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Molecular Phylogeny of Stingless Bees in the Special Region of Yogyakarta Revealed Using Partial 16S rRNA Mitochondrial Gene

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ABSTRACT

Systematics of bees has developed rapidly. Several studies have attempted to infer the kinship between each group of bee. One way is the use of phylogenetic analysis using molecular data. This study explains the phylogenetic relationship of stingless bees in the Special Region of Yogyakarta, Indonesia based on the 16S rRNA gene. The research has been carried out in five districts and cities in the region from June to September 2019. In the study, the stages implemented include; place determination of stingless bees sampling, sampling, and molecular identification (DNA extraction, DNA amplification, and sequencing), followed by the data analysis using NCBI database and MEGA X software. The result of this study indicated that among seven morphospecies from Yogyakarta, there are six species of stingless bees that have a closest genetic relationship with the same species data from Genbank, namely *Tetragonula laeviceps*, *T. iridipennis*, *T. sapiens*, *T. sarawakensis*, *Lepidotrigona terminata*, and *Heterotrigona itama*. Since the genetic distances of *T. laeviceps*, *T. iridipennis*, *T. sapiens*, are wider than 3,5%, the data indicate that there is a possibility that the three morphospecies are actually belong to a different species with a similar morphology. Meanwhile, for the morphospecies *T. biroi*, the closest hit is on *T. pagdeni* 16S rRNA DNA because the *T. biroi* 16S rRNA DNA data is not available on the database. This study is expected to contribute to the preservation and utilization of one of Indonesia's important biodiversity resources.

Keywords: 16S rRNA mitochondrial gene, Molecular phylogeny, Special Region of Yogyakarta, Stingless bees

Introduction

Indonesia has many species of stingless bees that widespread throughout the islands (Rasmussen, 2008). There are at least 46 species of stingless bees that are scattered in several islands (Kahono *et al.,* 2018), one of which is in Java in the Special Region of Yogyakarta (Trianto and Purwanto, 2020). The diversity of stingless bees also varies in the forest ecosystem and settlement area (Kelly *et al.,* 2014; Syafrizal *et al.,* 2014; Rahman *et al.,* 2015; Trianto and Marisa, 2020). In the settlement area, stingless nest can be found in parts of the house, such as a wall, roof, and door cavity (Erniwati, 2013; Suprianto *et al.,* 2020; Trianto *et al.,* 2020).

In the last decade, systematics of bees has developed rapidly. Several studies have attempted to infer the relationship between each group of bees. One way is the use of phylogenetic analysis (Peloso *et al.,* 2015). Phylogenetic analysis is a study that examines the relationship between various organisms with molecular and morphological analysis. Molecular analysis using the 16S rRNA gene has been proven successful in phylogenetic studies of bees, and the output is in the form of phylogenetic tree data (Rasmussen and Cameron, 2007). For examples, Baharuddin *et al.* (2014) confirm the identity of Malaysian *Trigona* spp. using partial 16S mitochondrial rRNA gene. A phylogenetic relationship of stingless bees (Meliponini) is constructed with samples from MARDI collection and further compared with other Malaysian sequences obtained from National Centre for Biotechnology Institute (NCBI) GenBank database. Meanwhile, Costa *et al.* (2003) succeeded in reconstructing the phylogenetic tree sequence data from the mitochondrial 16S rDNA of 34 species from 22 genera of stingless bees to investigate the phylogenetic relationships among the Meliponini.

The taxonomic renewal of bees has also been carried out with alteration from taxonomists. Before using DNA sequencing techniques, the morphological and morphometric descriptions became one of the mainstays in identifying species. Morphometric analysis is one way to determine the diversity of a species by testing the morphological character. Morphometric data can be used to explain the differences and similarities

between populations and describe the morphological population kinship (Efin *et al.,* 2019; Trianto and Purwanto, 2020). Meng *et al.* (2018) stated that the results of a morphometric analysis could provide a general description of the degree of taxa variability. Generally, each character observed is the result of gene interactions whose expression is influenced by the environment.

