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Characteristics Of Partial Mithchondrial Cytochrome Oxidase Sub Unit 1 Gene Of Honey Bee *Apis dorsata* Binghami [Hymenoptera: Apidae] From Minahasa, North Sulawesi, Indonesia

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Abstract

The aims of this study was to characterize mitochondrial cytochrome oxidase sub unit 1 gene (CO1) of *Apis dorsata* from Minahasa, North Sulawesi. *Apis dorsata* Binghami used in this study was obtained from the natural colony in Klabat forest, Airmadi forest and Kombi forest. From each native bee colony, ten workers were taken (n = 30). Femur hind legs used for subsequent DNA extraction were amplified by PCR, underwent electrophoresis and then sequenced. CO1 sequences then were matched with BLAST to obtain sequences from the NCBI that have high levels of homology (>90%). The results showed that the universal primer LCO -1490 and HCO - 2198 succes amplify the gene CO1 *Apis dorsata* of Minahasa. Based on phylogeny tree, *Apis dorsata* derived from forest Airmadidi and forest Kombi have the same CO1 sequences but have had differences with *Apis dorsata* from forests Klabat. BLAST analysis results showed that the partial mitochondrial CO1 sequences Apis dorsata of Minahasa has the highest rate of 94 % similarity with similar sequences recorded in the NCBI gene bank.

Key words: *Apis dorsata, CO1 Gene,* Minahasa,

INTRODUCTION

Apis dorsata in Minahasa North Sulawesi Indonesia has a striking morphological variation (Hadisoesilo, 2001). From morphometric analysis conducted by previous studies, it has been found that there are differences in morphological characters, especially in the proboscis length, body length, abdominal strip color and wing length (Mokosuli, 2013). *Apis dorsata* can be found in Minahasa (Kombi

indonesian. biodivers. j.
VoL.1. No.1: 1-13
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doi :

and Pangu) and in North Minahasa (Paslaten, Wasian, Kaweruan and mount Klabat slopes). Morphological variation is influenced by the characteristics of the feed-source plants as well as habitat altitude in both areas Ultrastructural scanning electron micrscope analysis revealed that the antennae were also different with *A. dorsata Fibricus* that live in Thailand and peninsular Southeast Asia (Mokosuli, 2013; Sawannapong *et al.* 2012).

Differences of morphological characteristics should be confirmed with molecular characterization because morphology can be explained as an expression of genes, whereas gene expression can be stimulated and influenced by the environment (Raffiudin, 2002) It is still a debate about the evolutionary relationships in *A. dorsata* originating from various locations in the world. *A. dorsata* is found in India, Thailand, Nepal, China, Malaysia, and several large islands in Indonesia including Sumatra, Kalimantan and Sulawesi (Hadisoesilo, 2001).

One method that can be used to trace the phylogenetic relationships of a species that has been universally accepted is by looking at their mitochondrial DNA (mtDNA). Genetic information contained in the mtDNA is derived from the parental females and do not undergo recombination with male parental genetic material or maternalistic. mtDNA is widely used to study the evolutionary relationships of animals and population diversity (Hebert *et al*, 2003; Roe and Sperling, 2007; Rivera *et al.* 2009). mtDNA is circular, double-stranded, spans about 15,000-16,000 base pairs with small molecular weight (MW) and consists of about 13 protein-coding genes (PCGS), 2 rRNA coding genes, 22 tRNA genes coding and regulatory regions (Hebert *et al*, 2003). Mitochondria are the venue for cellular respiration. Proteins encoded by mtDNA are proteins that act as enzymes and play an important role in cellular respiration system across eukaryotic aerobic organisms (Nelson and Cox, 2005). The genes in mtDNA encode enzymes such as cytochrome oxidase (CO1, CO2 and CO3), NADH dehydrogenase 1-6, and ATP synthetase (Hebert *et. al*, 2003). As genetic markers, it is easier to obtain amplicon of mtDNA because it is haploid and do not need to be cloned. mtDNA has a high evolutionary speed and the extremely small number of genes compared with the nuclear genome. Therefore, it is assumed that the entire molecule has the same genealogical history. mtDNA has been recommended in the study of molecular taxonomy with mtDNA sequence tags or barcodes (Hebert *et al.* 2003).

