Identification of leaf-cutting bees (Megachile sp.) from Northern Sulawesi of Indonesia: study of morphology, morphometric and genetic characteristics

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ABSTRACT

This study aims to identify of leaf-cutting bee (Megachile sp.) in Northern Sulawesi, Indonesia, based on morphology, morphometrics, and genetic characteristics. This study was done from September 2022 to March 2023. Species identification of the bees was carried out at the Museum Zoologicum Bogoriense (MZB). Morphometric measurements were used 25 characters of the body and then analysed using ANOVA. Molecular analysis used the partial Mitochondrial DNA COI sequence. Extract DNA was done from the thorax and amplified by primers LCO 1490 and HCO 2198. Data analysis and the phylogenetic tree were reconstructed using MEGA 11. The results showed that based on morphological characteristics, this species resembles to Megachile atrata Smith., but is not identical based on the wing colours and fine blackish-brown hairs on the abdomen. Hence, we use the term Megachile sp., hereinafter, for this bee. Morphometric analysis showed that 7 characters have significant differences between the populations. Molecular analysis showed that this bee had a low level of species similarity with other Megachile, ranging from 86.47% to 89.39%, even though the closed genetic distance was M. takaoensis (OR231111.1), and the farthest was M. opacifrons (OR796342.1). This study has contributed to the DNA barcoding of this bee in the gene bank database for further research in the future. Further research on this bee is required for conservation and management purposes because this bee is an effective pollinator for crops.

Keywords: COI, Leaf-cutting bees, Northern Sulawesi, Phylogenetic, Sequencing

Introduction

The leaf-cutting bee (Hymenoptera: Megachilidae) is a solitary bee that cuts the leaves of certain types of plants for nesting materials. This bee cuts the young leaves of plants in different shapes and sizes (Soh et al. 2019). Not all the leaves can be used by leaf-cutting bees to build their nest because those bees only choose leaves from certain plants that are hydrophobic and contain phytochemical properties such as antimicrobial, antiviral, and antiparasitic (Horne 1995; Kim 1997; Sinu & Bronstein 2018). The leaf-cutting bees use their instincts to select certain leaves that have bioactive content to protect bee eggs from microbial infections and parasitic insects that pose a threat to larval and pupa stadia (Sinu & Bronstein, 2018). Leaf-cutting bees make nests in the nests of other insects that have been abandoned. Leaf-cutting bees make nests in the ground, wooden holes, stone crevices, and bamboo cavities (Gonzalez et al. 2010). The nests are made from pieces of leaves or flowers that are rolled up to separate cells in a lined nest.

Studies on leaf-cutting bees from Southeast Asia are very limited and have not been widely explained. There are currently 150 known species of *Megachile* bees in Southeast Asia in dozens of subgenera (Ascher et al. 2016). However, there is still a need for additional studies on the distribution of species and their taxonomy. The larger *Megachile* bee species is reported to visit many plant species from the Fabaceae and Polygalaceae families (Corlett 2004; Momose & Karim 2005), while the smaller *Megachile* bee species in Southeast Asia are reported to visit many plant species from the Lamiaceae and Asteraceae families (Corlett 2004), while the report on the leaf-cutting bees was used leaves from Ebenaceae families for nesting are poor of study.

Information about the species of leaf-cutting bees and leaf species used as nest materials in Indonesia is very limited. The bees of *Megachile* mostly build nests in various preexisting cavities and are aligned with the source of materials that will be used to build their nest, such as resin or leaf pieces (Ascher et al. 2016). Meanwhile, *Megachile* bees from the subgenus *Creightonella* make nests in the ground using pieces of leaf arranged in layers of cell nest (Michener 2007). This study aims to identify of leaf-cutting bee (*Megachile* sp.) from the subgenus *Creightonella*, which uses the young leaves of *Diospyros rumphii* Bakh., (Ebenaceae) for nesting in the ground in Northern Sulawesi, Indonesia based on morphology, morphometric and genetic characteristics.