However, the use of morphometric data has limitations such as the low consistency values in showing phylogenetic relationships at the level of subspecies variation. So it needs to be compared with molecular data to draw maximum results. A study using these two approaches can provide broader information and help for systematic information and phylogeny reconstruction (Klingenberg and Gidaszewski, 2010).

This research focuses on the exploration of stingless bees species based on molecular character data in the Special Region of Yogyakarta. This study aims to explain the phylogenetic relationship of stingless bees based on the molecular character of 16S rRNA mitochondrial region. The results of this research can contribute to the preservation and utilization of one of Indonesia's important biodiversity resources, especially in the process of meliponiculture and conservation of stingless bees.

Materials and Methods

Time and location of study

This research was carried out from June to September 2019 for sampling and data analysis. Stingless bee sampling was performed in five regency in the Special Region on Yogyakarta (Figure 1).

Tools and materials

The tools used in this study are toolboxes, insect nets, digital camera, stationery, and observation sheets, loop and microscopes, tweezers, hand counters, macro lens, masks, ovens, gauges, insect needles, plastic seals, pipette tips (10, 200, 1000 microliters), microtube, PCR tube, jar, styrofoam, identification book, freezer, micropipette, centrifuge, autoclave, thermo cycle, digital scale, microwave, gel mold, electrophoresis chamber, UV transilluminator.

The materials used in this study were a specimens of stingless bees species (five individuals/location), ethanol 96%, chloroform, isopropanol, TE buffer, CTAB 3%, DNA loading dye. The 16S rRNA DNA mitochondrial primer pairs employed in the study is LR13107-F as a forward primer: 5'-TGG CTG CAG TAT AAC TGA CTG TAC AAA GG-3' and LR12647-R as reverse primer: 5'-GAA ACC AAT CTG ACT TAC GTC GAT TTG A-3' (Thummajitsakul *et al.,* 2013), PCR Kit (Go Taq Green©), Nuclease free water, DNA Ladder 1 kb and 100 bp, Promega Wizard sv gel© and PCR clean/up system kit©, 1% electrophoretic gel, TAE buffer, EtBr.

Collection and mounting specimen of stingless bees

The observation of stingless bees was conducted by roaming sampling. Further, a survey of sampling sites in each observation area was carried out by roaming sampling. Stingless bees are collected by spraying sugar water on bushes