indonesian. biodivers. j.
VoL.1. No.1: 1-13
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doi :

There have been numerous research about the phylogeny of invertebrate and vertebrate either using CO1 gene of mtDNA as a marker or genetic barcode (Bravo *et al*, 2008; Hajibabaei *et al*, 2005; Harmon et al, 2006; Hebert *et al.* 2004; Hulcr *et al.* 2007). DNA barcoding is able to separate most species of animals. CO1 is a gene that can be used as genetic marker in to study the genetic characteristics between species and between individuals. CO1 gene used for species identification through DNA barcodes lengths 648-700 bp. DNA barcoding has been successfully identify and distinguish the various species of invertebrates and vertebrates from birds to Lepidoptera in different areas (Hajibabaei *et al.* 2007). The results showed that the animal identification system based on the CO1 gene is quite effective. Mitochondrial genomes of related species in the diverse phylum have been demonstrated to be sufficient to distinguish between these animals (Bucklin *et al.* 2003).

Mitochondrial DNA genes have evolutionary rate of 1-10 x faster than the chromosomal genes (Avisa *et al.*, 1987; Caccone *et al.* 2004). Mapping of mitochondrial genome has been developed so it can be used as a reference or comparison between species (Friedrich and Muqim 2003; Kim *et al.* 2010; Liu *et al.* 2008; Liao *et al.* 2010; Morlais and Severson 2002;). CO1 has a special characteristic suitable as a tool in evolutionary study, as follows: (1) the last catalysts in the mitochondrial respiratory chain, so CO1 is widely studied at the biochemical level and has been demonstrated to be conserved in all eukaryotic aerobic species (Lunt *et al.* 1996); (2) traces of amino acids correlated with the function of each part of the CO1, thus showing the characteristics of the species that possess it (Lunt 1996; Roe and Sperling, 2007); (3) sequences 658 basepair (bp) at the 5' has been proposed as animal barcodes (Hebert et al, 2003). The barcode has successfully proven its ability to differentiate between species in the Lepidoptera (Hebert et al, 2003; Hajibabaei *et al.* 2005), beetles (Funk *et al.* 1995), some insect pests (Toda and Murai, 2007), moth *Hamona mermerodes* (Hulcr *et al.* 2007), and mosquitoes (Cywinska *et al.* 2006). CO1 sequences have also been used to examine the co-evolution of phytofage insects with their host plants (Rivera *et al.* 2009). The research has done to characteristics of CO1 gene from *A. dorsata* who live in Minahasa North Sulawesi, Indonesia and compare them with CO1 gene sequences recorded in the NCBI gene bank.

indonesian. biodivers. j.
VoL.1. No.1: 1-13
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doi :

MATERIALS AND METHOD

Sample

Samples were collected from *A. dorsata* colonies in mount Klabat Forest and forest of Airmadidi at North Minahasa and and Kombi forest at central Minahasa. From each native bee colonies, 10 workers were taken (n = 30). This study examined the part of the hind legs of worker bees. The research was conducted at the Laboratory of Medical and Biological Science Department of Molecular Biology, Manado State University, Indonesia. DNA sequencing were analyzed using an ABI PRISM 3730xl sequencer engine, Genetic Analyzer from Applied Biosystems by develop USA at First BASE Laboratories Sdn Bhd, Malaysia.

Equipment and Materials

The materials were used include: ethanol (Merck), chloroform (Merck), DNeasy Blood and Tissue Kit (Qiagen), PCR kit (Big Dye), and Terminator v3.1 Cycle Sequencing Kit. The primer of CO1 are LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: TAAACTTCAGGGTGACCAAAAAATCA (Folmer *et al.* 1994).

