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HIGH GENETIC VARIATIONS OF THE STINGLESS BEE *Tetragonula laeviceps* BASED ON MITOCHONDRIAL DNA OF CYTOCHROME C OXIDASE SUBUNIT 1 (*CO1*) GENE IN SUMATRA AND JAVA, INDONESIA

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ABSTRACT

The stingless bee *Tetragonula laeviceps* (Hymenoptera: Apidae) is frequently traded from its native regions to many areas across Indonesia for meliponiculture purposes. This species has a high morphological variation that make it difficult to identify. Identification at the molecular level based on the cytochrome c oxidase subunit 1 (*COI*) gene from mitochondrial DNA is needed to support morphological identification. However, there is a lack of *COI* gene data for these bees in the GenBank. Thus, this research aimed to characterize *T. laeviceps* in Indonesian native and traded regions using the *CO1* gene. Stingless bee samples were collected from two native regions: Batanghari (Jambi, Sumatra) and Lebak (Banten, Java), while the traded bee colonies were from Bogor (West Java, Java). The *COI* gene primers were manually designed based on the complete *CO1* gene of *Melipona bicolor* (AF466146). Twenty-four nucleotide variations from 13 individuals of *T. laeviceps CO1* gene sequences were found and classified into six haplotypes (Hap_1–Hap_6). The substitution of nucleotide number 157 of *T. laeviceps CO1* gene altered to a single putative amino acid from serine to phenylalanine. A high (3.7%) genetic distance was found between the Hap_1 haplotype (Batanghari and Bogor) and other haplotypes (Hap_4 and Hap_6). The Hap_1 performed a single clade split from other haplotypes with 82% bootstrap value in the phylogenetic tree, meaning that the Hap_1 of *T.*

laeviceps presumably has a high genetic differentiation. Extensive studies using *T. laeviceps* from a wider distribution area are needed to explain the haplotype variation thoroughly.

Keywords: DNA barcode, *CO1* gene, intraspecies variations, stingless bees, *Tetragonula laeviceps*.

ABSTRAK

Lebah kelulut *Tetragonula laeviceps* (Hymenoptera: Apidae) sering diperdagangkan dari daerah asal ke pelbagai daerah lain di Indonesia bagi tujuan meliponikultur. Spesies lebah ini memiliki variasi morfologi yang tinggi sehingga sukar untuk dicamkan. Pengecaman spesies pada peringkat molekul berdasarkan jujukan gen sitokrom oksidase c subunit 1 (*CO1*) diperlukan bagi menyokong pengecaman morfologi. Namun, data jujukan gen *CO1 T. laeviceps* di GenBank sangat terhad. Kajian ini bertujuan untuk mencirikan *T. laeviceps* yang berasal dari daerah asal dan daerah perdagangan menggunakan jujukan gen *CO1*. Lebah disampel dari dua daerah asal: Batanghari (Jambi, Sumatra) dan Lebak (Banten, Jawa), Indonesia sedangkan lebah yang diperdagangkan berasal dari Bogor (Jawa Barat, Jawa), Indonesia. Pencetus gen *CO1* dicipta secara manual berdasarkan gen *CO1* lengkap *Melipona bicolor* (AF466146). Sejumlah 24 variasi nukleotida dari 13 jujukan gen *CO1 T. laeviceps* dijumpai dan dikelaskan menjadi enam haplotip (Hap_1–Hap_6). Substitusi nukleotida pada site 157 dari gen *CO1 T. laeviceps* mengubah satu asam amino putatif dari Serin kepada Fenilalanin. Jarak genetik yang tinggi (3,7%) dijumpai antara haplotip Hap_1 (Batanghari dan Bogor) dan haplotip lainnya (Hap_4 dan Hap_6). Haplotip Hap_1 membentuk klad tunggal yang terpisah dari haplotip lain dengan nilai butstrap 82% pada pohon filogenetik, ini bermakna haplotip Hap_1 nampaknya mempunyai pembezaan genetik yang tinggi. Kajian meluas menggunakan *T. laeviceps* dari kawasan taburan yang lebih luas diperlukan untuk menerangkan variasi haplotip secara menyeluruh.

