AND DISCUSSION

Morphological characteristics of stingless bees

In this study, 49 colonies of stingless bees were found in different types of habitats (teak forest, secondary forest, evergreen, savannah, and mangrove), and one colony was observed from a meliponiculture on a nearby village/settlement. All colonies had been identified as *Lepidotrigona terminata* (24 colonies) and *Tetragonula laeviceps* (26 colonies).

Lepidotrigona terminata

Worker bees of this species have predominantly black and yellow bodies, a black head with white hair on the front, and a black clypeus that is also covered with white hair. The antennae are geniculate, and each one consists of 11 flagellomeres. In addition, this species has a yellowish gray socket and black scape with a brown base. The pedicel and flagella are blackish browns. Meanwhile, the compound eyes are greenish black, the ocelli are black and large, and the malar space has the same distance as the flagella. Its mandible has two small teeth, the basal half is black, and the other half is brown. *Lepidotrigona terminata* also possesses dark brown tegulae, bright black monotone wings with six hamuli on the hind wings, and a mesoscutum that is entirely black with yellow tessela. The scutellum tends to be short, reaching only its metanotum,

and is black with a golden yellow scale-like at the posterior margin. This stingless bee is also known for its black and hairy propodeum, yellow abdomen with black stripes, black legs, and brownish-black tarsus. Moreover, the hind tibia hair is simple, the corbicula in the hind tibia is pear-shaped, and the basitarsus hind tibia lacks a silica patch (Figure 2).

Tetragonula laeviceps

Tetragonula laeviceps has a smaller body size than *L. terminata*. In addition, it has a shiny brownish black whole body and a black head that is covered with short white hair. This species also has brownish-black compound eyes and black and large ocelli. The clypeus is black and covered with white hair completely. The antenna has 10 flagellomeres and is blackish brown in color. Moreover, *Tetragonula laeviceps* has a yellowish-brown scape, gray socket, and brown pedicel. The malar space is of this species is very short, and its mandible has two small brown teeth that are slightly black at the base. This stingless bee is also characterized by its brown tegula and monotone bright black membrane with dark brown venation. The hind wings have five hamuli. The mesoscutum and scutellum are black and completely covered with yellowish setae. Furthermore, *Tetragonula laeviceps* has a mesoscutum with hairbands and no tessellation. The scutellum is long and hanging. In addition, this stingless bee has a glossy black propodeum, a blackish brown abdomen with black stripes, slightly black legs, and a pear-shaped corbicula. The hind tibiae are black and short, sparsely covered with long setae at the apex but short at the base. A silica patch exists on the inside of the basitarsus hind tibia (Figure 3).

Meanwhile, PC2 had an eigenvalue of around 0.09 with a percent of variance of about 1.32. Thus, PC1 showed the body features that were used to determine species

differentiation. The features with the five highest scores were Hamuli number (HN), length of forewing including tegula (WL1), forewing length (FWL), hind wing length (HWL), hind tibia length (HTL), and body length (BL) as shown in the bar height in the loading graphic (Figure 4). These features are the most dominant characteristics that affect the variation of the clustering pattern of individual stingless bees.

Nest entrance features

The nest entrance of stingless bee showed some differences in shape, color, diameter, length, and height from the ground (Table 2). The nest in the natural habitat was found on various trees, such as *Ficus* sp., *Azadirachta indica*, *Protium javanicum*, *Schleichera oleosa*, *Tectona grandis*, *Cordia dichotoma*, *Saccopetalum horsfieldii*, *Streblus asper*, or *Schoutenia ovata*. Several stingless bees of the same species have different nest entrance structures. The stingless bee *Lepidotrigona terminata* can have an irregular, round pipe, oval, or wide funnel-shaped nest entrance, with colors that tend to be light brown or dark brown. Meanwhile, the *T. laeviceps* nest entrance has an irregular, round, pupa-like, or flattened-shaped nest entrance with a black color (Figure 5). The nest entrance of *T. laeviceps* in its natural habitat has a diameter and depth smaller than *L. terminata*. Furthermore, the height of the nest from the ground is shorter. The texture of the nest entrance of *L. terminata* is soft and dry, unlike that of *T. laeviceps* which tends to be wet and stickier. The position of the nest entrance in a particular tree is at different heights in each habitat. The nest in a more covered habitat, such as in a secondary forest or evergreen, can be found in a trunk lying low or high from the ground. Meanwhile, in arid places such as a savanna, the nest entrance is found in wood grooves and protected from being sighted.