DNA Extraction

Honey bee DNA extraction was performed according to protocol DNeasy Blood and Tissue Kit (Qiagen). As many as 5 mg legs crushed honey bee using tissue ruptor apparatus in tube 5 ml. The tissue of honey bee from the legs in the tube then was placed in a termoblock with a temperature of 56 ° C for 30 for minutes. The lysis: added 200 µ l of Lysis buffer 20 µ l TLS and proteinase K into the tube containing the leg chain honey bee. The tube put back into in termoblock for 24 hours with a temperature of 56^0 C (based on previous study, immersion time modified protocols which are soaked for 30 minutes). After 24 hours of soaking, the tube will centrifuged at 10,000 g (12,000 rpm) for 1 minute. Supernatan subsequently moved in a 1.5 ml new tube. The next stage is the stage of bindin: the supernatan on the tube recently added buffer Lysis stage results TBS as much as 200 µ l vortecs in 15 seconds. Spin filter input into the tubes, and then centrifuged 10,000 g (12,000 rpm) for 1 minute. After

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

centrifused, the tube replaced with new tube, whereas spin filter fixed and go on next stage. Stage of washing: washing solution added HS 400 µ l (in new tube), centrifused at 10,000 g (12,000 rpm) for 30 seconds. The tube replaced spin the filter anyway. Add washing solution MS 750 µ l, centrifuged at 10,000 g (12,000 rpm) for 1 minute. Move on a new tube, spin filter then centrifused at 10,000 g (12,000 rpm) for 30 seconds. The final stage, Elution phase move the washing step results in a new tube while the spin filter fixed. Add 100 mL of elution buffer, then incubated for 5 minutes followed by centrifuged at 6000 g (800 rpm) for 1 minute. Furthermore, the spin filter is issued while elutionnya tube can be stored in a state suu -20ºC , do the analysis of the purity and concentration of extracted DNA using nano spectrofotometer.

PCR Amplification

The process of PCR performed 3 stages: initiation, cycling, and a final extension. Initiation process for 3 minutes at a temperature of 95 ˚ C, followed by denaturation i.e. cycling stages 94 ºc for 30 seconds, Annealing 50 º C for 50 seconds, the extention of 72 º C for 50 minutes with the number of cycles as much as 35 times and the last stage of the final extention 72 º C for 5 minutes. Master mix PCR tube i.e. taq master top kit 2 x as much as 12.5 µ l, primary forward N4N 1.5 µ l, reverse primer N5J 1.5 µ l, µ 2 l template DNA, and as many as 7.5 ddH2O µ l with a total volume of 25 µ l. Time required for PCR method for 1 hour 40 minutes.

Electrophoresis

5 mL of PCR products were mixed with 1 mL of loading dye and 2 mL of gel staining and then loaded into the well of 1.5 % agarose gel electrophoresis. One of the well was filled with marker 100 bp by 5 µl. An electric charge of 100 volts were applied for 40 minutes. Electrophoresis results were viewed using UV light and documented by Gel Documentation.

Sequencing and Analyses

Sequencing were performed using BI PRISM 3730xl Genetic Analyzer (Applied Biosystems by Develop, USA). The obtained sequencing results were viewed and edited using software Geneous 5.6.4 (

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

Drummond *et. al.* 2012), to check for their quality, trimmed off early and end signal losses, ambiguous and unusable base pairs before further analyses. The CO1 gene sequences of 3 samples were aligned with National Center of Biotechnology Information (NCBI) BlastN database by using BLAST (Basic Local Alignment Search Tool) algorithm in the internet for comparison and identification (Altschul *et al.* 1997; Waiho *et al.* 2013). Phylogenetic analyses using Neighbour-Joining search with Kimura 2-parameter as model was conducted using eneous 5.6.4 (Drummond *et. al.* 2012),. The tree was bootstrapped using 1000 subreplicates. A total of 7 sequences with similarity rate of more than 90% were used for the formation of the phylogeny tree and one sequence was used as a comparison outgroup. Outgroups is *Bombus terrestris* (JG 843662.1) were selected from GenBank database.

Concentration and purity of DNA

DNA extraction were performed on each 10 worker bees from the hive in Klabat Mount Forest, Woodland and Forest Airmadidi Tondano. The results were then analyzed for DNA extract purity and concentration using nanospectrophotometer at A260/A280 nm wavelength (Table 1).

Table 1. Concentration and purity of DNA

No	Sample	Concentration	Purity
1	Klabat Forest (AKA)	68,10 µg/ml	1,7
2	Airmadidi Forest (AKI)	70,90 µg/ml	1,8
3	Tondano Forest (AKL)	72,80 µg/ml	1,9

From the research the purity of the extracted DNA sample influenced pre- treatment before extraction. The sample pre- treatment is soaking in alcohol to remove the water content which can interfere with the process of extraction kits. Sample size which produces grinded and time immersion in proteinase K resulting better concentration and purity of total DNA. Soaking longer than clues extraction kits produce higher mtDNA content, after amplification by PCR and visualized by electrophoresis.