Katakunci: Barkod DNA, gen *CO1*, variasi intraspesies, kelulut, *Tetragonula laeviceps.*

INTRODUCTION

The stingless bee (Hymenoptera: Apidae) is a eusocial bee that provides an excellent ecosystem service by pollinating natural plant communities and crops. The metabarcoding of stingless bee *Heterotrigona itama* collected from 12 meliponiculture in Peninsular Malaysia (Selangor, Terengganu, Perak, Kedah, and Perlis) and Borneo (Sabah and Sarawak) revealed that this species foraged 262 plant species from 70 families (Fahimee et al. 2021). In a local farm in North Bandung, West Java, Indonesia, the stingless bees *Tetragonula laeviceps* and *T. irridipenis* have a high rate of visitation and improve fruit set of agricultural commodities such as hot pepper (*Capsicum annum*) and tomato (*Lycopersicon esculentum*) (Putra et al. 2014, Putra & Kinasih 2014). Stingless bees have a large distribution area throughout the tropical and subtropical regions covering Afrotropical, Australasian, Indo-Malayan, and Neotropical Regions (Michener 2000). As part of the tropical Indo-Malayan ecoregion (Olson et al. 2001), Indonesia has a high stingless bee diversity. At least 46 stingless bee species from 10 genera have been recorded (Kahono et al. 2018). The most common stingless bee in Indonesia is *Tetragonula laeviceps*, with the largest distribution area covering the Indonesian Archipelagoes (Kahono et al. 2018).

Besides the large distribution area, the bees were also recorded as the most common species in Indonesian meliponiculture (Buchori et al. 2022). The demand for *T. laeviceps*

colonies has increased in Indonesian meliponiculture due to their high adaptation to diverse and disturbed environments such as rural and suburban areas (Sakagami et al. 1984). In addition, *T. laeviceps* produce high economic products such as honey and a larger amount of propolis with a higher antioxidant content than honeybees (Chanchao 2013; Muruke 2014). Cultivation of *T. laeviceps* in the coffee plantation showed a sustainable production of propolis and honey with high flavonoid and vitamin C content against free radicals (Abduh et al. 2020). Thus, *T. laeviceps* colonies are frequently traded from their native area to the other region of Indonesia for meliponiculture purposes.

Unfortunately, *T. laeviceps* has an unclear taxonomic status due to its high number of intraspecific variations in morphological characteristics, especially in size, color, and hair pattern on the specific body parts (Sakagami 1978). Morphometric analysis of *T. laeviceps* from Kalimantan (Purwanto et al. 2022) and Java Island (Trianto et al. 2020; Purwanto & Trianto 2021) revealed that *T. laeviceps* from different locations have different body length. Furthermore, body color analysis of *T. laeviceps* from Malaysia divided *T. laeviceps* into four different groups with different color of antennae scape and thorax (Ador et al. 2023). Thus, *T. laeviceps* is difficult to identify using morphological characteristics and needs to be assisted by molecular identification tools such as DNA barcoding.

The standardized cytochrome oxidase subunit 1 (*CO1*) gene from mitochondrial DNA is the primary barcode sequence for animal phyla, including stingless bees (Herbert et al. 2003). The *CO1* gene in the stingless bee successfully separated two morphospecies of *Meliponula ferruginea,* black and reddish brown, from Africa (Ndungu et al. 2018). The high haplotype diversity of *H. itama* from Malaysia was also found based on the *CO1* gene sequence (Mohd Yusof et al. 2018). The *CO1* gene was also successfully separated three species of *Tetragonula* from Sulawesi Island, Indonesia (Sayusti et al. 2021). Furthermore, Phylogenetic analysis based on *CO1* gen in *T. laeviceps* from Malaysia showed that *T. laeviceps* clustered to the three different groups with high 97-100% bootstrap value (Ador et al. 2023). However, the *CO1* gene data of *T. laeviceps* from Indonesia is currently limited. Therefore, this study aimed to analyze the intraspecies variations of *T. laeviceps* based on the *CO1* gene sequence by comparing native and traded *T. laeviceps* colonies in Indonesia.

MATERIALS AND METHODS

Stingless Bees Sampling

Stingless bee samples were collected from two native regions: Batanghari (Jambi, Sumatra) and Lebak (Banten, Java), Indonesia, and one traded region: Bogor (West Java, Java), Indonesia (Table 1). The origin of the bee colonies traded to Bogor is Lebak.

Table 1. The cytochrome c-oxidase subunit 1 (*CO1*) gene sequences of stingless bee ingroup (*Tetragonula* bees) and outgroup used in bioinformatic analysis

No	Species	Location	Sample code	Accession number	Reference
Ingroup					
1	*T. laeviceps*	Batanghari, Jambi, Sumatera, Indonesia (S 2°.06'50" E 103°18'45")	T.lv_BTH1	LC657572	Current study
2	*T. laeviceps*	Bogor, West Java, Java, Indonesia (S 06°.33' 13,0 " E 106°.43'13,2")	T.lv_BGR1 T.lv_BGR2 T.lv_BGR3	LC657573 LC657574 LC657575	Current study