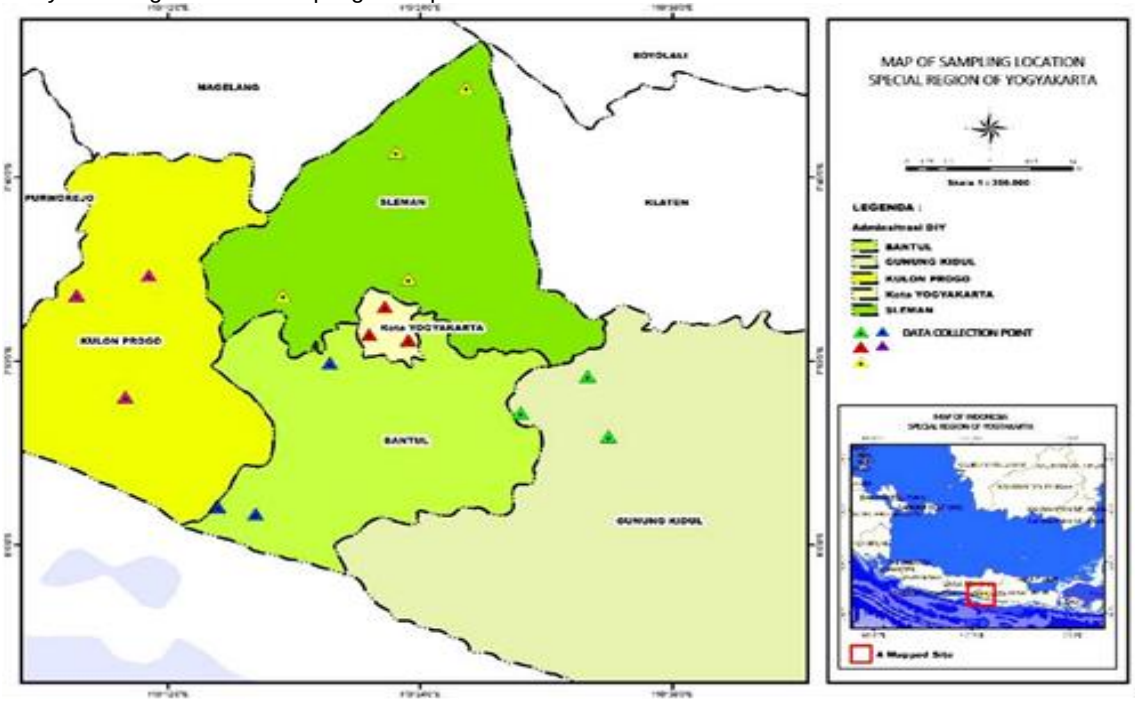

Figure 1. Study sites employed for the sampling of Stingless Bees in Special Region of Yogyakarta Province (Trianto and Purwanto, 2020).

as sampling points (Salim *et al.,* 2012; Trianto and Purwanto, 2020). The identification of the stingless bees using its morphological and morphometric characters as had been carried out in previous studies, so the stingless bees samples from Yogyakarta will be assigned as morphometric species (Trianto and Purwanto, 2020). For molecular identification (five individuals of stingless bees per location) were put into a bottle containing 90% ethanol and stored at 4°C.

DNA extraction

For DNA extraction, all parts of the bee (except head and wings) are used for the DNA extraction process. DNA extraction of stingless bee was extracted using CTAB method described by (Thummajitsakul *et al.,* 2011). One individual stingless bee/species was grinded using ice-cold pestle with 500 ul CTAB buffer and then transferred into 1.5 mL microtube. Sample was incubated at 55-65°C for 30 mins, then 500 ul chloroform was added, then the DNA preparations were shaken at 120 rpm for 30 mins. After shaking, the preparations were centrifuged at 5.000 rpm for five mins. Supernatans were transferred into new microtube. Then, an equal volume of isopropanol (1:1, v/v) was added into the tube. The DNA preparations were then incubated at room temperature for 10 mins. The samples were centrifuged at 5.000 rpm for 5 mins and subsequently the supernatant was removed. DNA pellets were washed using 70% ethanol, then centrifuged at 5.000 rpm for 5 mins. Finally, the DNA pellets were dried for 15 mins and resuspended by using 50 ul TE buffer.

DNA amplification and sequencing

The amplification of Polymerase Chain Reaction (PCR) and DNA Sequencing was conducted using the mitochondrial 16S rRNA gene primers as described previously (Thummajitsakul *et al.,* 2013). The PCR were completed in 35 cycles of 30 µl reaction volume, based on Na-Nokorn *et al.* (2006) and Mahendran *et al.* (2006). PCR reactions were performed using GoTaq Green master mix (Promega) under following conditions: pre-denaturation 95°C for 2 mins; 35 cycles of denaturation 95°C for 30 s; annealing 50°C for 30 s; extension 72°C for 30 s; hold 4°C. Amplified DNA (amplicon) was resolved on 1% agarose gel and visualized under UV transilluminator. The amplicons were sent to 1st Base DNA sequencing facility.

Bioinformatics analysis

The DNA sequences data from the sequencing facility were checked and edited using the Gene Studio software. Then, the DNA sequences were compared to the Genebank database using the Nucleotide BLAST (Madden, 2013) on the NCBI website (https://blast.ncbi.nlm.nih.gov/Blast.cg). The results showed the most closely related stingless bees to the samples. The phylogram reconstruction (phylogenetic tree) is completed using the Neighbor-Joining method with 1000 bootstrap value with the Kimura 2-Parameter (K2P) model in the MEGA X program (Kumar *et al.,* 2012).

Results and Discussion

Similarity of DNA sample of stingless bees

The obtained of thirty-eight sequential DNA sequences was then searched the similarity using the Nucleotide BLAST on the NCBI website (https://blast.ncbi.nlm. Nih.gov/Blast.cgi). Identity values indicate the results of Nucleotide BLAST analysis, such as query cover values and similarity values. The higher value of this parameter shows, the more similar the sequence of sample bases within the database (Apriliyanto and Sembiring, 2016). In this study, the results of alignment using a BLAST with the highest similarity value were in the sample *Tetragonula sarawakensis* Yogyakarta and GeneBank sequence (Accession Number: DQ790435.1) (Figure 2).