RESULTS AND DISCUSSION

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

PCR and Electrophoresis

Apis dorsata CO1 gene amplicons are amplified by PCR process visualized on electrophoresis chromatogram. Visualization results showed that band 1 (AKL) is not clear, while band 2 (AKI) and band 3 (AAI) is very clear. Thus the concentration of amplicons AKI and AAI more than AKL (Figure 1). AKI and AAI samples used were freshly obtained samples from nests in the forest and extracted after soaking with ethanol for > 24 hours, while the maternal sample is the sample that has been soaked in ethanol < 48 hours. CO1 gene Apis dorsata Binghami of three regions in Minahasa North Sulawesi amplified by the length of 679 base pairs

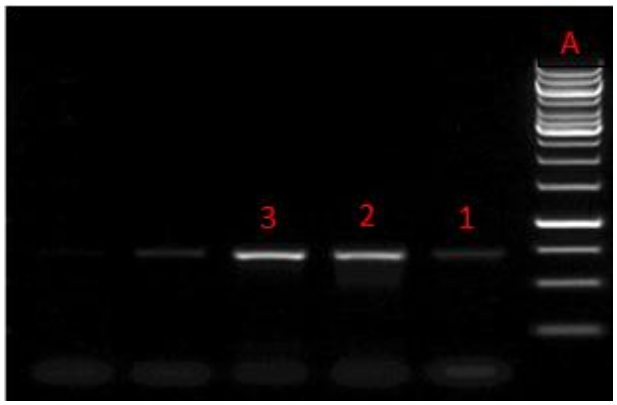


Figure 1. Visualization of mitochondrial DNA was amplified with primers CO1 (679 bp) . Note: 1 = DNA from *Apis dorsata* Klabat (AKL) , 2 = DNA from *Apis dorsata* Airmadidi (AAI) and 3 = DNA from *Apis dorsata* Tondano (AKI), A = mass of DNA marker.

BLAST Analyses

Cytochrome oxidase sub unit 1 gene sequence of A. dorsata, analyzed alignment on NCBI website (www.ncbi.nlm.nih.gov) using the BLAST program (Basic Local Allignment Search Tools). BLAST analysis was conducted on all sequencing data of *Apis dorsata* Binghami by comparing the partial mitochondrial *CO1* gene sequences of *Apis dorsata* Binghami with the online database of GenBank. All of the BLAST hits showed significant similarity (E-value < e-10) (Chini et al., 2006) with the 3 individual *Apis dorsata* Binghami partial *CO1* gene sequences. All of the hits retrieved from GenBank database were nucleotide sequences of partial mitochondrial *CO1* gene regardless of species, hence verifying the CO1 origin of *Apis dorsata* Binghami samples BLAST results obtained data from 47 banks that have high levels of gene homology (>84%). For further analysis, the research only focused on the level of homology > 90 %. The highest level of homology is 94%, which is *A. dorsata* haplotype 1 (AF153113.1). Based on

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

the results of the view that there is a red line, it indicates that the two sequences had very similar sequences of more than 200 nucleotides (Figure 2) .

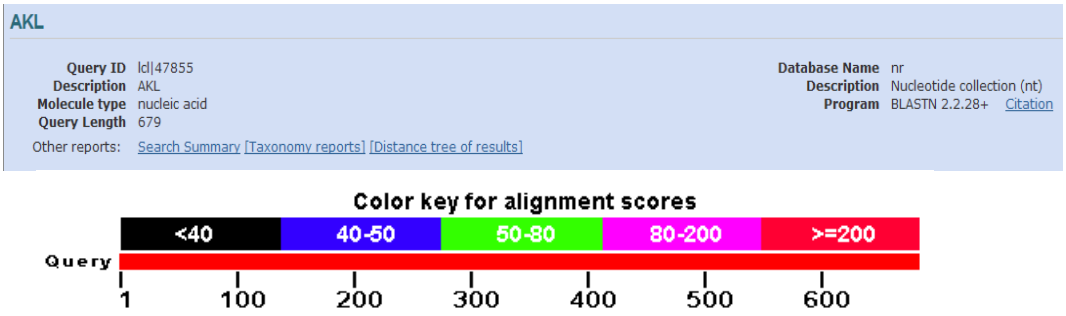


Figure 2. Levels CO1 gene sequence similarity (679 bp) *Apis dorsata* (AKL) with the data in the gene bank of NCBI.