			T.lv_BGR4	LC657576	
			T.lv_BGR5	LC657577	
			T.lv_BGR6	LC657578	
3	*T. laeviceps*	Lebak, Banten, Java, Indonesia (S 06°.46'73,43" E 106°.06'08,88")	T.lv_LBK1	LC657579	Current study
			T.lv_LBK2	LC657580	
			T.lv_LBK3	LC657581	
			T.lv_LBK4	LC657582	
			T.lv_LBK5	LC657583	
			T.lv_LBK6	LC657584	
4	*T. fuscobalteata*	North Luwu, South Sulawesi Province, Indonesia	T.fs_LWU	LC440357	Sayusti et al. (2021)
5	*T. clypearis*		T.cl_LWU	LC440358	
6	*T. sapiens*		T.sp_LWU	LC440356	
7	*T. iridipenis*	India	T.rd_IND	KT960851	Makkar et al. (2016)
8	*T. hockingsi*	Australia	T.hc_AUS	KM112224.1	Cunningham et al. (2014)
9	*T. carbonaria*	Australia	T.cr_AUS	KM112244.1	
Outgroup					
1	*Lepidotrigona termianta*	North Luwu Indonesia	L.tr_LWU	LC440359	Sayusti et al. (2021)
2	*Melipona bicolor*	Brazil	M.bc_BRZ	AF466146	Silvestre et al. (2008)
3	*Apis mellifera*	Australia	A.ml_AUS	L06178.1	Crozier & Crozier (1993)
4	*Bombus hypocrita*	Korea	B.hp_KOR	NC_011923	Hong et al. (2008)

T.lv = *T. laeviceps*, T.fs = *T. fuscobalteata*, T.cl = *T. clypearis*, T.sp = *T. sapiens*, T.rd = *T. iridipenis*, T.hc = *T. hockingsi*, T.cr = *T. carbonaria*, M.bc = *M. bicolor*, A.ml = A. mellifera, BTH = Batanghari, BGR = Bogor, LBK = Lebak, LWU = Luwu, IND = India, AUS = Australia, BRZ = Brazil

DNA Extraction and PCR Amplification

Genomic DNA was extracted using the 0.2% cetyltrimethylammonium bromide (CTAB) extraction method (Sambrook et al. 1989) with modification of double step in using PCI (Phenol-Chloroform-Isoamil alcohol) and CIAA (Chloroform-Isoamil alcohol) (Raffiudin & Crozier 2007). Two pairs of *CO1* gene primers were manually designed to amplify *T. laeviceps CO1* gene based on the template of *COI* gene of *Melipona bicolor* mitochondrial whole sequence (Accession number AF466146 (Silvestre et al. 2008). Primers were designed by searching for primer regions on the DNA template of the closest taxa that match the primer criteria i.e., primer length (18–24 bases), 56–62°C melting temperature (Dieffenbach et al. 1993), 50–60% GC content, ideal 2–3 GC clamp (Patel & Prakash 2013), and avoiding hairpins dan self-dimer (Patel & Prakash 2013). Primer specificity was tested using BLAST tools at http://blast.ncbi.nlm.nih.gov/Blast.cgi, while primer efficiency was tested using *oligoanalyzer* tools in Integrated DNA Technology (IDT) (https://sg.idtdna.com) by looking at the formation of hairpins and self-dimer. One pair of primer was obtained, i.e., F_Mbc_CO1 (forward primer) and R_Mbc_CO1 (reverse primer) (Table 2) that was used to amplify the first *T. laeviceps* samples from Batanghari region (T.lv_BTH1). However, the *CO1* gene amplicon using our

designed primer resulted in double band DNA. The above DNA band was then cloned and sequenced as the first 908 bp of *T. laeviceps CO1* gene sequence. The second primer pair F_Tlv_CO1 (forward primer) and R_Tlv_CO1 (reverse primer) (Table 2) were then designed using the first *T. laeviceps* sequences and was used to amplify the *CO1* gene for other *T. laeviceps* samples.

Table 2. Nucleotide sequences of primers for *T. laeviceps CO1* gene amplification

No	Primer Name	Sequences (5'-3')	Primer Position	DNA Template used for primer design
1	F_Mbc_CO1	**TGCTTTATG ATCAGGAAT AATTGG**	45–68	Complete *CO1* gene of *M. bicolor* AF466146.1 (Silvestre et al. 2008)
	R_Mbc_CO1	**TAAAATATG CTCGTGTAT CAATAT**	872–895	
2	F_Tlv_CO1	**CTCTCTAGTT ACAGGTCAT GCA**	99–120	Partial *CO1* gene of *T. laeviceps* LC657572 (Current study)
	R_Tlv_CO1	**CTCGTGTAT CAATATCGA GTCC**	820–841	