The result of this study (Table 1) indicated that among seven morphospecies from Yogyakarta, there are six species of stingless bees that have a closest genetic relationship with the same species data from Genbank, namely *Tetragonula laeviceps*, *T. iridipennis*, *T. sapiens*, *T. sarawakensis*, *Lepidotrigona terminata*, and *Heterotrigona itama*. Since there is no any data on the 16S rRNA DNA of *T. biroi* available on database, this study cannot establish whether the morphospecies of *T. biroi* from Yogyakarta is actually belong to species. Except that, the species that identified as *T. biroi* from Yogyakarta did not have blast results on *T. biroi*. Instead, the closest 16S rRNA DNA to the *T. biroi* fragment was from *T. pagdeni* (Accession Number DQ790413.1) (Table 1).

Phylogenetic tree reconstruction

In this study, *Apis cerana* and *Apis dorsata* 16S rRNA DNA mitochondrial DNA are used as the *out-group*. In contrast, members of each species from different locations in Rasmussen and Cameron (2007) studies are used as comparative data in *the in-group*. The results of phylogenetic analysis based on 16S rRNA mitochondrial gene nucleotide sequences (Figure 3) showed that several clades were formed with each species in the same clade supported by a significant bootstrap value between 91-100%. It also had a small genetic distance below 3.5% (Table 2), which indicates that the individual is still in the same species. According to Zemlak *et al.* (2009), the genetic distance threshold for a species should be around 3.5%. Meanwhile, according to an analysis of BOLD Systems that uses the COI gene, the

Figure 2. The result of Alignment using BLAST sample *Tetragonula sarawakensis* Yogyakarta and GeneBank sequence (Accession Number: DQ790435.1).

Table 1. The result of the 16S rRNA mitochondrial gene BLAST analysis

Sample	Sequence in Genebank	Accession Number	Query Qover	Identity	Author
H. itama Yogyakarta	*H. itama*	DQ790396.1	95%	96.89%	Rasmussen and Cameron, 2007
L. terminata Yogyakarta	*L. terminata*	DQ790399.1	95%	95.28%	Rasmussen and Cameron, 2007
T. iridipennis Yogyakarta	*T. iridipennis*	DQ790423.1	95%	94.17%	Rasmussen and Cameron, 2007
T. sapiens Yogyakarta	*T. sapiens*	DQ790427.1	95%	92.72%	Rasmussen and Cameron, 2007
T. sarawakensis Yogyakarta	*T. sarawakensis*	DQ790435.1	95%	99.35%	Rasmussen and Cameron, 2007
T. laeviceps Yogyakarta	*T. laeviceps*	DQ790438.1	95%	94.16%	Rasmussen and Cameron, 2007
T. biroi Yogyakarta	*T. pagdeni*	DQ790413.1	95%	96.61%	Rasmussen and Cameron, 2007

genetic distance threshold for a species is 3%. If the genetic distance of the two individuals or groups exceeds this value, they are not included in the same species (different species).

Based on phylogenetic tree reconstruction (Figure 3), it appears that the seven species of stingless bees analyzed have the closest genetic relationship with the same species from which the data of GenBank, except for *T. biroi*. The first clade shows that the samples of *H. itama* Yogyakarta 1 and *H. itama* Yogyakarta 2 are in the same clade as the *H. itama* sample from Malaysia (Genbank accession number DQ790396.1) with a bootstrap value of 100% (Figure 3). The genetic distance between *H. itama* Yogyakarta 1 and *H. itama* Yogyakarta 2 is 1.3% (Table 2), which indicates that both subpopulation are still belong to the same species. Meanwhile, the genetic distance between *H. itama* Yogyakarta and *H. itama* from Malaysia is 3.8% (Table 2), indicates that the sample of *H. itama* Yogyakarta is a different type or at least comes from a separate population. Sakagami *et al.* (1990) reported that this species was found in Java.

