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Molecular Characterization of Mitochondrial Gene CO1 Barcode of Several Insect Species Carriers of *Phytophthora palmivora* at Cocoa Plantation in North Sulawesi Province

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Abstract : The existence of ants and termites that were very active in cocoa plants in the field was suspected as one of the causes of the spread of *Phytophthora palmivora* pathogen. The spread of pathogens occurs along with the activity of insects in which pathogens stick to the insect such as on the hairs of the feet, antennae, head, thorax and abdomen. Identification through molecular marker of DNA barcode to insect species acting as carriers of cocoa blight need to be performed. The study aimed to examine and analyze the nucleotide sequence of DNA of several insect species of ants and termites by amplifying CO1 mitochondrial genes. Molecular identification of the Mitochondrial CO1 Barcode genes of several insect species carrying *Phytopthora palmivora* in cocoa plantations in North Sulawesi Province were as follows: black ant (STHT) has a single DNA fragment measuring of 638 base pairs, very close as *Dolichoderus rugocapitus* type (*Query ID: lcl | Query_98113*), has a 94.8 % spacing. Taxonomically, it is included in the order Hymenoptera, subordo Apocrita, family Formicidae, and genus *Dolichoderus*. The red ant (STMK) has a single DNA fragment measuring of 658 base pairs, very close as *Monomorium destructor* type (*Query ID: lcl | Query_38701*), has a 100% spacing. Taxonomically, it belongs to the order Hymenoptera, subordo Apocrita, family Formicidae, and genus *Monomorium*. Termite (STMB) has a single DNA fragment measuring of 604 base pairs, very close as *Nasutitermes corniger* type (*Query ID: lcl | Query_18869*), has a 94.8% spacing. Taxonomically, it belongs to the order Isoptera, family Termitidae, and genus *Nasutitermes.*

Keywords : *Phytopthora palmivora, Dolichoderus rugocapitus, Monomorium destructor, Nasutitermes corniger,* Mitochondrial CO1 Barcode, PC.

I. Introduction

Cocoa fruit rot disease caused by the fungus *Phytophthora palmivora* becomes the main disease of cocoa plants worldwide, and in Indonesia it is the most important disease because the disease spread widely in all cocoa plantation area. Sporangium *P. palmivora* can be carried away by water splash or wind and reaching higher fruits. The fungus in the ground can also be transported by insects, such as ants that can reach higher

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fruits. From the higher fruits, sporangium can be carried by water to the fruit below. Infection of *P. palmivora* can occur directly to healthy fruits from diseased fruits through rain splashes and insects as carriers[1].

Mostly insects that are caught on the cocoa plantations are insects known as disease vectors, in which 56.1% consisting of Coleoptera (family: Nitidulidae, Scolytidae, Curculionidae and Tenebrionidae), Hymenoptera (family: Formicydae, Chalcididae), and Hemiptera (family: Reduviidae, Aphididae)[2]. The activity of predatory insects that feed from one plant to another cause spores of pathogen to quickly spread from diseased cocoa plants to healthy cocoa plants; those predatory insects are mainly from the Order Hymenopetra, formicidae family, namely weaver ants (*Oecophyllas maragdigna*) and black ants (*Dolichoderus thoracicus*)[3]. Feeding behavior of ants as a predator greatly helps farmers to control insect pests of cocoa plants, however, beside as predators, ants are most likely to play a role in spore dispersal of *P. palmivora*, *Oncobasidium thebromae* and *Corticium salmonicolor*[3].

Attempts to identify insect species in classification will greatly help to determine the status of the insect to its habitat in which it lives. The identification techniques can be carried out with various methods developed today, a morphologic or a molecular approach. Currently, the molecular identification technology for insects use the mitochondrial gene of cytochrome oxidase subunit 1 (CO1)[4]. For genetic analysis of insect species, the use of DNA Barcode is considered the most advantageous in terms of speed and precision[5].

II. Research methods

1. Time and Place of Research

The research was conducted from July to September 2015. The black ants, red ants and termites were collected from the field of Pungkol Village as the center of cocoa plant development in South Minahasa Regency of North Sulawesi Province. The extraction of insect samples for DNA characterization analysis was performed at the Laboratory of Faculty of Mathematics and Natural Sciences of Sam Ratulangi University, Manado.