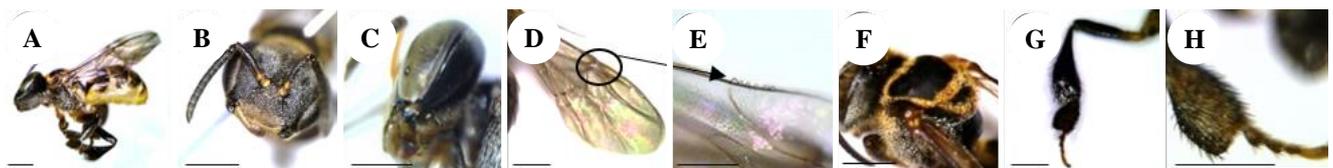


Figure 2. Morphological characteristics of *Lepidotrigona terminata*: A. whole body; B. head width; C. eye width; D. monotone wing membrane; E. six hamuli on the anterior margin of the hind wing; F. mesoscutum with tessellation; G. length of hind tibia; and H. width of basitarsus hind tibia. Scale bar: 1 mm

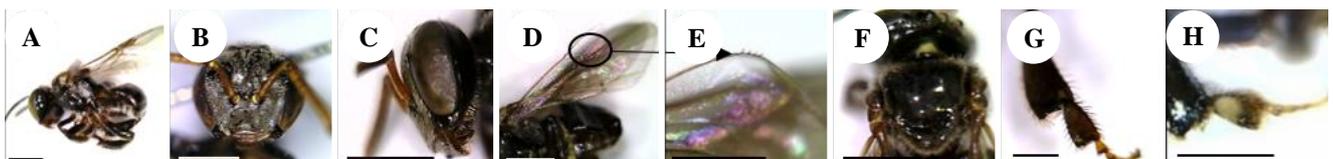


Figure 3. Morphological characteristics of *Tetragonula laeviceps*: A. whole body; B. head width; C. eye width; D. monotone wing membrane; E. five hamuli on the anterior margin of the hind wing; F. mesoscutum without tessellation; G. length of hind tibia; and H. width of basitarsus hind tibia. Scale bar: 1 mm

Table 1. Morphometric characteristics of stingless bees *Lepidotrigona terminata* and *Tetragonula laeviceps* in BNP, East Java, Indonesia

Body characters	Morphometry of stingless bees (mm)							
	<i>Lepidotrigona terminata</i> (N: 20)				<i>Tetragonula laeviceps</i> (N: 20)			
	Min	Max	\bar{x}	SD	Min	Max	\bar{x}	SD
Body length (BL)	4.71	5.46	5.12	0.33	3.67	4.37	3.92	0.33
Head length (HL)	1.72	1.95	1.78	0.12	1.35	1.41	1.38	0.02
Head width (HW)	2.18	2.26	2.24	0.04	1.61	1.66	1.64	0.02
Mandible length (ML)	0.72	0.80	0.74	0.04	0.62	0.66	0.63	0.02
Mandible width (MW)	0.24	0.26	0.25	0.01	0.22	0.28	0.25	0.03
Clypeus length (CL)	0.57	0.58	0.58	0.01	0.42	0.46	0.45	0.02
Lower interocular distance (LID)	1.27	1.29	1.28	0.01	0.86	0.88	0.87	0.01
Upper interocular distance (UID)	1.58	1.62	1.59	0.02	0.94	1.11	1.01	0.07
Eye width (EW)	0.48	0.60	0.52	0.06	0.39	0.44	0.41	0.02
Eye length (EL)	1.39	1.42	1.40	0.01	1.01	1.05	1.03	0.02
Maximum interorbital distance (MOD)	1.67	1.71	1.68	0.02	1.12	1.19	1.14	0.03
Lower interorbital distance (LOD)	1.42	1.43	1.42	0.01	0.85	0.92	0.88	0.03
Interantennal distance (IAD)	0.29	0.32	0.30	0.01	0.16	0.21	0.18	0.02
Interocellar distance (IOD)	0.59	0.63	0.61	0.02	0.19	0.24	0.20	0.02
Ocellular distance (OOD)	0.44	0.51	0.48	0.03	0.22	0.26	0.23	0.02
Antennocellar distance (AD)	0.67	0.72	0.69	0.02	0.61	0.63	0.62	0.01
Antennocular distance (AOD)	0.40	0.46	0.43	0.03	0.28	0.33	0.31	0.02
Gena width (GW)	0.38	0.43	0.40	0.02	0.24	0.26	0.24	0.01
Length of flagellomere IV (FL)	0.16	0.17	0.17	0.00	0.14	0.16	0.15	0.01
Width of flagellomere IV (FW)	0.14	0.14	0.14	0.00	0.11	0.17	0.15	0.03
Malar length (ML)	0.12	0.13	0.12	0.01	0.07	0.10	0.09	0.01
Mesoscutum length (MCL)	1.47	1.52	1.49	0.02	0.84	0.90	0.87	0.03
Mesoscutum width (MCW)	1.22	1.26	1.24	0.02	1.27	1.36	1.33	0.04
Length of forewing including tegula (WL1)	5.51	5.54	5.53	0.02	3.55	3.61	3.60	0.03
Distance between M-Cu bifurcation (WL2)	1.64	1.68	1.66	0.02	1.14	1.16	1.15	0.01
Fore wing length (FWL)	5.19	5.26	5.23	0.04	3.58	3.60	3.59	0.01
Fore wing width (FWW)	1.92	2.01	1.98	0.05	1.23	1.27	1.24	0.02
Hind wing length (HWL)	3.92	4.10	4.00	0.08	2.43	2.45	2.44	0.01
Hind wing width (HWW)	0.90	0.96	0.93	0.03	0.60	0.61	0.60	0.01
Hamuli number (HN)*	8.00	8.00	8.00	0.00	5.00	5.00	5.00	0.00
Hind femur length (HFL)	1.20	1.27	1.24	0.03	0.97	1.08	1.00	0.05
Hind tibia width (HTW)	0.73	0.77	0.74	0.02	0.42	0.48	0.45	0.02
Hind tibia length (HTL)	2.12	2.15	2.14	0.02	1.32	1.39	1.37	0.03
Hind basitarsus width (HBW)	0.46	0.49	0.47	0.01	0.26	0.34	0.29	0.04
Hind basitarsus length (HBL)	0.68	0.74	0.72	0.03	0.56	0.61	0.59	0.02