DNA amplification was conducted in a 25 µL final volume of the PCR mix containing 8 µL distilled water (dH_2O), 1.5 µL of 25 mM $MgCl_2$, 1.0 µL of 10 µM forward primer, 1.0 µL of 10 µM reverse primer, 12.5 µL of MyTaq™ Red Mix (Bioline Reagents Ltd, United Kingdom), and 1 µL of the DNA template with the DNA concentration of 20–60 ng/µL. PCR was conducted in 30 cycles under predefined conditions: 2.5 min of pre-denaturation at 95°C, 15 s of denaturation at 95°C, 15 s of annealing at 44–53°C, 1.5 min of elongation at 72°C, and 2 min of post-elongation at 72°C. Visualization of PCR products was conducted by electrophoresis using 1% agarose gel and stained using Diamond Nucleic Acid Dye (Promega, Madison, USA).

Bioinformatic Analysis

A total of 13 *CO1* gene sequences of *T. laeviceps* were analyzed using the nucleotide Basic Local Alignment Search Tool (BLAST-N) (http://blast.ncbi.nlm.nih.gov/blastn). Putative amino acid analysis was applied to all nucleotide sequences using Genetyx-win 4.0 (genetyx.co.jp) and aligned using the Clustal X sequence alignment program (Thompson et al. 1997). Polymorphism analysis within current *T. laeviceps* was conducted using DnaSP6 (Rozas et al. 2017) to determine their haplotype diversity. The genetic distance and phylogenetic tree of *T. laeviceps* and other *Tetragonula* bees from GenBank (Table 1) were constructed using the maximum likelihood approach implemented in MEGA 6.06 (Tamura et al. 2013) with 1000 bootstraps. The substitution rates of the putative codon position were analyzed using the Tamura-Nei model (Tamura & Nei 1993).

RESULTS

Thirteen *CO1* gene sequences of *T. laeviceps* have been submitted to GenBank (Table 1). Our *T. laeviceps CO1* gene sequences were A-T rich, with 65.8% of the A-T composition. Twenty-four nucleotide variations were found out of 581 bp multiple alignment sequences, with the highest 21 nucleotide variations belonging to *T. laeviceps* from Batanghari and Bogor (Table 3). The conserved and parsimony-informative sites represented 557 and 24 nucleotides, respectively. BLAST-N analysis further revealed that the closest relative of *T. laeviceps* was *Lepidotrigona flavibasis* (MN747147.1) with 82.09%–82.61% and 99%–100% of similarity and query cover value, respectively.

The DnaSP polymorphism analysis identified six haplotypes (Hap_1–Hap_6) out of the 13 *T. laeviceps CO1* gene sequences. The Hap_1 haplotype was found in bee samples from Batanghari and Bogor, while Bogor and Lebak shared the same haplotypes, i.e., Hap_2–Hap_4. The specific haplotype of Hap_5 and Hap_6 was only found in Lebak. The Hap_1 haplotype consisted of samples with the highest 21 nucleotide variations from the native Batanghari (Tlv_BTH1) and those traded to the Bogor region (Tlv_BGR1, Tlv_BGR3) (Table 1). Using the combined current data with *Tetragonula CO1* from Genbank (447 bp), the interspecies genetic distance ranged from 16.9% to 31.5% (Table 4). The genetic distance within *T. laeviceps* ranged from 0.2%–3.7%. The lowest 0.2% genetic distance was observed between Hap_2 and Hap_3, Hap_4 and Hap_5, and Hap_4 and Hap_6. In contrast, a high 3.7% genetic distance was shown between Hap_1 and the other five haplotypes (Table 4).

Despite a high genetic distance between *Tetragonula*, the constructed phylogenetic tree showed that *Tetragonula* species belonged to a single cluster. All *CO1* gene sequences of *T. laeviceps* also performed a single cluster with a high 99% bootstrap value and the high intraspecific variation of Hap_1 split from the other five haplotypes with 82% bootstrap value in the constructed *CO1* gene phylogenetic tree (Figure 1). Putative codon position analyses of the *T. laeviceps CO1* gene revealed that transition exceeded the transversions (Figure 2a), with the highest substitution number observed at the third codon position (Figure 2 b). In addition, the high nucleotide variation of Hap_1 altered one putative amino acid from serin to phenylalanine (Table 5).