In the second clade, it is showed that the *L. terminata* Yogyakarta sample has a closest genetic relationships with the *L. terminata* sample from Thailand (LC307137.1) with a bootstrap value of 99% compared to the sample from Sulawesi (DQ790398.1) with a bootstrap value of 98% (Figure 3). It is also supported by a smaller genetic distance value of 1.7% (Table 2) for the sample of *L. terminata* Yogyakarta with Thailand and 5.3% (Table 2) with a sample from Sulawesi. The clade indicates that there are genetic variations among the three populations.

The third appears that there are three species of stingless bees grouped in the clade, namely *T. pagdeni*, a data from GenBank with accession number LC307143.1 originating from Thailand and DQ790413.1 originating from Malaysia with 100% bootstrap value (Figure 3) and genetic distance 9.6% (Table 2). Meanwhile, the *T. pagdeni* sample from GenBank has a bootstrap value of 96% with *the T. biroi* Yogyakarta (Figure 3). Genetic distance values of 4.6% also support it in both (Table 2). It indicates that the *T. biroi* sample from Yogyakarta is not a *T. pagdeni* species (Table 1). Since we cannot find any 16S rRNA DNA of *T. biroi* sequences on the databases, this study cannot establish the actual identity of *T. biroi* from Yogyakarta base on its molecular characters. Furthermore, *T. iridipennis* Yogyakarta and *T. iridipennis* from India (GenBank access numbers DQ790423.1) have bootstrap values of 97% (Figure 3) with a genetic distance of 7.0% (Table 2). Similar to the *T. biroi* situation, the result indicates that the population identified as of *T. iridipennis* of Yogyakarta may belong to a different species.

The fourth clade showed that *T. laeviceps* Yogyakarta has the closest genetic relationship to *T.* laeviceps originating from Malaysia (GenBank access number DQ790438.1), with a bootstrap value of 93% (Figure 3) and genetic distance 4.9% (Table 2). Similar to the *H. itama* and *T. iridipennis* species, the *T. laeviceps* Yogyakarta species is suspected to be a native species of the Special Region of Yogyakarta as reported by Sakagami *et al.* (1990)

that the species was found in Java. Many reports have shown that due to the wide distribution of *T. laeviceps* and the wide variety observed, *T. laeviceps* is actually a complex species (Rasmussen and Michener, 2010).

The fifth clade showed that *T. sapiens* Yogyakarta has the closest kinship to *T. sapiens* from GenBank data with access numbers DQ790427.1 from Australia and DQ790425.1 from Papua New Guinea, with a bootstrap value of 98% (Figure 3) and genetic distance 9, 1% (Table 2). It indicates that the sample of *T. sapiens* Yogyakarta is a different species or comes from a separate distance population.

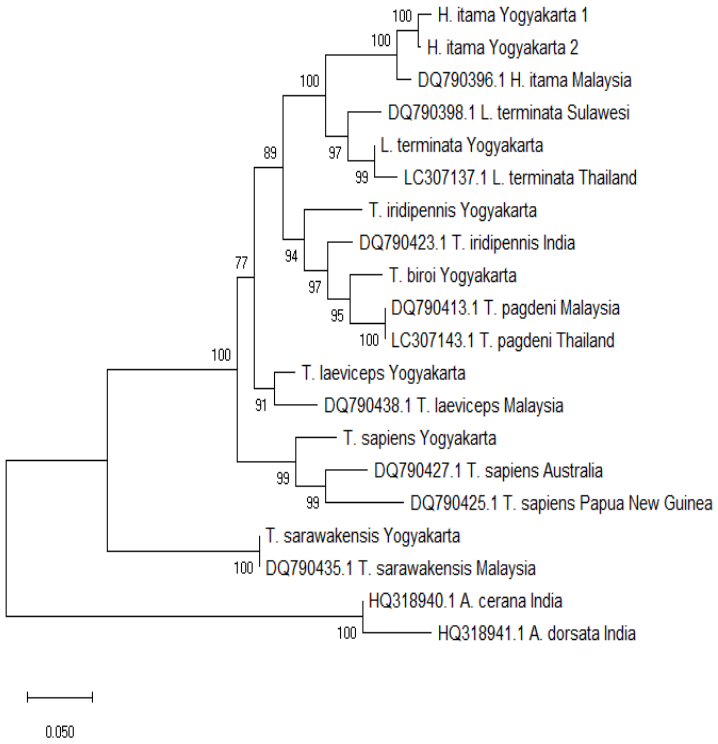

Figure 3. Phylogeny reconstruction of 16S rRNA gen in stingless bees.