Note: N: number of samples; *The unit is not in millimeters

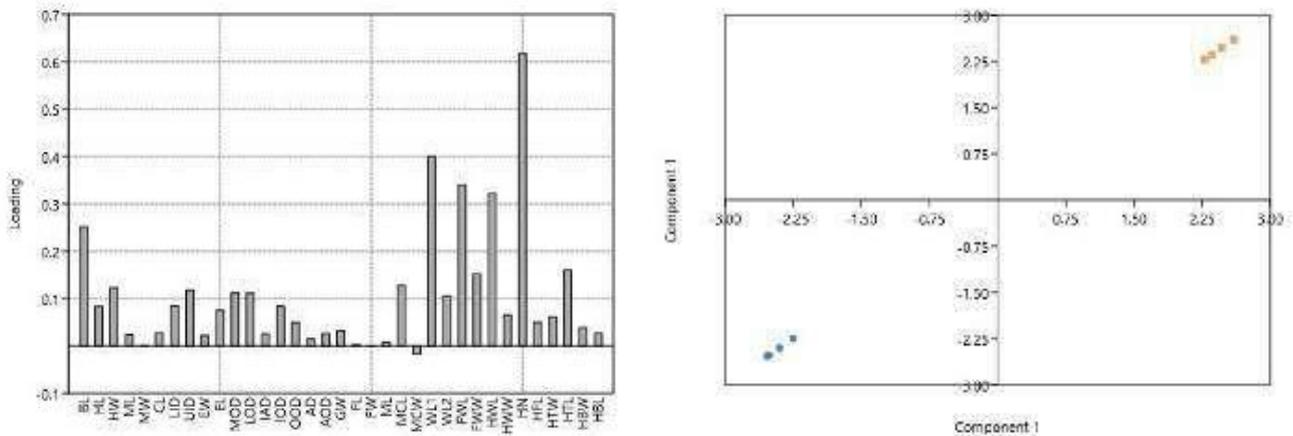
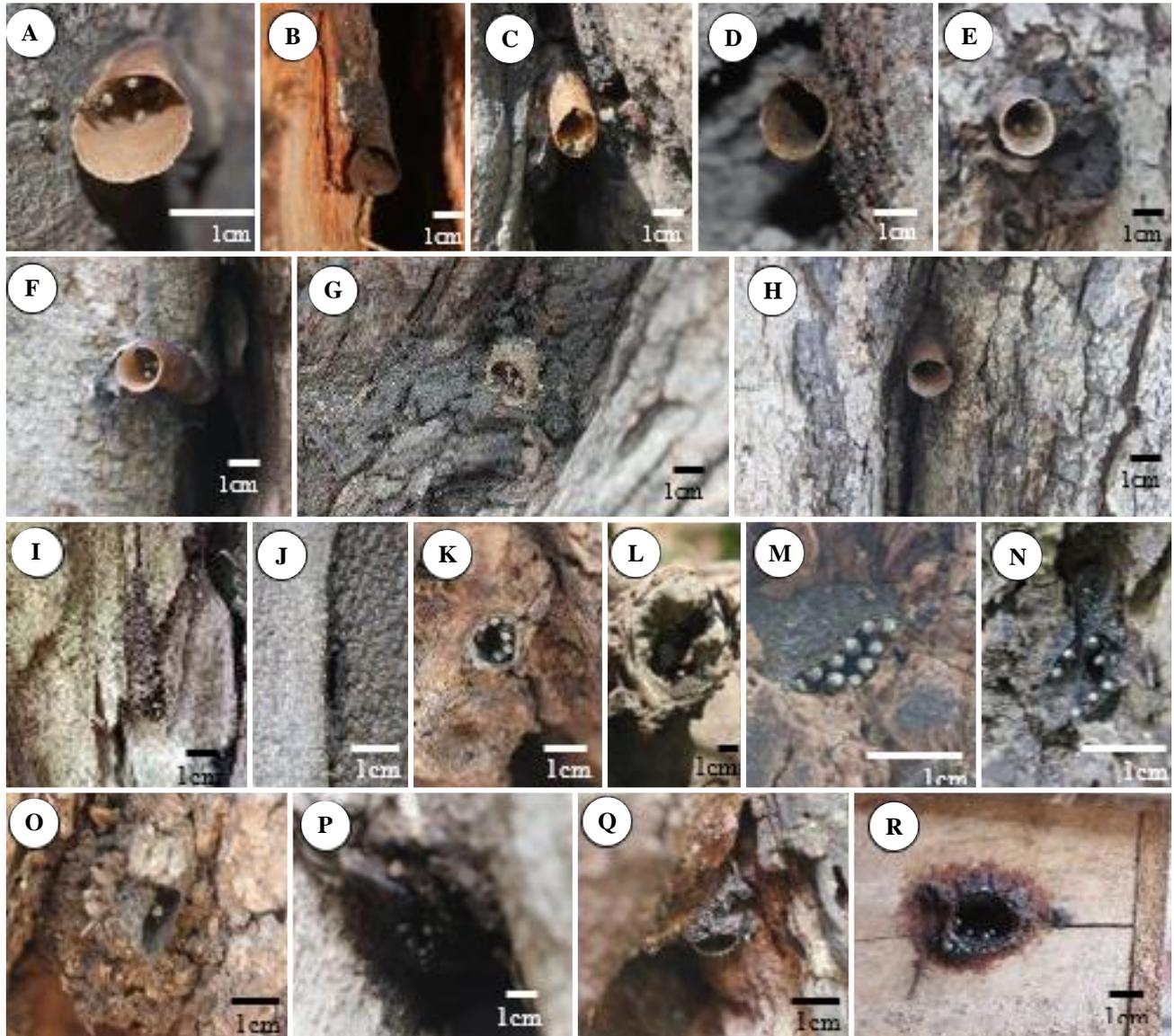