2. Total DNA Extraction

Insect samples of ants and termites were the workers that were preserved in 95% ethanol. Total DNA Extraction were done by using DNA Extraction Kit *Geneaid*. The extract stages were as follows: one specimen of ant or termite was put into a 1.5 ml micro tube then 400 µl GP1 Buffer were added, inverted five times then incubated for 3 hours at 60º C using thermoblock and then centrifuged at 12,000 rpm for 1 minute. The supernatant was piped into a new microtube and mixed with 100 µl of GP2 Buffer, then inverted and incubated for 1 minute in ice and centrifuged at 12,000 rpm for 1 minute. The supernatant was transferred to a new eppendorf tube and added GP3 Buffer of 750 µl and it was inverted. The sample was inserted into a spin filter with mounted container tube and centrifuged as before. The filtrate was removed, and the container tube was reassembled. 400 µl W1 Buffer was inserted and centrifuged as above. The filtrate was removed, and the container tube was reassembled. A total of 600 µl of Wash Buffer was inserted and centrifuged as above. The filtrate was removed, and the container tube was reassembled. To dry the filter, the empty tube was centrifuged again for 2 minutes and then the filtrate along with the container tube was removed. The spin filter was transferred into a new eppendorf tube, dried for 2 minutes, 100 µl Elution Buffer were added, allowed to stand for 2 minutes and centrifuged. Spin filter was removed, and the DNA was stored at -10º C. In general, total DNA extraction process of insect specimens can be seen in Figure 1.

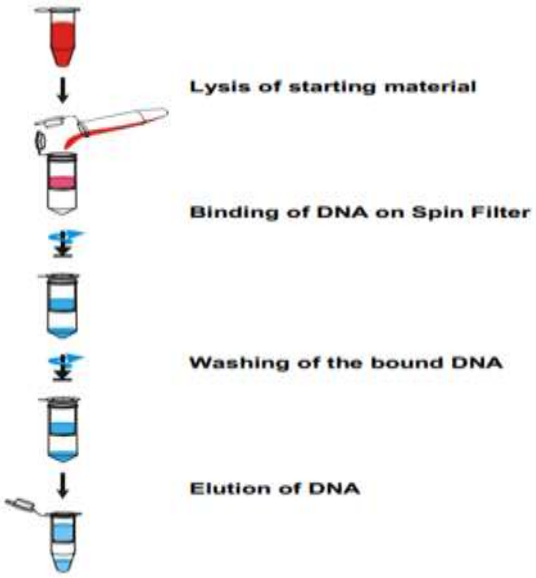

Figure 1. Outline of Total DNA extraction process of insect specimens

3. Polymerase Chain Reaction (PCR) of COI gene

PCR reaction used KIT 2X KapaTaq PCR Master Mix (Kapa Biosystem), each reaction of 40 µl has 15 pmol of each primer and DNA template. The COI gene was amplified using PCR[6], with specific primers of LCO1490 (5'-ggt caaatc ataaagatattg g-3') and HC02198 (5'-taa act tcagggtgaccaaaaaat ca-3'). The reaction conditions for the first PCR using both primers were 94° C denaturation (5 minutes) followed by 35 denaturation cycles of 94° C (30 seconds), 50° C of annealing (40 seconds), and 72° C of extension (50 seconds).

4. Agarose Gel Electrophoresis

The PCR results were separated by 1% agarose gel electrophoresis (in 1x TBE buffer) and observed using UV-Transilluminator. Agarose gel of 1% was prepared by boiling 1 gram of dissolved agarose to 100 ml TBE 0.5x. The hardened gel is immersed in a 0.5x TBE solution. The agarose gel was printed and 10 µl of PCR products were loaded into the gel wells. The 100 Volt electrical current was applied to the gel for 30 minutes. Then, the gel was soaked into an ethidium bromide solution for 10 minutes. The DNA of PCR products was visualized by using UV-Transilluminator and the success of PCR was detected in the presence of a single DNA band of 700 bp.

5. COI Gene Sequencing

Sequencing used two primers on the second PCR. PCR results and both primers were sent to First Base (Malaysia) for sequencing. The results obtained were chromatograms containing DNA sequences.

6. Data analysis

The obtained DNA sequences were edited and compared using the algorithm ClustaW. Identification used GenBank database (www.ncbi.nlm.nih.gov)[7]. The phylogenetic trees were made using Geneious v 5.6 software with the Neighbor-Joining algorithm[8].