Materials and methods

Materials

The bee sample collection was done on September to November 2022 from three populations in the northern part of Sulawesi, namely: (1) population of Bolaang Mongondow at 123 m asl (Lat: 0.724941 and Long: 124.105181), (2) population of Manado at 378 m asl (Lat: 1.563093 and Long: 124.833078) and (3) population Karakelang Island at 8 m asl (Lat: 4. 5453 and Long: 126. 7418) (Figure 1).

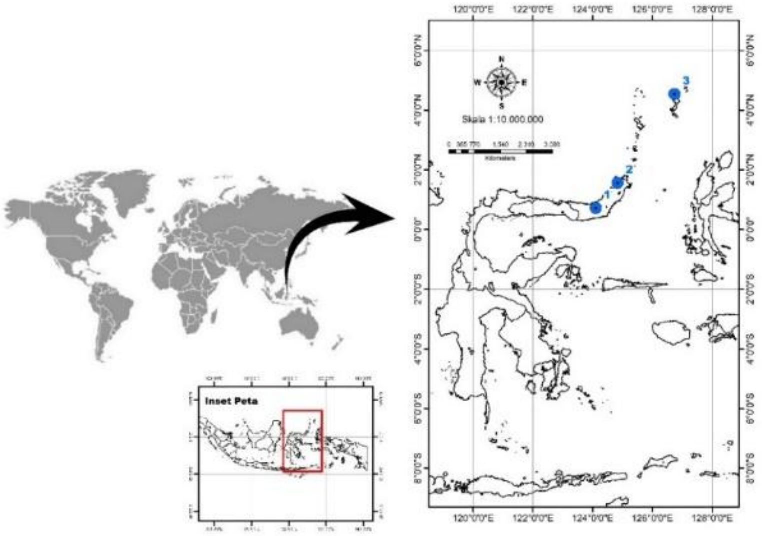

Figure 1. Map of research location in Northern Sulawesi, Indonesia. (1) Bolaang Mongondow, (2) Manado, (3) Karakelang Island

Methods

The sampling method used in this study is the purposive sampling method.

Morphology analysis

Morphology analysis and identification of the bee species were conducted at Muzeum Zoologicum Bogoriense (MZB), National Research and Innovation Agency, Indonesia, from

November to December 2022. The comparative specimens used in the identification of the species are 40 collections of leaf-cutting bee specimens (*Megachile*) with the collection number MZB. Hymn. 35577 to MZB. Hymn. 35621. Identify bee species based on morphological characteristics using identification keys (Michener 2007; Sheffield et al. 2011; Bzdyk 2012; Ascher et al. 2016; Gonzalez et al. 2019; Soh et al. 2019).

Morphometric analysis

Morphometric measurement follows Bzdyk with modification (Bzdyk 2012). Morphometric analysis was conducted in December 2022, at the Entomology Laboratory, Agriculture Faculty of Sam Ratulangi University, and the Forensic Laboratory of the North Sulawesi Police. We have modified morphometric measurements from the previous study conducted by Bzdyk with additional points of measurement on Antennal space length (AL), Mandible length (ML), and Abdomen length (ABL). Morphometric characters observed in this study are the length of the following body parts: Body Length (BL), Head Length (HL), Head Width (HW), Clypeus Width (CW), Anntenocullar Distance (AD), Interantennal Distance (ID), Distance from antennal socket to anterior ocelus (ASO), Wing cells length (WCL), Marginal cell length (MCL), Stigma length (SL), Hind Wing Length (HWL), Length to vannal lobe (LTV), Length to jugal lobe (LTJ), Coxa length (CL), Trochanter length (TL), Femur length (FL), Tibia length (TBL), Tibial spur length (TSL), Tarsus length (TRL), Basitarsus length (BTL), Distitarsus length (DTL), Antennal length (AL), Antenall Space (SL), Mandible length (ML), Abdomen length (ABL). Morphometric measurements were done using Hirox Digital Microscope HR-2500 on a millimeter scale. The data from the morphometric measurements was then analyzed using oneway ANOVA, and followed by the Duncan test using SPSS ver 27.