Figure 4. Loading and scatter plots of PC1

Table 2. Nest entrance characteristics of stingless bees *Lepidotrigona terminata* and *Tetragonula laeviceps* in BNP, East Java, Indonesia

Parameter	<i>Lepidotrigona terminata</i> (n: 24)	<i>Tetragonula laeviceps</i> (n: 26)
Shape	Irregular, round pipe, oval, wide funnel	Irregular, round, pupa-like, flattened
Color	Light brown or dark brown	Black
Diameter (cm)	1.28-1.48	0.47-1.5
Length/depth (cm)	0.5-2.3	0-1.7
Height from the ground (m)	0.59-2.5	0.3-1.6
Texture/ornament	Soft and tend to dry	Sticky, some with propolis

Note: n: number of colonies

**Figure 5.** Nest entrance of stingless bee nests in BNP: (A-H) *Lepidotrigona terminata* nests found in (A-D) a secondary forest, (E-F) evergreen, and (G-H) savanna; (I-R) *Tetragonula laeviceps* nests found in (I-N) secondary forest, (O) teak forest, (P-Q) savanna, and (R) meliponiculture adjacent to the park. Scale bar: 1 cm

Distribution of colonies in different habitats

Figure 6 presents the number of stingless bee colonies found at the sampling site. Based on these data, the *T. laeviceps* colonies were present in all sampling sites with an FR value of 100%. Meanwhile, only 55% of *L. terminata* was distributed. This species was absent in four locations, namely, Balanan, Labuhan Merak, Bitakol, and Batangan. Fourteen colonies of *L. terminata* were found in Perengan, and it was the highest number compared with the other sites. Meanwhile, one colony was found in Watu Numpuk. The Perengan forest area was dominated by tall plants with a dense canopy. The nests of *L. terminata* were mostly located in *Ficus* sp., *Azadirachta indica*, *Protium javanicum*, and *Schleichera oleosa* trees. The highest number of *T. laeviceps* colonies was observed in Labuhan Merak and Bama. The forest in these areas was divided into patches. Therefore, some bushy flowering plants flourish in the area. The nests of *T. laeviceps* in these areas were located in the trunk of *Azadirachta indica*, *Protium javanicum*, *Schleichera oleosa*, and *Cordia dichotoma* trees.

Figure 7 shows that the stingless bee colonies were mostly found in secondary forests rather than other habitats. A

total of 21 colonies of *L. terminata* and 16 colonies of *T. laeviceps* are present in this type of forest. This habitat is located in the Perengan, Bama, and Labuhan Merak. Meanwhile, the lowest number of stingless bee colonies was observed in the teak forest, in which two colonies of *T. laeviceps* can be found. By contrast, the *L. terminata* could not be detected in either mangrove or teak forests.

Molecular identification

The DNA sequences of six samples from the different nests were compared with the GenBank database using Nucleotide BLAST. The sequence of sample similarity was evaluated by its percent identification and query cover (Table 3). All sequences of 16S rRNA gene samples were identified as two species of stingless bees that have the closest genetic relationship with the same species data from GenBank, namely, *Lepidotrigona terminata* and *Tetragonula laeviceps*. The highest similarity values were observed in sample 4 *Lepidotrigona terminata* (Acc. Num MG543810.1), followed by sample 6 *Tetragonula laeviceps* (Accession Number KU571748.1).