Table 3. Variable sites and haplotypes of *T. laeviceps* of *CO1* gene sequence

Haplotypes	Samples	Nucleotide Position																							
		3	2 4	7 3	7 5	1 2 3	1 3 5	1 5 3	1 5 7	1 6 2	1 9 8	2 0 4	2 6 4	3 2 1	3 5 1	3 6 6	3 6 9	3 8 1	3 9 6	4 5 0	4 5 9	4 7 1	4 9 8	5 0 7	5 5 5
Hap_1	T.lv_BTH1, T.lv_BGR1, T.lv_BGR3	G	T	T	A	T	C	T	C	T	G	A	T	T	A	G	C	A	A	G	A	C	C	G	G
Hap_2	T.lv_BGR4, T.lv_LBK1	A	C	C	G	.	.	C	T	C	A	G	.	C	G	A	A	G	G	A	G	T	T	A	A
Hap_3	T.lv_LBK4, T.lv_BGR5, T.lv_BGR6	A	.	C	G	.	.	C	T	C	A	G	.	C	G	A	A	G	G	A	G	T	T	A	A
Hap_4	T.lv_BGR2, T.lv_LBK2, T.lv_LBK5	A	.	C	G	C	A	C	T	C	A	G	C	C	G	A	A	A	G	G	G	T	T	A	A
Hap_5	T.lv_LBK3	A	.	C	G	.	A	C	T	C	A	G	C	C	G	A	A	A	G	G	G	T	T	A	A
Hap_6	T.lv_LBK6	A	.	C	G	C	A	C	T	C	A	G	C	C	G	A	G	A	G	G	G	T	T	A	A

T.lv = *T. laeviceps*, BTH = Batanghari, BGR = Bogor and LBK = Lebak

Table 4. The estimated genetic distance of *T. laeviceps* based on the *CO1* gene sequence. Abbreviations refer to Table 1

No	Samples	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]	[13]	[14]	[15]	[16]	[17]	[18]	[19]
1	T.lv_BGR4_Hap_2																			
2	T.lv_LBK4_Hap_3	0.002																		
3	T.lv_BGR5_Hap_3	0.002	0.000																	
4	T.lv_BGR6_Hap_3	0.002	0.000	0.000																
5	T.lv_LBK1_Hap_2	0.000	0.002	0.002	0.002															
6	T.lv_BGR2_Hap_4	0.011	0.009	0.009	0.009	0.011														
7	T.lv_LBK2_Hap_4	0.011	0.009	0.009	0.009	0.011	0.000													
8	T.lv_LBK3_Hap_5	0.009	0.007	0.007	0.007	0.009	0.002	0.002												
9	T.lv_LBK5_Hap_4	0.011	0.009	0.009	0.009	0.011	0.000	0.000	0.002											
10	T.lv_LBK6_Hap_6	0.014	0.011	0.011	0.011	0.014	0.002	0.002	0.004	0.002										
11	T.lv_BGR1_Hap_1	0.035	0.033	0.033	0.033	0.035	0.037	0.037	0.035	0.037	0.037									
12	T.lv_BGR3_Hap_1	0.035	0.033	0.033	0.033	0.035	0.037	0.037	0.035	0.037	0.037	0.000								
13	T.lv_BTH1_Hap_1	0.035	0.033	0.033	0.033	0.035	0.037	0.037	0.035	0.037	0.037	0.000	0.000							
14	T.cr_AUS	0.236	0.232	0.232	0.232	0.236	0.232	0.232	0.228	0.232	0.232	0.219	0.219	0.219						
15	T.hc_AUS	0.209	0.205	0.205	0.205	0.209	0.199	0.199	0.202	0.199	0.199	0.178	0.178	0.178	0.194					
16	T.rd_IND	0.214	0.211	0.211	0.211	0.214	0.208	0.208	0.208	0.208	0.208	0.190	0.190	0.190	0.315	0.223				
17	T.fs_LWU	0.230	0.227	0.227	0.227	0.230	0.226	0.226	0.223	0.226	0.226	0.218	0.218	0.218	0.251	0.245	0.249			
18	T.sp_LWU	0.181	0.178	0.178	0.178	0.181	0.172	0.172	0.169	0.172	0.172	0.169	0.169	0.169	0.227	0.223	0.228	0.222		
19	T.cl_LWU	0.209	0.206	0.206	0.206	0.209	0.209	0.209	0.205	0.209	0.209	0.181	0.181	0.181	0.244	0.251	0.231	0.218	0.202	