The similarity species between populations based on morphometric characteristics were calculated using the Sorensen formula (Odum 1996) modified with the following formula:

$$\mathrm{IS}=\frac{2\ \mathrm{C}}{(\mathrm{A}+\mathrm{B})}$$

Notes:
IS = Similarity Index
A = The number of different morphometric characters in sample location A
B = The number of different morphometric characters in sample location B
C = The same number of morphometric characters (not different) in sample locations A and B

Molecular analysis

Molecular analysis was conducted at Genetika Science Indonesia Laboratory from January to March 2023. Total DNA extraction from the thorax using ZR Tissue and Insect DNA MiniPrepTM (Zymo Research). The DNA genome is cleaned to produce high-quality DNA for PCR using DNA Clean and ConcentratorTM -5 (DCCTM-5). The COI region DNA barcode was amplified using the Toyobo KOD FX Neo PCR Master Mix with the LCO 1490 primary pair as the forward primer (5'-GGTCAACAAATCATAAAGATATTGG-3') and the HCO 2198 primary as the reverse primer (5'TAAACTTCAGGGTGACCAAAAAACA-3') (Folmer et al. 1994; Tallei et al. 2017). PCR includes 2-minute initial denaturation at 98^0C for 10 seconds, annealing at 54^0C for 30 seconds, elongation at 68^0C for 45 seconds, and 5-minute additional extension at 68^0C. PCR products are sequenced bidirectionally using the same PCR primary pair at the 1st base DNA Sequencing Service Malaysia.

Chromatograms follow standard procedures. The clean sequence of COI is stored in the NCBI Gene Bank (http://www.ncbi.nlm.nih.gov). Species identification was carried out using the Basic Local Alignment Search Tool (BLAST). The clean sequence was aligned in MEGA 11 (Tamura et al. 2021) with the 10 best matched *Megachile* COI sequences taken from the gene bank. The evolutionary history was inferred by the Maximum Likelihood (ML) method based on the Kimura 2-parameter (K2P) model (Kimura 1980). Phylogenetic analysis using MEGA 11 (Tamura et al. 2021).

Results and Discussion

Morphology characteristics

The results of species identification in MZB show that the bee species in this study have similarities with *Megachile atrata* Smith,1853 but are not identical to the previous collection of leaf-cutting bee specimens in MZB (MZB 35577- MZB 35621), so in this study, we use the name *Megachile* sp. For the first time, the leaf-cutting bees which use the young leaves of *D. rumphii* for nesting in the ground (*Megachile* sp.), were recorded in northern Sulawesi, Bioregion Wallacea, Indonesia (Figure 2). This species resembles to *M. atrata* Smith., but differs in the color of the wings and fine blackish-brown hairs on the abdomen. The bee of *M. atrata* has orange color in wings shape (Soh 2014; Ascher et al., 2016; Ascher & Pickering 2024), while *Megachile* sp. in this study has pale to dark brown in the color of wings (Figure 4). The bee of *M. atrata* known as the leaf-cutting bee, has a black and large body stature described for the first time from the Philippines (Smith 1853). The bees of *M. atrata* are very similar to *M. frontalis* (Fabricius). Both of these bees (*M. atrata* and *M. frontalis*) have a wide occurrence from Malay Peninsula to the Solomon Islands (Michener & Ivany 1960; Michener 2007).

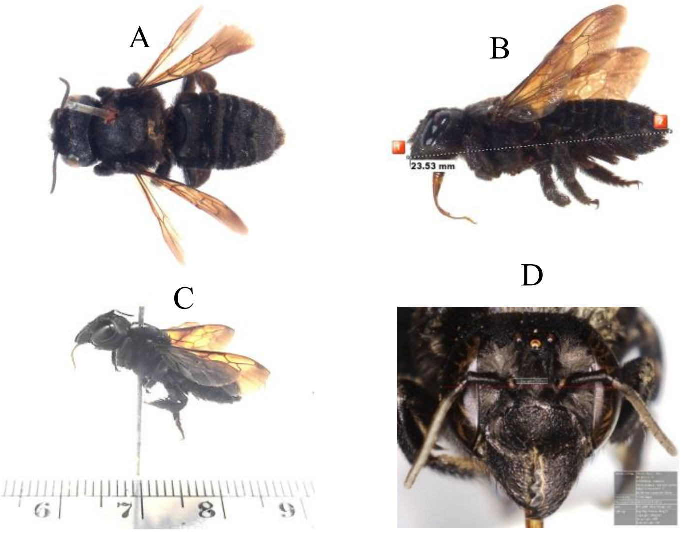

Figure 2. Adult female of *Megachile* sp.,

Notes : (A) dorsal view (B,C) lateral view (D) frontal view

Description:

Female (♀): Black body with fine brown hairs. Wings are light brown to dark brown. Body length size 22, 52 – 25,97 (mm); head length 3,85 – 5,18 (mm); head width 5,71 – 7,27 (mm);

clypeus width 3,07 – 4,08 (mm); annteocullar distance 0,80 – 1,43 (mm); interantennal distance 1,12 – 1,46 (mm); distance from antenna socket to anterior ocelus 1,04 – 1,38 (mm); antenna length 3,23 – 4,30 (mm); the number of antenna segments 9,00 – 10,00 (mm), mandible length 1,76 – 2,40 (mm); wings are pale to dark brown. wing cell length 10,94 – 11,88 (mm); marjinal cell length 3,21 – 5,19 (mm); stigma length 0,91 – 1,19 (mm); hind wing length 9,17 – 11,03 (mm); vannal lobe length 5,43 – 6,71 (mm); jugal lobe length 3,02 – 4,08 (mm); coxa length 0,92 – 1,34 (mm); trochanter length 0,57 – 1,30 (mm); femur length 2,71 – 3,23 (mm); tibia length 2,68 – 3,59 (mm); tibia spur length 0,69 – 3,49 (mm); tarsus length 3,99 – 5,80 (mm); basitarsus length 2,05 – 2,92 (mm); distitarsus length 0,68 – 0,83 (mm); abdomen is black with fine brown hair on the surface, and the length 8,16 -10,12 (mm).

Male (♂): Unknow

The morphological characteristics of leaf cutting bees (*Megachile* sp.) can be influenced by differences in their habitat. Environmental factors that can cause morphological variations in leaf cutting bees include temperature, humidity, availability of food sources and natural selection pressures can affect variations in body size, wing shape and color, proboscis length and mandibular structure used to cut leaves. Adaptation to the environment can also lead to morphometric changes that assist leaf cutting bees (*Megachile* sp.) in foraging for food and building nests. The leaf cutting bees that live in densely vegetated areas have larger body sizes and stronger mandibles compared to sparsely vegetated areas.

Morphometric characteristics

Morphometric analysis has been widely applied to Hymenoptera for species identification and classification. More traditional approaches have sensitivity to geometric morphometry, so an approach that combines genetic and morphometry methods is a more optimal approach to answer questions about taxonomy and species identification in Hymenoptera. The results of morphometric measurement were then analysed for the variance using one-way ANOVA and followed by the Duncan test based on the data from morphometric measurements of each population (Table 1).

Table 1. Results of morphometric measurements of *Megachile* sp., based on the population

No	Body Characters	Bolaang Mongondow		Manado		Karakelang island	
		Min-Max (mm)	Mean ($\bar{x}$)	Min-Max (mm)	Mean ($\bar{x}$)	Min-Max (mm)	Mean ($\bar{x}$)
1	Body Length (BL)	25,97 ± 24,62	25,25 $^{*)}$	24,08 ± 22,52	23,30 ^{ns}	25,65 ± 20,61	23,18 ^{ns}
2	Head Length (HL)	4,17 ± 3,23	3,85 ^{ns}	3,97 ± 3,80	3,89 ^{ns}	5,18 ± 3,80	4,50 ^{ns}
3	Head Width (HW)	6,15 ± 5,64	5,93 ^{ns}	5,71 ± 5,55	5,63 $^{*)}$	7,27 ± 5,81	6,66 $^{*)}$
4	Clypeus Width (CW)	3,28 ± 3,16	3,23 ^{ns}	3,14 ± 2,99	3,07 $^{*)}$	4,08 ± 3,18	3,65 $^{*)}$
5	Anntenocullar Distance (AD)	0,90 ± 0,80	0,84 $^{*)}$	0,87 ± 0,80	0,84 ^{ns}	1,43 ± 0,92	1,14 $^{*)}$
6	Interantennal Distance (ID)	1,18 ± 1,15	1,17 $^{*)}$	1,26 ± 1,12	1,19 ^{ns}	1,46 ± 1,21	1,37 $^{*)}$