Analysis of DNA sequence alignment AKI, AKLand AAI showed some differences in the nucleotides. The order of nucleotide at 93 of Apis dorsata CO1 sequence : AKA was found cytosine (C), while AKL were thymine (T). The order of nucleotide to the AKA at 194 was found thymine (T), while AKL was found adenine (A). The order of nucleotide bases to the AKL at 281 was found cytosine (C) , while AAI and AKI were thymine (T). The order of nucleotide bases to the AKL at 443 was found cytosine (C), while AAI and AKI were thymine (T) . Thus there were 4 polymorphic sites in *A. dorsata* colonies based on the location or origin of the samples. Of the four polymorphic sites, 2 sites experienced transposition and 2 sites experienced deletions (Table 1).

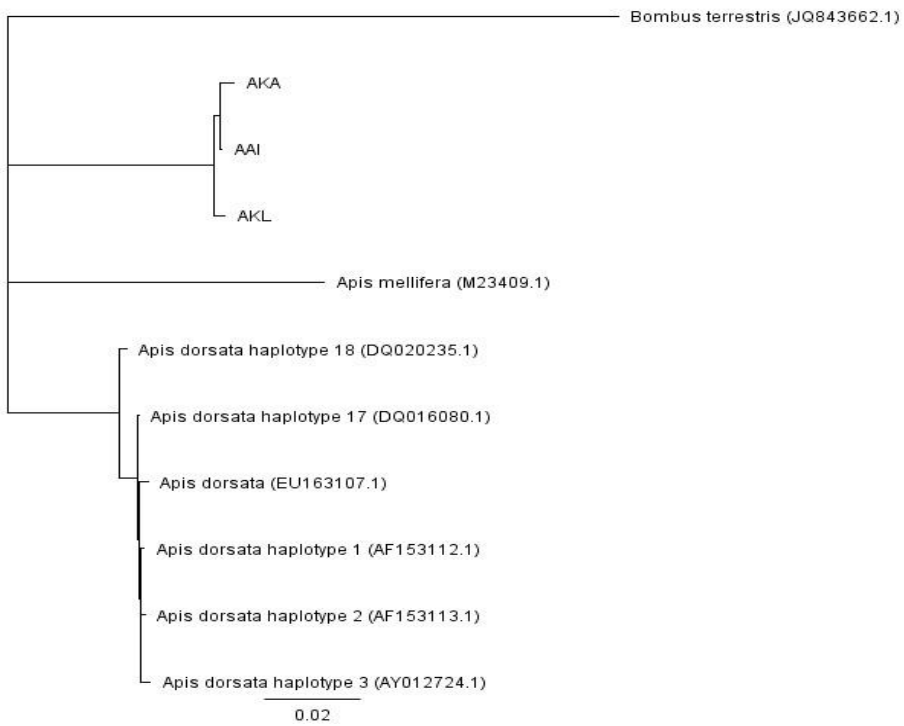
Table 2. Site nucleotides difference cytochrome oxidase subunit gene 1 (CO1) *Apis dorsata* from three different locations (AKA, AAI and AKL) in Minahasa , North Sulawesi , Indonesia

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

Description : AAI = Airmadidi Forest, AKA = Klabat mountain forest , AKL = Kombi forest

Phylogenetic tree

Of the three samples according to the location of the bees found *A. dorsata* which is taken from Airmadidi Forest and Kombi Forest form their own groups based on similarity apart with samples derived from Klabat Forest (Figure 3) .



indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
doi :

Figure 3. CO1 gene phylogenetic tree of *A. dorsata* Klabat Forest (AKA), Forest Airmadidi (AKI) and Forest Tondano (AKL) compared with data from NCBI with the closest similarity and comparison (outgroup)