III. Results and Discussion

The PCR results were separated using 1% agarose gel electrophoresis (in TBE buffer 1x) and observed by using UV-Transilluminator. The DNA of PCR products was visualized using UV-Transilluminator and the success of PCR was detected in the presence of a single DNA band of 700 bp (Figure 2)

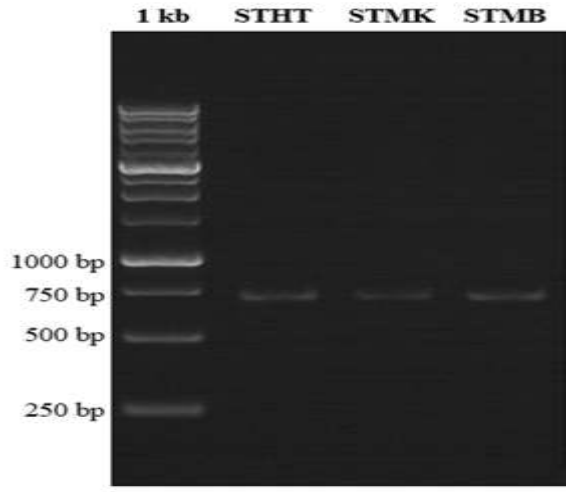

Figure 2. PCR Electrophoresis result of total DNA of insect samples

Figure 2 showed that the DNA of PCR products can be detected to correspond to the three species of insect sample; STHT is black ant, STMK was red ant, and STMB was termite.

1. STHT (Black Ant)

The sequencing of mitochondrial DNA genes using CO1-specific primers of LCO1490 (5'GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') produced a single DNA fragment of 638 base pairs. The sequence of DNA of black ant insect (STHT) produced a combination of base pairs as in Figure 3.

AATTTTATATTTTATTTTTGCTATTTGATCTGGGTTAATTGGGTCCTCAATAAGTATAATTATTCGTT
TAGAGTTAGGAACTTGTGATTCTATAATCAATAATGATCAAATTTATAATTCTATTGTTACCGGGC
ACGCCTTTGTTATAATCTTTTTTATAGTTATACCTTTTATAATTGGGGGATTCGGAAACTTTCTTGTT
CCTCTAATATTAGGAGCACCTGATATAGCATATCCTCGCATAAATAATATAAGATTCTGACTTCTA
CCCCCATCAATTCTTCTGTTATTAATTAGAAATTTTATTAGATCAGGAGTAGGGACAGGGTGAACT
GTATACCCCCCATTAGCCTCTAATATTTATCATAATGGTCCTTCTGTTGATCTGGCAATTTTTTCTCT
TCATATTGCCGGGGTATCATCAATTCTAGGGGCAATTAACTTTATTTCCACAATTATTAACATACG
AAATAAAAATTTATCTTTTGATAAGATTCCTTTATTAGTATGATCAATTTTAATTACAGCAATTTTA
CTACTTCTATCACTTCCGGTTTTAGCTGGTGCTATCACTATACTTCTAACCGACCGTAATATTAATA
CTTCATTTTTTGACCCTTCAGGAGGGGGAGATCCAATTCTTTATCAACACTTATTC

Figure 3. DNA sequence result of CO1 of STHT insect (black ant)

The confirmation result of mitochondrial DNA sequences of CO1 of black ant insect (STHT) in the databank base Gen BOLD (Barcode of Life Data System) obtained the result of phylogeny tree as in Figure 4.