7	Distance from antennal socket to anterior ocelus (ASO)	1,10 ± 1,04	1,06 $^{*)}$	1,15 ± 1,04	1,09 $^{*)}$	1,38 ± 1,13	1,29 $^{*)}$
8	Antenall length (AL)	3,79 ± 3,23	3,53 ^{ns}	3,42 ± 3,30	3,36 ^{ns}	4,30 ± 3,58	3,78 ^{ns}
9	Antenall Space (AS)	10 ± 9	9,67 ^{ns}	10 ± 9	9,50 ^{ns}	10 ± 9	9,33 ^{ns}
10	Mandible length (ML)	2,40 ± 1,96	2,17 $^{*)}$	1,90 ± 1,76	1,83 ^{ns}	2,37 ± 1,85	2,08 $^{*)}$
11	Abdomen length (ABL)	9,76 ± 9,07	9,39 $^{*)}$	8,26 ± 8,16	8,21 $^{*)}$	10,12 ± 9,13	9,68 $^{*)}$
12	Wing cell length (WCL)	11,88 ± 11,57	11,76 $^{*)}$	11,13 ± 10,75	10,94 $^{*)}$	11,48 ± 11,25	11,39 $^{*)}$
13	Marginal cell length (MCL)	3,65 ± 3,45	3,52 ^{ns}	3,37 ± 3,21	3,29 ^{ns}	5,19 ± 3,44	4,09 $^{*)}$
14	Stigma length (SL)	1,10 ± 0,91	1,03 ^{ns}	1,14 ± 1,05	1,10 ^{ns}	1,08 ± 0,98	1,03 ^{ns}
15	Hind Wing Length (HWL)	11,03 ± 10,40	10,79 $^{*)}$	9,48 ± 9,17	9,33 $^{*)}$	10,76 ± 9,85	10,35 $^{*)}$
16	Length to vannal lobe (LTV)	6,12 ± 5,43	5,80 $^{*)}$	6,71 ± 5,57	6,14 $^{*)}$	5,83 ± 5,48	5,71 $^{*)}$
17	Length to jugal lobe (LTJ)	3,44 ± 2,85	3,12 ^{ns}	4,08 ± 3,16	3,62 ^{ns}	3,37 ± 3,02	3,15 ^{ns}
18	Coxa length (CL)	1,25 ± 1,18	1,22 $^{*)}$	0,96 ± 0,92	0,94 $^{*)}$	1,34 ± 1,01	1,19 $^{*)}$
19	Trochanter length (TL)	0,86 ± 0,57	0,72 ^{ns}	0,75 ± 0,64	0,695 ^{ns}	1,30 ± 0,68	0,92 ^{ns}
20	Femur length (FL)	3,12 ± 2,77	2,96 ^{ns}	2,97 ± 2,71	2,84 ^{ns}	3,23 ± 2,91	3,05 $^{*)}$
21	Tibia length (TBL)	3,49 ± 2,68	3,21 ^{ns}	3,19 ± 3,02	3,105 ^{ns}	3,59 ± 3,37	3,46 ^{ns}
22	Tibial spur length (TSL)	3,49 ± 2,68	3,21 $^{*)}$	0,94 ± 0,80	0,87 $^{*)}$	1,12 ± 0,69	0,89 $^{*)}$
23	Tarsus length (TRL)	5,03 ± 4,64	4,78 ^{ns}	4,15 ± 3,83	3,99 ^{ns}	5,80 ± 4,34	4,90 ^{ns}
24	Basitarsus length (BTL)	2,64 ± 2,33	2,49 ^{ns}	2,11 ± 1,99	2,05 $^{*)}$	2,92 ± 2,29	2,53 $^{*)}$
25	Distitarsus length (DTL)	0,74 ± 0,69	0,72 ^{ns}	0,73 ± 0,70	0,715 ^{ns}	0,83 ± 0,68	0,78 ^{ns}