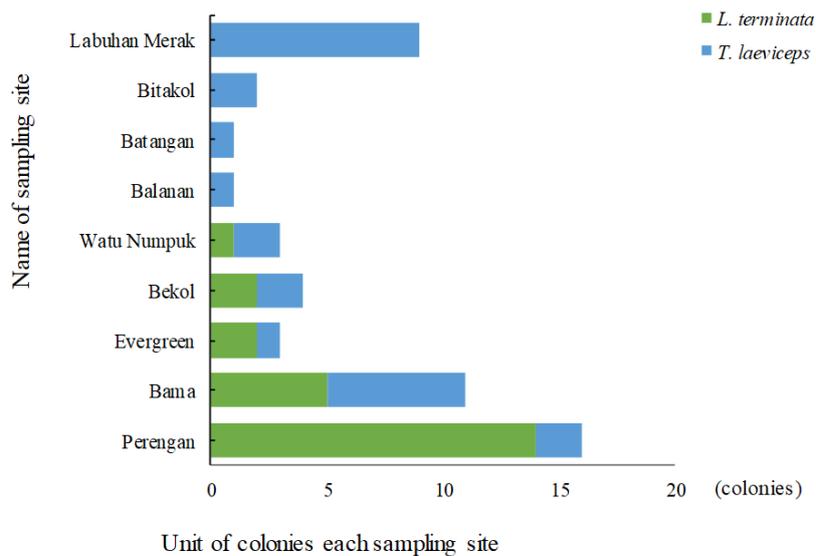


Figure 6. Graphic of colony distribution in all study sites

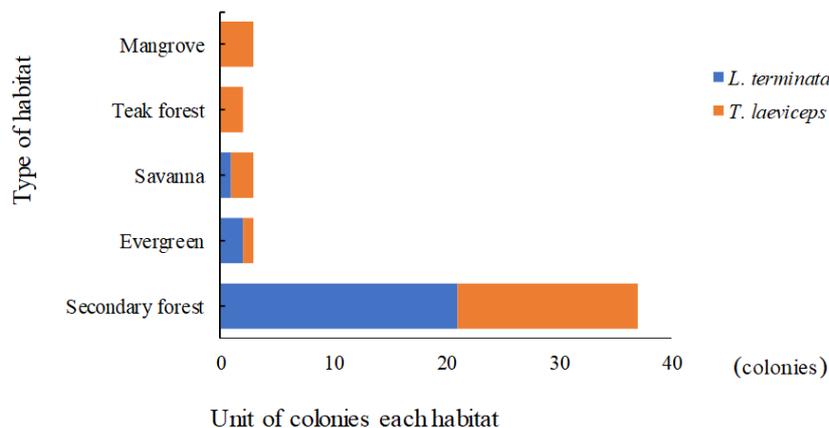


Figure 7. Graphic of colony distribution in all types of habitats

Table 3. Result of similarity Nucleotide BLAST NCBI analysis for 16S rRNA gene mtDNA of the stingless bees from Baluran NP, East Java

No. Sample	Per. Ident (%)	Query cover (%)	Species	Accession number
1	95.23	100	<i>Lepidotrigona terminata</i>	DQ790399.1
2	96.36	100	<i>Tetragonula laeviceps</i>	KU571748.1
3	96.25	100	<i>Tetragonula laeviceps</i>	KU571748.1
4	96.80	100	<i>Lepidotrigona terminata</i>	MG543810.1
5	96.27	100	<i>Tetragonula laeviceps</i>	KU571748.1
6	96.39	100	<i>Tetragonula laeviceps</i>	KU571748.1

Table 4. Nucleotide variations in the alignment of partial 16S rRNA gene sequences of the stingless bees from Baluran NP, East Java compared with the specimens from Yogyakarta (Trianto and Purwanto 2020b) and West Java (Purwanto and Trianto 2021)

Species	Nucleotide position										
	13	30	354	357	367	392	394	397	398	402	405
<i>Lepidotrigona terminata</i> East Java	G	A	A	C	T	T	A	C	T	A	A
<i>Lepidotrigona terminata</i> Yogyakarta	-
<i>Lepidotrigona terminata</i> West Java	-	G	G	T	C	A	C	T	C	C	T
	13	24	54	88	144	160	194	222	234	366	
<i>Tetragonula laeviceps</i> East Java	A	G	-	T	A	T	T	A	C	A	
<i>Tetragonula laeviceps</i> Yogyakarta	-	.	A	C	.	C	.	G	T	G	
<i>Tetragonula laeviceps</i> West Java	.	-	-	.	G	C	C	.	.	.	

Notes: (.): indicate identity with the first-lane DNA sequences; (-): indicate deletion base in the sequence

The stingless bee sequences used in this study were compared with the sequence samples from Yogyakarta (Trianto and Purwanto 2020b) and West Java (Purwanto and Trianto 2021). Table 4 provides the nucleotide variations among *L. terminata* and *T. laeviceps*, which were based on the molecular analysis using 16S rRNA, from three different provinces in Java. A total of 406 bp of *L. terminata* from the East Java sequence have 359 conserve sites and 10 variable sites compared with the samples from Yogyakarta and West Java. Base G was found at site 13 but not in the species from Yogyakarta and West Java. Nucleotide frequency analysis proved that the sequence consists of 36.2% T/U, similar to that in all samples, followed by 9.1% C, 39.9% A, and 14.8% G. The same amounts of C and A were observed in the Yogyakarta sample, but the G content was higher. Meanwhile, the *T. laeviceps* sequence length was about 441 bp. A total of 434 conserved sites and 7 variable sites with a deletion base were detected at site 54 for the East Java sample. All samples contained similar amounts of T/U, C, A, and G, with values of 37.6%, 9.8%, 38.5%, and 14.1%, respectively, in the East Java sample.