Discussion

Apis dorsata is forest honey bee with huge potential because it produces more honey than Apis mellifera. In addition, previous research has suggested that the honey produced by *Apis dorsata* contained richer bioactive because synthesized from nectar that is more diverse than the honey produced by *Apis mellifera* (Mokousli, 2013; Hadisoesilo, 1997). However *Apis dorsata* Binghami can not be artificially cultivated by humans. Therefore, research is still lacking notch species. The debate taxonomic position of the honeybee *Apis dorsata* Binghami which is a species endemic to Sulawesi has been started since 1950. Sulawesi honey bees were first collected by Alfred Russel Wallace was reported by Smith in 1859 and named *Apis zonata* based on morphological identification (Hadisoesilo, 1997). Morphological identification is no longer enough to obtain accurate data on the position of a species. Molecular identification using CO1 gene as a genetic barcode has been accepted universally (Herbert *et al.*, 2003)

In the present study showed that universal primers LCO-1490 and HCO-2198 is effective in amplifying the CO1 gene *Apis dorsata* Binghami. The BLAST results not found a 100 percent degree of similarity with the genus of *Apis*, CO1 sequences that have been recorded in the gene bank. The highest degree of similarity with sequences recorded in the NCBI gene bank is 94% that is *A. dorsata* haplotype 1 (GenBank Accession Number AF153113.1.). Thus *Apis dorsata* Binghami of Minahasa Sulawesi had a difference of 6% CO1 gene sequences. This Obtained CO1 gene sequence can serve as a reference partial CO1 gene sequences of *Apis dorsata* Binghami for any future studies such as identification, population studies, intraspecific and interspecific discrimination of Apis dorsata in the world.

Thus the sequence was more similar to AKL and AAI , compared with AKA or CO1 gene from *A. dorsata* Airmadidi more similar to *A. dorsata* from Klabat, than *A. dorsata* from Tondano (Figure 3). CO1 gene is maternalistic. CO1 gene has very rapidly mutate or have a high rate of sequence change and showed a sequence divergence in the same species (Herbert *et al.*, 2003; Bucklin *et al.*, 2003; Pareira *et al.*, 2010). CO1 gene has been used to determine the spread of *A. mellifera* L. in, the results of the

indonesian. biodivers. j.
VoL.1. No.1: 1-13
ISSN : 2722-2659
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analysis showed a high variation in *A. mellifera* CO1 sequences were introduced into the United States of Africa (Szalanski and Magnus, 2010).

Results of this study indicated that the origin of the colonies of *Apis dorsata* also affect CO1 gene sequences. Klabat forest and Airmadidi forest has a diversity of flowering plants as a sources of food for bees is higher than Kombi forest where the diversity of flowering plants as a source of food for bees is fewer and dominated by clove plants. The present study showed position based on phylogeny tree formed *Apis dorsata* of forest Klabat (AKL) have differences with *Apis dorsata* from Airmadidi Forest and Kombi Forest. Genetic variations in the CO1 gene also found on *Apis mellifera* in Turkey. Apis mellifera who lived in an adjacent area turns out to have formed two groups. Eastern European races were clustered together, whereas the Mellifera and Iberian haplotypes were clustered far apart (Özdil and İlhan, 2012). There has been no research publications *Apis dorsata* Binghami in North Sulawesi so that there is no reference for comparison. However, the geographical location greatly affects degree of diversity CO1 gene in many species of animals (Burton and Lee, 1994; Gan and Burton, 1995). Furthermore , previous studies of morphometric analysis of *A. dorsata* from Minahasa and Minahasa Utara showed significant differences in body length , long proboscis and long front wing . Thus the morphology of A. dorsata are derived from two regions in the Minahasa have differences. Results of this study underlying future research is the study of genetic diversity of *Apis dorsata* Binghami who live throughout the island of Sulawesi . However Sulawesi Island is the heart of the zone known as Wallacea region with high endemic species.

CONCLUSION

The universal primer LCO -1490 and HCO - 2198 can amplify the gene CO1 *Apis dorsata* of Minahasa. Based on phylogeny tree , *Apis dorsata* derived from forest Airmadidi and forest Kombi have the same CO1 sequences but have had differences with *Apis dorsata* from forests Klabat. BLAST analysis results showed that the partial mitochondrial CO1 sequences Apis dorsata of Minahasa has the highest rate of 94 % similarity with similar sequences recorded in the NCBI gene bank .

indonesian. biodivers. j.
VoL.1. No.1: 1-13
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