$^{*)}$ = Significant in α 95%
ns = not significant

Based on the result of morphometric analysis (Table 1) showed that there are 7 of 25 characters different from each other population namely: ASO, ABL, WCL, HWL, LTV, CL, and TSL. The results show that BL from Bolaang Mongondow ($\bar{x}$=25.25 mm) differed significantly from Manado ($\bar{x}$=23.30 mm) and Karakelang Island ($\bar{x}$=23.18 mm). In contrast, BL of Manado and the Karakelang island did not differ significantly. The results of these findings show that the body length of this bee from Bolaang Mongondow is larger than Manado and Karakelang island. The size of HW from Manado was significantly smaller ($\bar{x}$ = 5.63 mm), and Karakelang Island was significantly larger ($\bar{x}$= 6.66 mm). The size of CW from Manado was significantly smaller ($\bar{x}$= 3.07 mm), while Karakelang Island was significantly larger ($\bar{x}$= 3.65 mm).

The results of this finding show that the size of HW and CW of this bee in the northern peninsula of Sulawesi (Manado) is suspected to have undergone environmental adaptation so that its size becomes smaller than Bolaang Mongondow, in addition to this species that lives in isolated areas (Karakelang Island) is larger than the population of Bolaang Mongondow. The size of ID from the Bolaang Mongondow differed significantly smaller ($\bar{x}$=1.17 mm), than Manado and Karakelang Island, on the other hand, the bees from Karakelang Island differed significantly larger ($\bar{x}$=1.37 mm). The length size of BTL from the Manado differed significantly smaller ($\bar{x}$= 2.05 mm); on the other hand, the bees from Karakelang Island differed significantly larger ($\bar{x}$= 2.53 mm). The size of ML from Bolaang Mongondow was significantly larger ($\bar{x}$= 2.17 mm); on the contrary, the bees from Manado are the smallest ($\bar{x}$= 1.83 mm).

The environmental conditions in which the bees live play a role in shaping the morphometric characteristics of bees, as revealed in the study of Anaktototy et al. 2021 that morphometric parameters such as thorax width, propodeum length, and mesonotum size differ significantly between islands. Other studies also stated that the difference in environmental altitude between highlands and lowlands shows differences in bee morphometrics in the size of the proboscis and femur of the hind legs (Novita et al. 2013).

The results of this study show that the species Similarity Index (IS) between the three research sites is in the high to very high range (Table 2). Morphological characters that have similarities between populations based on the variables of body parts (morphometry) between the 3 populations compared, namely: HL, AL, AS, SL, LTJ, TL, TL, TRL, and DTL.

Table 2. Index similarity species (%) based on morphology characteristics from each population compared

Population	Bolaang Mongondow	Manado	Karakelang Island
Bolaang Mongondow	-----	85.71	69.23
Manado	85.71	-----	80.00
Karakelang Island	69.23	80.00	-----

Categories Similarity Index:

Very High: IS > 75 %

High: IS > 50 % - 75 %

Low: > 25 % - 50 %

Very Low: IS < 25 %

The results of morphological characteristics analysis (Table 2) show that very high species similarity index was shown by bee samples from Bolaang Mongondow and Manado (85.71%). The very high species similarity index was also shown by bee samples from Manado and Karakelang Island (80.00%), while Bolaang Mongondow and Karakelang Island were relatively high (69.23%).

Molecular characteristic

The COI sequence of leaf-cutting bees, which use the young leaves of *D. rumphii* for nesting in the ground (*Megachile* sp.), has been deposited in the gene bank NCBI with accession numbers PQ583403.1 (Bolaang Mongondow), PQ583404.1 (Karakelang Island), and PQ583405.1. (Manado). The ten best-matched DNA sequences are shown in Table 3. All the sequences have query cover range from 99 % - 100%, with the percentage of similar identity ranging from 86,47% - 89.39%.