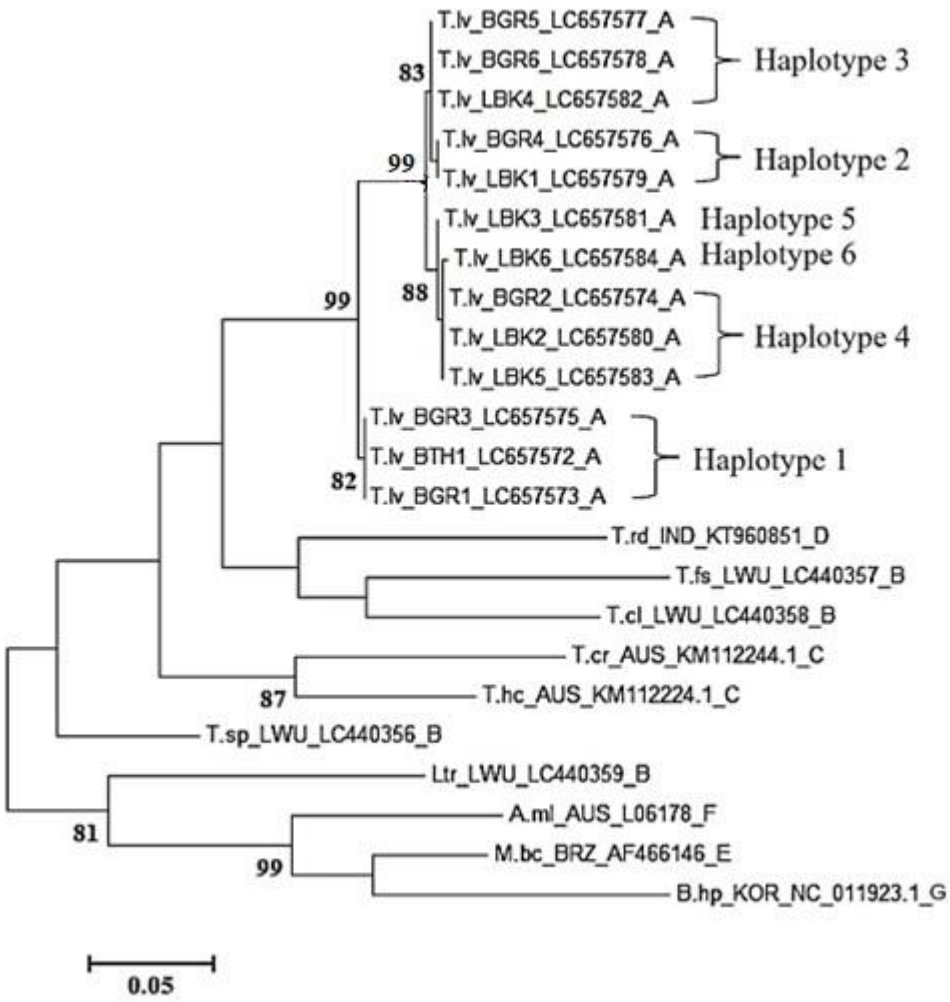

Figure 1. Phylogenetic tree of *Tetragonula* bees based on 447 bp *COI* gene sequences. The letters in the last indicate that the sample is from A. this study, B. Sayusti et al. (2021), C. Cunningham et al. (2014), D. Makkar et al. (2016), E. Silvestre et al. (2008), F. Crozier & Crozier (1993) and G. Hong et al. (2008). The number at the nodes denote 1000x bootstrap values. Abbreviations refer to Table 1