Table 2. Genetic distance of stingless bees in The Special Region of Yogyakarta Province

	H._itama_Yogyakarta_1	H._itama_Yogyakarta_2	DQ790396.1_H._itama_Mys	L._terminata_Yogyakarta	DQ790398.1_L._terminata_Sulawesi	LC307137.1_L._terminata_Thailand	T._iridipennis_Yogyakarta	DQ790423.1_T._iridipennis_India	T._laeviceps_Yogyakarta	DQ790438.1_T._laeviceps_Mys	DQ790413.1_T._pagdeni_Mys	LC307143.1_T._pagdeni_Thailand	T._sapiens_(S.FT)	DQ790427.1_T._sapiens_Australia	DQ790425.1_T._sapiens_Papua	T._sarawaekensis_(B.K)	DQ790435.1_T._sarawaekensis_Mys	T._biroi_Yogyakarta	HQ318940.1_A._cerana_India	HQ318941.1_A._dorsata_India
H._itama_Yogyakarta_1																				
H._itama_Yogyakarta_2	0,01																			
DQ790396.1_H._itama_Mys	0,04	0,03																		
L._terminata_Yogyakarta	0,13	0,12	0,12																	
DQ790398.1_L._terminata_Sulawesi	0,13	0,12	0,10	0,05																
LC307137.1_L._terminata_Thailand	0,13	0,12	0,12	0,02	0,06															
T._iridipennis_Yogyakarta	0,18	0,16	0,16	0,11	0,13	0,14														
DQ790423.1_T._iridipennis_India	0,18	0,17	0,18	0,13	0,18	0,15	0,07													
T._laeviceps_Yogyakarta	0,16	0,15	0,15	0,12	0,13	0,15	0,12	0,14												
DQ790438.1_T._laeviceps_Mys	0,17	0,16	0,14	0,14	0,12	0,17	0,14	0,12	0,05											
DQ790413.1_T._pagdeni_Mys	0,15	0,14	0,17	0,13	0,17	0,14	0,10	0,07	0,13	0,10										
LC307143.1_T._pagdeni_Thailand	0,19	0,18	0,18	0,14	0,14	0,15	0,06	0,05	0,14	0,14	0,10									
T._sapiens_(S.FT)	0,23	0,23	0,24	0,18	0,19	0,21	0,15	0,19	0,12	0,14	0,19	0,17								
DQ790427.1_T._sapiens_Australia	0,23	0,23	0,24	0,19	0,21	0,22	0,18	0,21	0,14	0,15	0,22	0,18	0,09							
DQ790425.1_T._sapiens_Papua_New_Guinea	0,28	0,28	0,29	0,23	0,25	0,22	0,21	0,26	0,17	0,19	0,26	0,19	0,11	0,09						
T._sarawaekensis_(B.K)	0,39	0,37	0,39	0,31	0,33	0,42	0,28	0,31	0,25	0,29	0,33	0,35	0,25	0,30	0,33					
DQ790435.1_T._sarawaekensis_Mys	0,39	0,37	0,39	0,31	0,33	0,42	0,28	0,31	0,25	0,29	0,33	0,35	0,25	0,30	0,33	0,00				
T._biroi_Yogyakarta	0,16	0,15	0,15	0,11	0,13	0,13	0,08	0,11	0,13	0,15	0,05	0,11	0,16	0,20	0,22	0,29	0,29			
HQ318940.1_A._cerana_India	0,56	0,53	0,53	0,49	0,53	0,57	0,50	0,54	0,45	0,49	0,52	0,60	0,49	0,58	0,64	0,44	0,44	0,49		
HQ318941.1_A._dorsata_India	0,63	0,61	0,60	0,59	0,61	0,62	0,58	0,62	0,55	0,58	0,60	0,68	0,57	0,66	0,70	0,52	0,52	0,59	0,03	