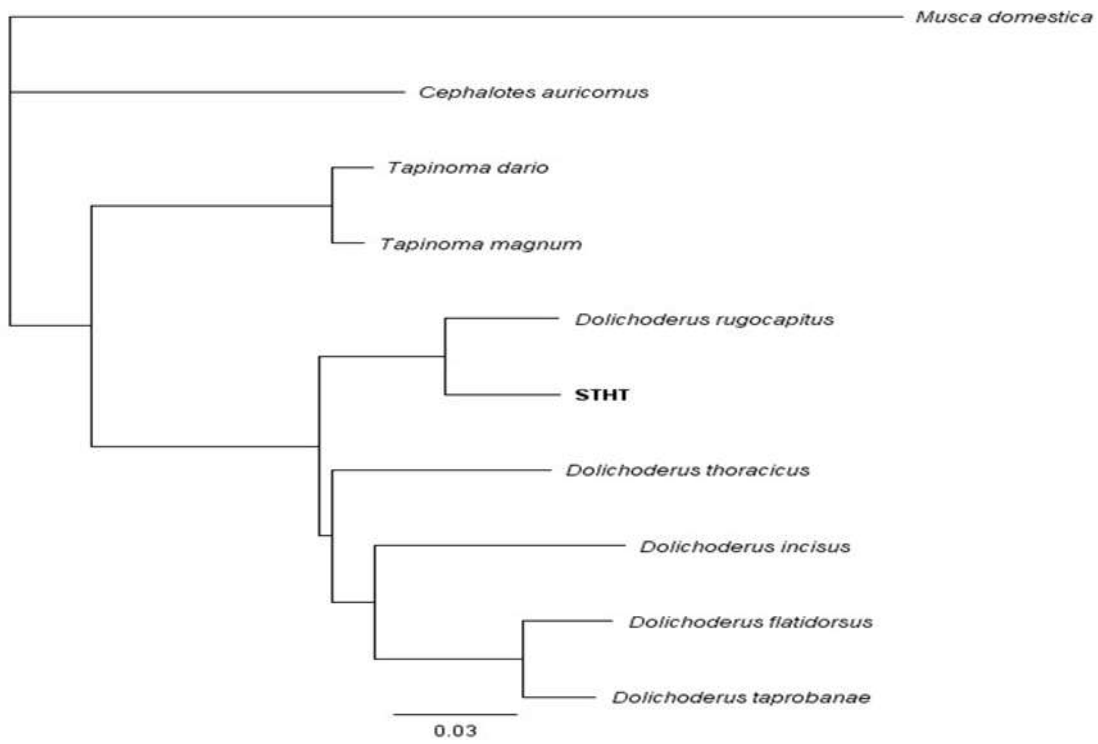

Figure 4. Phylogeny tree of STHT Insect (black ant) based on National Center for Biotechnology Information (NCBI) data base[9].

Figure 4 indicated the black ant insect (STHT) based on DNA sequences, editing using *Clusta W algorithm*. The identification of insect samples from the NCBI Gen Bank database using BLAST (*Basic Local Alignment Search Tool*) method (www.ncbi.nlm.nih.gov)[9] showed that the STHT insects (black ant) in the phylogeny tree was very close to *Dolichoderus rugocapitus* (*Query ID: lcl|Query_98113*). This ant species has close branch to *Dolichoderus thoracicus* species, *Dolichoderus incisus*, *Dolichoderus flatidorsus*, and *Dolichoderus taprobanae*. Comparison with other nearby insect species can be seen in Table 1.

Table 1. Distance of STHT (black ant) (*Query ID: lcl|Query_98113*) with a comparison to other nearby insects

No.	Specimen name	1	2	3	4	5	6	7	8	9	10
1	*Musca domestica*	**100**	74.8	74.6	74.7	73.5	72.1	72.1	71.6	71.8	72.3
2	*Cephalotes auricomus*	74.8	**100**	84.6	85.1	79	80.9	81.8	80.1	78.5	80.1
3	*Tapinoma dario*	74.6	84.6	**100**	98.3	85.1	84.3	84.1	83.4	82.7	82.4
4	*Tapinoma magnum*	74.7	85.1	98.3	**100**	84.8	83.5	84.1	83.8	83.2	83
5	*Dolichoderus thoracicus*	73.5	79	85.1	84.8	**100**	90	89.3	89	89.5	89.2
6	*Dolichoderus rugocapitus*	72.1	80.9	84.3	83.5	90	**100**	94.8	87.8	88.9	89.7
7	**STHT** (black ant)	72.1	81.8	84.1	84.1	89.3	94.8	**100**	88.1	88.9	89.2
8	*Dolichoderus incisus*	71.6	80.1	83.4	83.8	89	87.8	88.1	**100**	89.3	89.8
9	*Dolichoderus flatidorsus*	71.8	78.5	82.7	83.2	89.5	88.9	88.9	89.3	**100**	96.2
10	*Dolichoderus taprobanae*	72.3	80.1	82.4	83	89.2	89.7	89.2	89.8	96.2	**100**

Table 1 showed that black ant (*Query ID: lcl | Query_98113*) have 94.8% proximity to *D. rugocapitus* species, followed by *Dolichoderus thoracicus* (89.3%), *Dolichoderus incises* (88.1 %), *Dolichoderus flatidorsus* (88.9 %), *Dolichoderus taprobanae* (89.2%), *Tapinoma magnum* (84.1%), *Tapinoma Dario* (84.1%), *Cephalotes auricomus* (81.8%), and *Musca domestica* (72.1%).

2. STMK (red ant)

The sequencing result of mitochondrial DNA genes using CO1-specific primers of LCO1490 (5'GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') produced a single DNA fragment with 658 base pairs. The DNA sequence of red ant (STMK) generated a combination of base pairs as in Figure 5.