The pairwise genetic distances between three *L. terminata* bees from Java were between 0.00 and 0.02, and those for *T. laeviceps* were between 0.01 and 0.02. These findings indicated that no significant variance was observed among species from different provinces in Java. Given their genetic distance of 0.03, the *L. terminata* from East Java is more similar to the stingless bees from Thailand than Sulawesi, with which it shares a genetic distance of 0.04. The genetic distance between *L. ventralis* and *L. terminata* ranges between 0.06 and 0.08, which indicates that they belong to a different species. Similar to

Lepidotrigona, the genus *Tetragonula* showed a small variation in the genetic distance (0.01-0.08) (Table 5). The genetic distance can indicate how DNA transfers between populations (gene flow) and leads to microevolution (Nopriawansyah et al. 2019).

Furthermore, the genetic relationship was shown by a phylogenetic tree constructed using the neighbor-joining method with the K2P model. The sequences with NCBI accession numbers are published bee sequences retrieved from GenBank. The numbers above branches indicate the genetic distance, and the number below branches denotes the percentage bootstrap values of 1000 replicates. Evolutionary analysis was conducted in MEGA11® software (Tamura et al. 2021). The outgroups of the phylogenetic tree used another member of family Apidae in different tribes, i.e., *Apis cerana*, *A. dorsata*, and *A. mellifera* (Figure 8).

Discussion

We successfully collected two species of stingless bees in BNP. The existence of stingless bees in BNP can be caused by many factors, including a microclimate, which tends to be hot, and the presence of seasonal pollen-source plants. The stingless bees found in this study belong to two species based on the description of their morphological characteristics, morphometric measurements, and appearance of the nest entrance. The morphological and morphometric characteristics of the stingless bees in this study were then compared with the specimens described by Efin et al. (2019) from Pandeglang-Banten, Purwanto and Trianto (2021) from Ciamis-West Java, Trianto and Purwanto (2020a) from Yogyakarta, and Sayusti et al. (2020) from South and West Sulawesi.

Table 5. Estimates of genetic distance based on the 16S rRNA gene sequences between seven stingless bees species generated using Mega11® software (Tamura et al. 2021)

Species	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
1. <i>Apis cerana</i> Malaysia KX113623.1																				
2. <i>Apis dorsata</i> KX113621.1	0.10																			
3. <i>Apis mellifera</i> Malaysia KX113622.1	0.11	0.08																		
4. <i>L. terminata</i> East Java	0.26	0.26	0.28																	
5. <i>L. terminata</i> Yogyakarta	0.27	0.28	0.29	0.00																
6. <i>L. terminata</i> West Java	0.28	0.28	0.30	0.02	0.04															
7. <i>L. terminata</i> Sulawesi DQ790399.1	0.23	0.23	0.24	0.04	0.06	0.06														
8. <i>L. terminata</i> Thailand MG543810.1	0.24	0.25	0.27	0.03	0.05	0.04	0.05													
9. <i>L. ventralis</i> Malaysia DQ790400.1	0.24	0.22	0.25	0.06	0.08	0.08	0.07	0.06												
10. <i>T. drescheri</i> Malaysia MH453963.1	0.27	0.26	0.27	0.14	0.16	0.14	0.12	0.15	0.13											
11. <i>T. fuscobalteata</i> Malaysia DQ790416.1	0.26	0.24	0.24	0.13	0.14	0.14	0.12	0.13	0.11	0.09										
12. <i>T. laeviceps</i> East Java	0.25	0.23	0.24	0.12	0.14	0.14	0.10	0.13	0.12	0.03	0.08									
13. <i>T. laeviceps</i> Yogyakarta	0.28	0.25	0.26	0.13	0.14	0.15	0.12	0.14	0.14	0.05	0.10	0.02								
14. <i>T. laeviceps</i> West Java	0.26	0.23	0.24	0.13	0.15	0.14	0.11	0.13	0.13	0.03	0.08	0.01	0.02							
15. <i>T. laeviceps</i> KU571748.1	0.25	0.23	0.23	0.11	0.13	0.14	0.10	0.12	0.10	0.04	0.07	0.03	0.06	0.04						
16. <i>T. laeviceps</i> Malaysia DQ790438.1	0.26	0.24	0.25	0.15	0.16	0.17	0.12	0.15	0.12	0.04	0.09	0.04	0.06	0.05	0.04					
17. <i>T. minangkabau</i> MH453962.1	0.27	0.25	0.27	0.13	0.15	0.14	0.11	0.14	0.12	0.03	0.09	0.03	0.06	0.04	0.04	0.04				
18. <i>T. sapiens</i> Australia DQ790427.1	0.27	0.23	0.25	0.15	0.16	0.16	0.13	0.16	0.12	0.05	0.08	0.05	0.07	0.06	0.06	0.06	0.07			
19. <i>T. sarawakensis</i> Malaysia DQ790435.1	0.26	0.23	0.24	0.14	0.15	0.16	0.12	0.14	0.11	0.07	0.07	0.06	0.07	0.06	0.06	0.07	0.08	0.03		
20. <i>T. sirindhornae</i> Isthmus DQ790431.1	0.26	0.24	0.25	0.13	0.14	0.15	0.11	0.13	0.11	0.03	0.08	0.03	0.05	0.04	0.02	0.03	0.03	0.06	0.06	