The sixth clade showed that *T. sarawakensis* Yogyakarta has the closest genetic relationships to the *T. sarawakensis* from Malaysia (GenBank data access number DQ790435.1), supported by bootstrap value of 100% (Figure 3) and genetic distance 0% (Table 2). Considering the distance between the two population, the very close genetic similarity indicates that *T. sarawakensis* Yogyakarta is not a native species of the region like in *H. itama*, *T. laeviceps*, and *T. iridipennis* species, but it is indeed an introduced species. This assumption is also supported by information from the stingless-bee-keepers in Yogyakarta that admitted of introducing the *T. sarawakensis* species from Kalimantan to Yogyakarta.

Based on the above analysis, it is found that in general, the morphological and morphometric analyzes that have been carried out in previous studies (Trianto and Purwanto, 2020), when compared with molecular analyzes, result in the same species identification. However, there are two samples from the 38 samples that had been sequenced, that differs from the results of the morphological and morphometric analysis. The morphology and morphometric of the two samples that were identified as *T. biroi*, while based on the results of molecular analysis, the closest related hit was on *T. pagdeni* 16S rRNA DNA. Even though with a wide genetic distance. It occurs because of the limited data on GenBank consist of no *nucleotide* data for the *T. biroi* species. So that, when the sequence is analyzed using the online Nucleotide BLAST on the NCBI website (https://blast.ncbi.nlm.ih.gov/Blast. cgi), what appears is not the *T. biroi* species data but the data of other species that have the closest relationships to the query, which is *T. pagdeni*. Will and Rubinoff (2004) explained the lack of molecular methods in the process of identifying an organism, one of which is that some nucleotide data from several species are not found in GenBank. Therefore, it needs across morphological and morphometric data to ensure species identification of the organism. When examined through morphological analysis data, the species of *T. biroi* and *T. pagdeni* species are different even they are still in the same genus. The difference is on the species *T. biroi* that has a dark brown characteristic on the venation, and stigma; and black hair on the scutum and scutellum (Smith, 2012; Trianto and Purwanto, 2020). Whereas, *T. pagdeni* species have faded red brick to dark brown characteristics in the venation and stigma as well as pale yellowish hairs on scutum and scutellum (Smith, 2012). A further study is needed to clarify the confusion.

The phylogenetic analysis in this study used the 16S rRNA mitochondrial gene. It is because, in the analyzing processes, the 16S rRNA mitochondrial gene has several advantages over other genes, especially in the order Hymenoptera. Whitfield and Cameron (1998) supported that the mitochondrial 16S rRNA gene is the most informative gene for phylogenetic analysis between closely related species or populations, between tribes, subfamilies, and families. Furthermore, the use of the 16S rRNA gene could also provide solutions to the morphological problems that occurred, such as cryptic species and sibling species.

In addition, the selection of the 16S rRNA mitochondrial gene was based on the availability of stingless bees' database on the NCBI website (https://www.ncbi. Nlm.nih.gov/) and the BOLD System (http://boldsystems.org/). In NCBI data, the sequences of the type of stingless bees consists only sequences that use the 16S rRNA gene instead of other genes, so that it can be used as a source of analysis or comparison data of sequences. Furthermore, on BOLD System, the sequential data for stingless bees is only limited to a few species (December 5, 2019) such as *Tetragonula carbonaria*, *T. hockingsi*, *T. davenporti*, *T. pagdeni* and *T. iridipennis*. Therefore, it cannot be used as a source of data analysis or comparison for sequences.

Conclusions

The result of this study indicated that among seven morphospecies from Yogyakarta, there are six species of stingless bees that have a closest genetic relationship with the same species data from Genbank, namely *Tetragonula laeviceps*, *T. iridipennis*, *T. sapiens*, *T. sarawakensis*, *Lepidotrigona terminata*, and *Heterotrigona itama*. The genetic distances of *T. laeviceps, T. sapiens,* and *T. iridipennis* indicate the possibility that the three morphospecies are actually belong to a different species. Since there is no any data on the 16S rRNA DNA of *T. biroi* available on database*,* this study cannot establish whether the morphospecies of *T. biroi* from Yogyakarta is actually belong to species.

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