Table 3. The ten best-matched DNA sequences in the NCBI gene bank database

No	Scientific name	Max. score	Total score	Query cover (%)	E value	Percent identity (%)	Accsession
1	*M. takaoensis*	828	828	100%	0.0	89.39%	OR231111.1
2	*M. albisecta*	765	765	99%	0.0	87.69%	OR796820.1
3	*M. albisecta*	749	749	99%	0.0	87.23%	OR796529.1
4	*M. ramakrishnae*	737	737	99%	0.0	86.97%	KT960849.1
5	*Megachile* sp. CHL14	730	730	99%	0.0	86.85%	KX820968.1
6	*M. newberryae*	732	732	99%	0.0	86.78%	HQ929881.1
7	*M. rotundata*	732	732	99%	0.0	86.78%	KJ836559.1
8	*M. diabolica*	726	726	99%	0.0	86.63%	KX580374.1
9	*M. rotundata*	726	726	99%	0.0	86.63%	KJ837183.1
10	*M. opacifrons*	721	721	99%	0.0	86.47%	OR796342.1

This study shows that, based on the results of BLAST with the sequence data of leaf-cutting bees (Hymenoptera: Megachilidae) stored in the NCBI gene bank database, from 10 best match sequences (Table 2) show a very low level of species similarity (< 95 %). The closest species based on BLAST results is *M. takaoensis* (OR231111.1) with query cover 100%, and the farthest is *M. opacifrons* (OR796342.1). However, the percent identity of the 10 best-matched sequences is very low, ranging from 86,47 % to 89,39 %. The molecular differences between *M. takaoensis* and *Megachile* sp., are also very contrasting in their stature. *M. takaoensis* has a brownish-black body stature (Morris 2024), while *Megachile* sp., in this study, has a pitch-black body stature with smooth brownish hair.

The molecular phylogenetic analysis by the ML method is based on the K2P model, and the phylogenetic tree reveals two main clades (Figure 3).

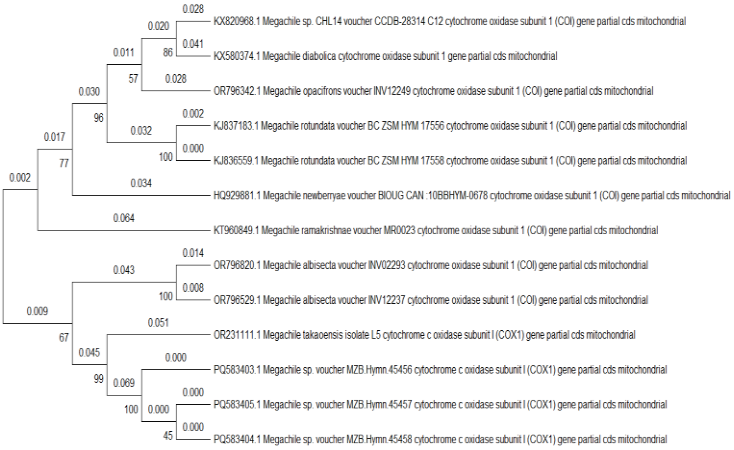

Figure 3. Molec model.

Results of the phylogenetic analysis showed that the bees of *Megachile* sp. under study, are closed related and come from the same ancestor, but have been separated (Figure 4). This study shows that this bee comes from Bolaang Mongondow (PQ583403.1) and then spreads to Manado (PQ583405.1), and the others migrate to Karakelang Island (PQ583404.1) with different morphological characteristics. These findings are appropriate to the statement by Wilson et al. 2013 that different geographical conditions can cause morphological differences. The lack of data collection on various species and the dependence of the morphological identification process have led to debate about species due to genetic variation (Dayrat 2005; Agnarsson & Kuntner 2007). The greater the value of genetic distance between individuals or populations, the more isolated they are from one another. Genetic distances indicate the possibility of the influence of geographical isolation on a population (Schmitt & Haubrich 2008; Laltanpuii et al. 2014).