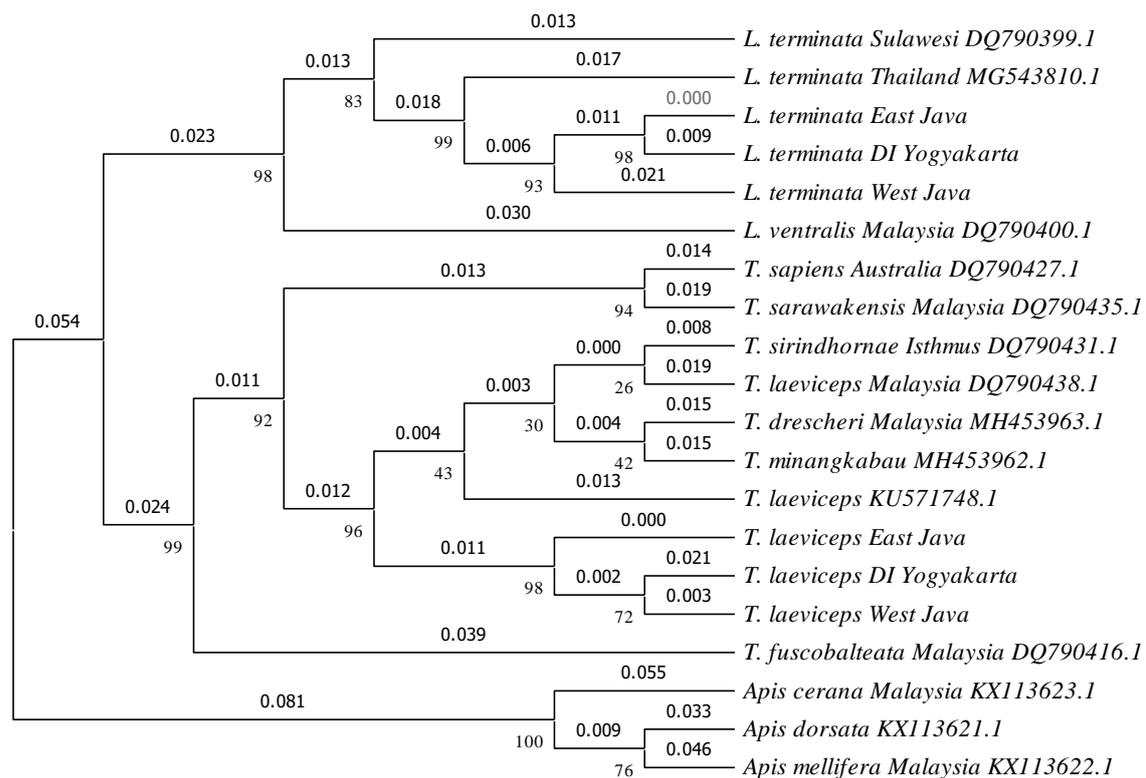


Figure 8. Phylogenetic tree of 20 bee species (17 stingless bees and 3 honeybees) based on 16S rRNA gene sequences constructed using MEGA11® software (Tamura et al. 2021). *Lepidotrigona terminata* East Java and *Tetragonula laeviceps* East Java are sequences from this study

The morphological differences between *Lepidotrigona terminata* and *Tetragonula laeviceps* can be observed by their body color and body shape features. *Tetragonula laeviceps* is brownish black, has a narrower malar space, hind wings with 5 hamuli, a scutellum protruding to the back (hanging), and a silky patch on the inner hind tibia basitarsus (Smith 2012; Engel et al. 2018). Meanwhile, *L. terminata* is characterized by a yellow and black pattern, a mesonotum covered by a yellow scale-like border (tessellation), hind wings with 6 hamuli, a short scutellum just reaching the metanotum, reticulate propodeal dorsum, and simple hair on the posterior hind tibia (Smith 2012; Engel et al. 2018; Sayusti et al. 2020). All specimens found in BNP had the same morphological features as the same species from another area.

The morphometric characteristics of the two species based on PCA dominantly differed in terms of HN, followed by WL1, FWL, HWL, BL, and HTL. The *L. terminata* found in BNP had 6 hamuli, same as the samples found by Sayusti et al. (2020). Meanwhile, the *L. terminata* found in West Java by Purwanto and Trianto (2021) and Yogyakarta by Trianto and Purwanto (2020a) had 8 hamuli. The *T. laeviceps* found in BNP had 5 hamuli, which is the same as those found by Efin et al. (2019); Trianto and Purwanto (2020a); Purwanto and Trianto (2021). *Lepidotrigona* and *Tetragonula* have 6-8 and 5 hamuli, respectively (Smith 2012; Engel et al. 2018). Hamuli are hook-like setae on the anterior margin of the

hind wing. They allow two wings to flight function by interlocking with the recurved posterior edge of the forewing. Each species of Hymenoptera has automorphic hamuli (Basibuyuk and Quicke 1997). Larger stingless bees have larger wings and have more hamuli in the hind wings to make a tighter bond on their wings when they fly.