AATTTTATATTTTATTTTTGCAATTTGAGCAGGAATAATTGGATCTTCTATAAGTATAATTATCCGA
CTAGAATTAGGCACATGTGACTCCATTATTAATAATGATCAAATTTATAACTCCTTAGTAACTAGC
CACGCATTTATTATAATTTTCTTCATAGTAATACCTTTCATAATCGGAGGATTTGGTAATTTCCTTG
TACCCCTTATACTAGGATCCCCCGATATAGCTTACCCCCGAATAAATAATATAAGATTTTGACTTC
TACCCCCTTCTCTAACCCTCCTAATTATTAGAAGATTCATCAATACAGGTGTAGGAACAGGATGAA
CTATCTACCCCCCCCTAGCATCTAACATCTTTCATAATGGACCTTCTGTTGACTTATCTATCTTTTCT
CTCCATATTGCAGGAATGTCCTCTATCTTAGGAGCAATCAATTTCATTTCTACTATTATCAATATAC
ATCAAAAAAATTTATCCATAGATAAAATTCCTTTATTAGTATGATCTATCCTAATTACTGCTATTCT
CCTTCTACTTTCCCTTCCTGTTCTAGCCGGAGCCATTACAATGTTATTAACTGACCGAAACTTAAAT
ACCTCTTTTTTTGATCCCTCAGGAGGAGGTGATCCAATTTTATACCAACACTTATTC

Figure 5. DNA sequence result of CO1 of STMK insect (red ant)

DNA sequence confirmation result of Mitochondrial CO1 of red ants (STMK) in the Gen BOLD gene bank data base (*Barcode of Life Data System*) obtained the phylogeny trees as in Figure 6.

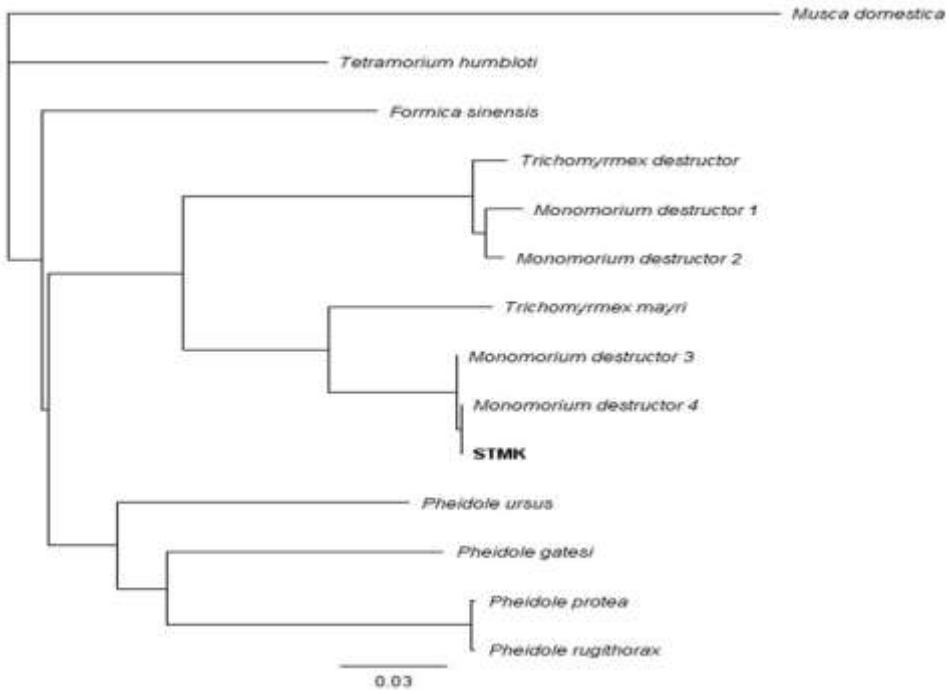

Figure 6.STMK insect (black ant) phylogeny tree based on National Center for Biotechnology Information (NCBI) data base[9].

Figure 6 showed the presence of red ant (STMK) based on DNA sequence, editing using *ClustaW algorithm*. Identification of insect sample according to Gen Bank NCBI database using BLAST (*Basic Local Alignment Search Tool*) method (www.ncbi.nlm.nih.gov) [9] showed that STMK insects (red ant) in phylogeny trees was very close to *Monomorium destructor* 4 (*Query ID: lcl | Query_38701*). This red ant species showed close branches to *Monomorium destructor* 3, *Trichomyrmex mayri*, *Trichomyrmex destructor*, *Monomorium destructor* 1, *Monomorium destructor* 2, and further branches to *Pheidole* spp., *Formica sinensis*, *Tetramorium humbloti* and *Musca domestica*. Comparison with other nearby insect species can be seen in Table 2.