Estimation of genetic distance among *Megachile* bees based on the ten best matched sequences in gene bank NCBI shown in Table 4

Table 4. Estimation of evolutionary divergence between selected haplotypes

No	Haplotype	1	2	3	4	5	6	7	8	9	10	11	12	13
1	PQ583405.1													
2	PQ583404.1	0,000												
3	PQ583403.1	0,000	0,000											
4	OR796820.1	0,138	0,138	0,138										
5	OR796529.1	0,144	0,144	0,144	0,022									
6	OR796342.1	0,153	0,153	0,153	0,117	0,120								
7	OR231111.1	0,119	0,119	0,119	0,140	0,129	0,149							
8	KX820968.1	0,151	0,151	0,151	0,135	0,131	0,072	0,160						
9	KX580374.1	0,151	0,151	0,151	0,124	0,127	0,087	0,153	0,070					
10	KT960849.1	0,151	0,151	0,151	0,129	0,124	0,118	0,151	0,120	0,140				
11	KJ837183.1	0,151	0,151	0,151	0,127	0,131	0,068	0,168	0,084	0,092	0,129			
12	KJ836559.1	0,149	0,149	0,149	0,126	0,129	0,067	0,166	0,082	0,090	0,127	0,002		
13	HQ929881.1	0,149	0,149	0,149	0,104	0,099	0,096	0,136	0,092	0,099	0,113	0,090	0,089	

The estimation of substitution using the maximum composite likelihood method is shown in Table 5. Each entry shows the probability of substitution (r) from one base (row) to another base (column). Substitution patterns and rates were estimated under the K2P model (Kimura 1980). The sum of the values of (r) was equal to 100. The nucleotide frequency consists of A = 25.00%, T/U = 25.00%, C = 25.00%, and G = 25.00%. This analysis involved 14 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 658 bp.

Table 5. Estimation of the nucleotide substitution pattern using the maximum composite likelihood method.

	A	**T/U**	**C**	**G**
A	-	6,7931	6,7931	**11,4139**
T/U	6,7931	-	**11,4139**	6,7931
C	6,7931	**11,4139**	-	6,7931
G	**11,4139**	6,7931	6,7931	-

The ability of COI gene sequence to differentiate leaf-cutting bees (*Megachile* sp.) that use the young leaves of *D. rumphii* (Ebenaceae) from different locations in northern Sulawesi, Indonesia, has been shown in this study. This molecular marker was also able to identify leaf-cutting bees (*Megachile* sp.) successfully based on a sequence similarity with other *Megachile* species.

The closest genetic distance was 0.1190 between haplotype *Megachile* sp. from northern Sulawesi, Indonesia (PQ583403.1, PQ583404.1, PQ583405.1) and *M. takaoensis* (OR231111.1). The farthest genetic distance was 0.1529, between haplotype *Megachile* sp. and *M. opacifrons* (OR796342.1). This finding shows that molecularly, these bees are different species from other species recorded in the NCBI gene bank database. Due to that, the genetic data recorded for this species is the first record for this species in the NCBI gene bank database.

4. Conclusions

For the first time, leaf-cutting bees (*Megachile* sp.), that use the young leaves of *D. rumphii* for nesting in the ground were identified. Based on morphological characteristics, this species resembles *M. atrata*, but is not identical based on the wing colours and fine blackish-brown hairs on the abdomen. The results of the morphometric analysis showed that 7 of 25 characters have significant differences between the populations compared. The results of the molecular analysis showed that this species had a low level of species similarity with other *Megachile* sp. (Megachilidae), ranging from 86.47% to 89.39%. The closed genetic distance was *M. takaoensis* (0.1190), and the farthest was *M. opacifrons* (0.1529). Molecular phylogenetic analysis showed that leaf-cutting bees under study were classified in similar clusters with *M. takaoensis* and *M. albisecta*. This study has contributed to a better understanding of the species of leaf-cutting bees that nest in the ground using young leaves of *D. rumphii* for nesting material in Northern Sulawesi, Indonesia. This study has also contributed to the DNA barcoding of this bee in the gene bank database for further research in the future. Further research on this bee is required for conservation and management purposes because this bee is an effective pollinator for crops.

Author contribution

J.K. design, collected and analysed data and wrote the manuscript. M.T., J.F.W., J.M.E.M., R.W.T., E.A., designed the research and supervised all the process. W.P.B. analysed data, wrote the manuscript and supervised the process.

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Conflict of Interest

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

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