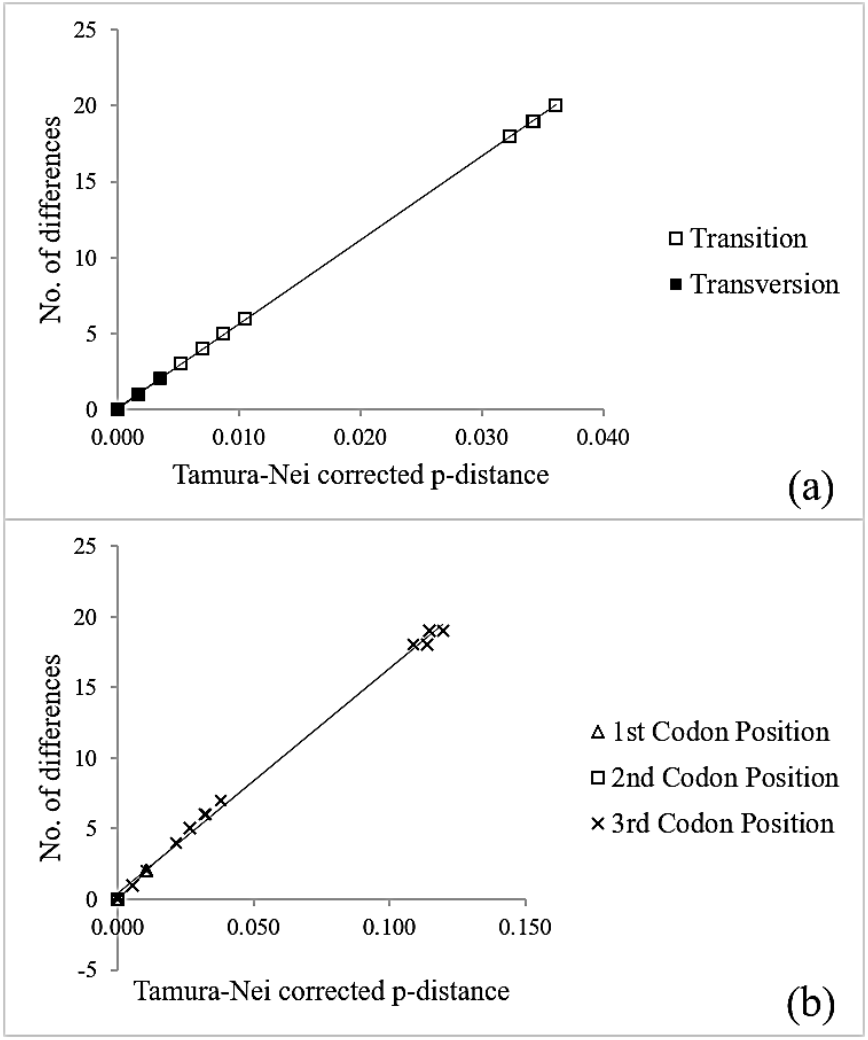

Figure 2. Substitution rates of the *CO1* gene putative codon position of *T. laeviceps*: (a) The relative transition and transversion of *T. laeviceps CO1* gene, (b) The different substitution numbers of the three codon positions of *T. laeviceps CO1* gene

Table 5. Multiple alignment of *T. laeviceps COI* putative amino acid sequences. Dot (.) indicates conserved amino acid sequences. Abbreviations refer to Table 1

Haplo-types	**Samples**	**Amino Acid Position**																		
		41	42	43	44	45	46	47	48	49	50	51	52	53	54	56	67	58	59	60
Hap_2	Tlv_BGR4, Tlv_LBK1	T	V	Y	P	P	L	S	S	Y	L	Y	H	S	S	P	S	V	D	F
Hap_3	Tlv_LBK4, Tlv_BGR5, Tlv_BGR6	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_5	Tlv_LBK3	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_4	Tlv_BGR2, Tlv_LBK2, Tlv_LBK5	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_6	Tlv_LBK6	.		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Hap_1	Tlv_BTH1, Tlv_BGR1, Tlv_BGR3	.	.	.	.	.	.	.	.	.	.	.	.	P	.	.	.	.	.	.

DISCUSSION

The trading of *T. laeviceps* colonies in Indonesia is becoming increasingly common. Thus, the bee colonies are frequently transferred from the native area to the other Indonesian region by human translocation. This study aimed to analyze the intraspecies variations of *T. laeviceps* based on the *CO1* gene sequence by comparing the native and traded *T. laeviceps* colonies. A high intraspecies variation of *T. laeviceps* from the Indonesian native and traded region is the main finding of this study. Six haplotypes of the *T. laeviceps CO1* gene out of the 13 analyzed sequences have a high 24 nucleotide variations. The Hap_1 haplotype from Batanghari and Bogor has the highest 21 nucleotide variations compared to the other five haplotypes. The Hap_2–Hap_4 haplotypes occurred both in Lebak and Bogor, while Hap_5–Hap_6 was the specific haplotype only found in Lebak. However, carefulness is needed to determine the common and specific haplotype in *T. laeviceps* since they are frequently traded from the native to other region of Indonesia for meliponiculture purposes. Thus, the colony origin is a must-recorded information in the haplotype variation study of *T. laeviceps*. Those high intraspecies variation of *T. laeviceps* also occurred in *T. laeviceps* from Malaysia, which also found a high number of 36 haplotypes based on the *CO1* gene (Ador et al. 2023). The same phenomenon was also found in the stingless bee *Heterotrigona itama* from Malaysia, which has six haplotype variations based on the *CO1* gene sequence with high 143 nucleotide variations (Mohd Yusof et al. 2018).

The genetic distance analysis of *T. laeviceps* was further conducted using the other *Tetragonula CO1* gene from GenBank. The interspecies genetic distance between *Tetragonula* bees ranged from 16.9% to 31.5%. This finding confirms the results from a previous study revealing a high genetic distance (20.9%–22.8%) between *Tetragonula* species based on the *CO1* gene (Sayusti et al. 2021). The intraspecies genetic distance within *T. laeviceps* ranged from 0.2% to 3.7%, with the highest 3.7% between the Hap_1 haplotype and the other five haplotypes. It followed the results of nucleotide polymorphism analysis that showed the highest variations in the Hap_1 haplotype. A similar high genetic distance within species up to 4.3% was also previously found in the *CO1* gene of the cryptic stingless bee *Hypotrigona gribodoi* from Africa (Ndungu et al. 2018). The high and remarkable 7.3% genetic distance of the *CO1* gene sequence also successfully separated two morphospecies of *Meliponula ferruginea*, black and reddish brown, from Africa (Ndungu et al. 2017).