The *T. laeviceps* found in BNP, East Java, had an average WL1 of approximately 3.60 ± 0.03 mm, similar to the specimens from Yogyakarta (3.63 mm) (Trianto and Purwanto 2020a). However, this value is smaller than those of specimens from Ciamis, West Java (3.70 ± 0.02 mm) (Purwanto and Trianto 2021) and Banten (4.481 ± 0.068 mm) (Efin et al. 2019). The FWL was approximately 3.59 ± 0.01 mm, similar to those of specimens from West Java and Yogyakarta but smaller than those of specimens from the work of Efin et al. (2019) (4.265 ± 0.081 mm). The HWL and HTL of the specimens in this study were about 2.44 ± 0.031 and 1.37 ± 0.03 mm, respectively, which are similar to those of the specimens from West Java and Yogyakarta. These sizes are smaller than those obtained by Efin et al. (2019) (2.855 ± 0.053 and 1.73 ± 0.019 mm for HWL and HTL, respectively). The *T. laeviceps* from BNP had an average BL of approximately 3.92 ± 0.33 mm, which is larger than those of the specimens from Yogyakarta (3.65 mm) and West Java (3.42 ± 0.02 mm). Meanwhile, the specimens from Banten were larger in terms of BL (4.445 ± 0.072 mm).

The *L. terminata* in BNP had an average WL1 of approximately 5.53 ± 0.02 mm, FWL of 5.23 ± 0.04 mm, HWL of 4.00 ± 0.08 mm, and HTL of 2.14 ± 0.02 mm. These sizes are similar to those of the specimens from West Java and Yogyakarta. The average BL of *L. terminata* was 5.12 ± 0.33 mm, similar to those of the specimens from Sulawesi (5.0 ± 0.4 mm) but slightly larger than those of the specimens from West Java (4.63 ± 0.01 mm) and Yogyakarta (4.63 mm).

Variations in the morphological and morphometric characteristics of stingless bees are the result of long adaptation from previous generations. They adapted to suit the environment to optimize their fitness. Environmental conditions force bees to adjust their morphology to facilitate flying and foraging activities in the environment (Novita and Sutriyono 2013). Each species of stingless bee has variations in body features that can affect their ability to form a nest. The mandibles are variously modified to collect resin or manipulate a particular material. The body size is more elongated to build a nest inside narrow burrows or cavities (Engel et al. 2020). Stingless bees build nests inside crevices or tree trunks. They lack an involucre, and their cells are arranged in clusters that spread out across the cavity (Engel et al. 2020).

The nest of stingless bees in the natural habitat is easily noticed by its nest entrance. The color and shape of the nest entrance within one species can be different, as observed in the nest entrance of *L. terminata* and *T. laeviceps*. However, the reason why specific types of nest entrances can only be found in certain habitats remains unclear. The variation in nest entrance color in nature is suspected due to the different resin sources and external factors. The nest texture becomes harder if the wax concentration is higher than that of resin (Pangestika et al. 2018). Other factors that influence the structure of nest entrances include nest age, bee genetics, light, rainfall, and predation (Sakagami et al. 1982). The nest entrance contributes additional information to describe the nest of stingless bee species only, and such knowledge cannot be used as the benchmark for identification because the nest entrance can change due to the conditions of the surrounding environment (Sakagami et al. 1982). Notably, the position of the nest entrance can differ depending on the habitat of the stingless bee. In open habitats, some nest entrances are located on the lower part of the trunk or in hidden tree grooves. These characteristics can be a form of the bees' adaptation to survive in their environment.

The distribution of bee colonies in BNP can be influenced by the availability of plants suitable for nesting, nectar, and as pollen sources. Colonies of stingless bees are mostly found in secondary forests. This type of habitat consists of diverse vegetation and a dense canopy, which provide suitable conditions for bee survival (Rohman et al. 2020). The colonies of *T. laeviceps* are distributed in all types of habitats under study. This condition indicates that *T. laeviceps* colonies adapt well to the environment. Gadhayah and Pastagia (2019) reported that *T. laeviceps* could visit flowers of many plant species, including vegetable, fruit, oil seed, pulse, forage, and weed crops. Their small body allows them to build a nest in a small

space inside the ground, rock crevices, in dry or rotten wood, pithy stems, plant galls, and tree branches or attach their nest to stems or rock surfaces (Engel et al. 2020). The nest entrance of *T. laeviceps* tends to be sticky because it is covered with propolis which maintains the hive's temperature. The propolis also protects the hive from external threats, such as microbes, predators, and extreme weather (Roubik 2006).

The first report on the characteristics and distribution of the stingless bees *Lepidotrigona terminata* and *Tetragonula laeviceps* found from BNP adds data on the biology and distribution of stingless bees in Indonesia. In general, bees play a significant role in maintaining the ecosystem (Rosli et al. 2020); thus, their existence in nature needs to be preserved. We believe that the significant role of bees also applies to the stingless bees on BNP. Therefore, the presence of stingless bees in BNP must be monitored to maintain the balance of the national park ecosystem, and this step includes preventing the introduction of bees, limiting the harvesting of honey and bee colony from the park, and developing sustainable meliponiculture in the settlement around the national park. On a larger scope, we must prevent damage and disturbance in the national park area to solve the problem in maintaining the existence of stingless bees.

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