Despite a high genetic distance between *Tetragonula* bees, the constructed maximum likelihood phylogenetic tree showed that *Tetragonula* species belonged to a single cluster with the *Tetragonula sapiens* placed in the basal position. The phylogenetic tree also showed that the minute stingless bee *T. fuscobalteata* is the sister species of *T. clypearis*, and the complex Australian stingless bee *T. carbonaria* is the sister species of *T. hockingsi*. It is congruent with the Bayesian phylogenetic relationship of the old-world stingless bees using mitochondrial and nuclear genes that clustered the *Tetragonula* bees with the same sister species between *T. fuscobalteata*, and *T. clypearis*, *T. carbonaria*, and *T. hockingsi* as well (Rasmussen & Cameron 2007). In addition, all *CO1* gene sequences of *T. laeviceps* also performed a single cluster with a high 99% bootstrap value, and the high intraspecific variation of the Hap_1 haplotype split from the other five haplotypes with 82% bootstrap value. The high 97% bootstrap value also occurred in the clustering of *T. laeviceps* from Malaysia based on the *CO1* gene (Ador et al. 2023). The clustering based on *CO1* gene was congruent with the clustering based on the morphological character of antennal scape color and thorax color (Ador et al. 2023). Using the Cytochrome b gene, the Neighbor-Joining phylogenetic tree showed that *T.*

laeviceps from Malaysia and Thailand were also separated into different clusters (Christy et al. 2019).

Putative codon position analyses of the *T. laeviceps CO1* gene also revealed that transition exceeded the transversion (Figure 2a), with the highest substitution number observed at the third codon position (Figure 2b). This result supported the finding that transition in the *CO1* gene was more common than transversion in the genus *Tetragonula* (Sayusti et al. 2021). In addition, the high nucleotide variation of Hap_I resulted a single un-synonymous putative amino acid substitution from serin to phenylalanine that have different side chain type (Table 5). Serin has a polar neutral side chain, while phenylalanine has an aromatic hydrophobic side chain (Lide 1991). Un-synonymous amino acid substitution of NADH dehydrogenase subunit 2 gene (ND2) in the mitochondrial DNA of subspecies *A. mellifera ligustica* and *A. mellifera caucasica* was also occurred from threonine that has a polar neutral side chain to isoleucine that has an aliphatic hydrophobic side chain (Ilyasov et al. 2019). The high substitutions of the *T. laeviceps* Hap_1 haplotype indicate that they are presumably undergoing a continuous evolutionary process.

CONCLUSION

The present study provides the genetic variation analysis of the stingless bee *T. laeviceps* in Indonesia. Based on the *CO1* gene, *T. laeviceps* has a high genetic variation with six haplotypes across the three sampling regions in Indonesia. Our study highlights the Hap_1 *T. laeviceps* from the native region of Batanghari and traded region of Bogor that have a high 3.7% genetic distance with the Hap_4 and Hap_6 and experienced a single amino acid alteration. Thus, our preliminary study will have a major impact on further research of *T. laeviceps* particularly in assisting morphological identification. Combining several genes and morphological characters will be applied for future research to confirm *T. laeviceps* haplotype variations among wider native and traded areas in Indonesia.

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AUTHORS DECLARATIONS

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Conflict of Interest

The authors declare that they have no conflict of interest.

Ethics Declarations

No ethical issue is required for this research.

Data Availability Statement

Data were synthesized and re-analyzed from three Bachelor thesis of the students in Department of Biology, IPB University, Indonesia, entitled: Characterization of Stingless Bee Species from Bogor, Lebak, and North Luwu Based on *Cytochrome C Oxidase I* Gene (2019), Differentiation of The *Cytochrome Oxidase I* (*COI*) Gene in *Trigona laeviceps* and *Trigona reepeni* (2016), Variation of *Cytochrome Oxidase I* (*COI*) Gene of *Trigona* Bee from Desa Batu Kucing and Desa Bungku, Jambi (2014).

Authors' Contributions

RR, TA, REP, RCHS and HP designed the study; TS, CNA, FRL, and RAB conducted the laboratory works under supervision of RR; TS prepared and re-analyzed the combined data under supervision of RR; RR and TS led the writing of the manuscript; All authors contributed to the drafts and approved the final version of the manuscript.

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