Table 2. Distance table of STMK (red ant)*, Query ID: lcl|Query_38701*, compare to other insects

No.	Specimen name	1	2	3	4	5	6	7	8	9	10	11	12	13	14
1	*Musca domestica*	100													
2	*Trichomyrmex destructor*	71.7	100												
3	*Monomorium destructor 1*	71.5	97.7	100											
4	*Monomorium destructor 2*	71.7	98.2	98.5	100										
5	*Trichomyrmex mayri*	72.5	85.1	84.4	85.1	100									
6	*Monomorium destructor 3*	71.4	86.6	86.6	87.2	92.7	100								
7	*Monomorium destructor 4*	71.4	86.6	86.6	87.2	92.5	99.8	100							
8	**STMK** (red ant)	71.4	86.6	86.6	87.2	92.5	99.8	100	100						
9	*Formica sinensis*	75	82.6	81.9	81.9	80.6	81.4	81.4	81.4	100					
10	*Tetramorium humbloti*	75	81.8	80.8	81.6	81.8	82.3	82.1	82.1	82.4	100				
11	*Pheidoleursus*	74.2	80.3	80.6	80.6	80.1	81.8	81.6	81.6	83.1	82.8	100			
12	*Pheidolegatesi*	71.9	79.8	79.4	79.4	80.8	81.8	81.8	81.8	83.1	81.3	86.1	100		
13	*Pheidoleprotea*	71	79.6	79.1	79.8	82.4	82.4	82.3	82.3	80.9	83.9	83.3	85.6	100	
14	*Pheidolerugithorax*	71	79.6	79.1	79.8	82.4	82.4	82.3	82.3	80.9	83.9	83.3	85.6	99.8	100

Table 2 showed that red ant (Query ID: lcl | Query_38701) have similarities with *Monomorium destructor 4* species by 100%, followed by other insect species of *Monomorium destructor 3* (98.8%), *Trichomyrmex mayri* (92.5%), *Monomorium destructor 1* (86.6%), *Trichomyrmex destructor* (86.6%), *Formica sinensis*(81.4%), *Tetramorium humbloti* (82.1%), *Pheidole spp.* (81.6 - 82.3%), and *Musa domestica* (71.4% %).

3. STMB (termite)

The DNA sequence result of Mitochondria gene using CO1 using specific primers of LCO1490 with sequence (5'GGTCAACAAATCATAAAGATATTG-3') and HCO2198 (5'TAAACTTCAGGGTG ACCAA AAAATCA-3') produced a single DNA fragment of 604 base pairs. The DNA sequence of STMB insect (termite) produced a combination of base pairs as shown in Figure 7.

AACTTTATATTTCGTATTTGGAGCTTGATCAGGAATAGTGGGAACGTCCCTAAGAATGCTCATCCG
AACAGAGCTAGGACAACCTGGATCCCTAATTGGAGATGATCAAATCTACAACGTCATTGTCACAG
CCCATGCCTTCGTTATAATTTTCTTCATAGTTATACCAATCATAATTGGAGGCTTCGGAAACTGATT
AGTACCCTTAATGCTAGGGGCACCAGATATGGCATTCCCACGAATAAACAACATAAGATTCTGGC
TACTTCCTCCATCCCTAACTCTTCTTCTTACTAGTAGTACAGTAGAAAGCGGGGTAGGAACAGGAT
GAACTGTTTACCCCCCTCTTGCTAGAGGAATTGCCCACGCCGGAGCCTCTGTAGACCTAGCAATCT
TCTCATTACACTTAGCGGGAGTCTCATCAATTCTGGGAGCAGTAAACTTTATTACAACCACAATTA
ACATAAAGCCAAAGAGCATAAAGCCCGAACGAATCCCACTATTTGTATGATCGATCGCTATTACA
GCCCTACTCTTGCTACTCTCCCTCCCAGTTCTGGCAGGAGCAATTACTATACTACTAACAGACCGT
AATCTAAATACATCGTTTTTCGATCCAGCAGGAGGGGGAGACCCAATCCTATACCAACACTTATTC

Figure 7. DNA sequence result of CO1 of STMB insect (termite)

The result of DNA sequence confirmation of CO1 mitochondria of termite (STMB) from the Gen BOLD (Barcode of Life Data System) gene bank database obtained the phylogeny tree as shown in Figure 8.

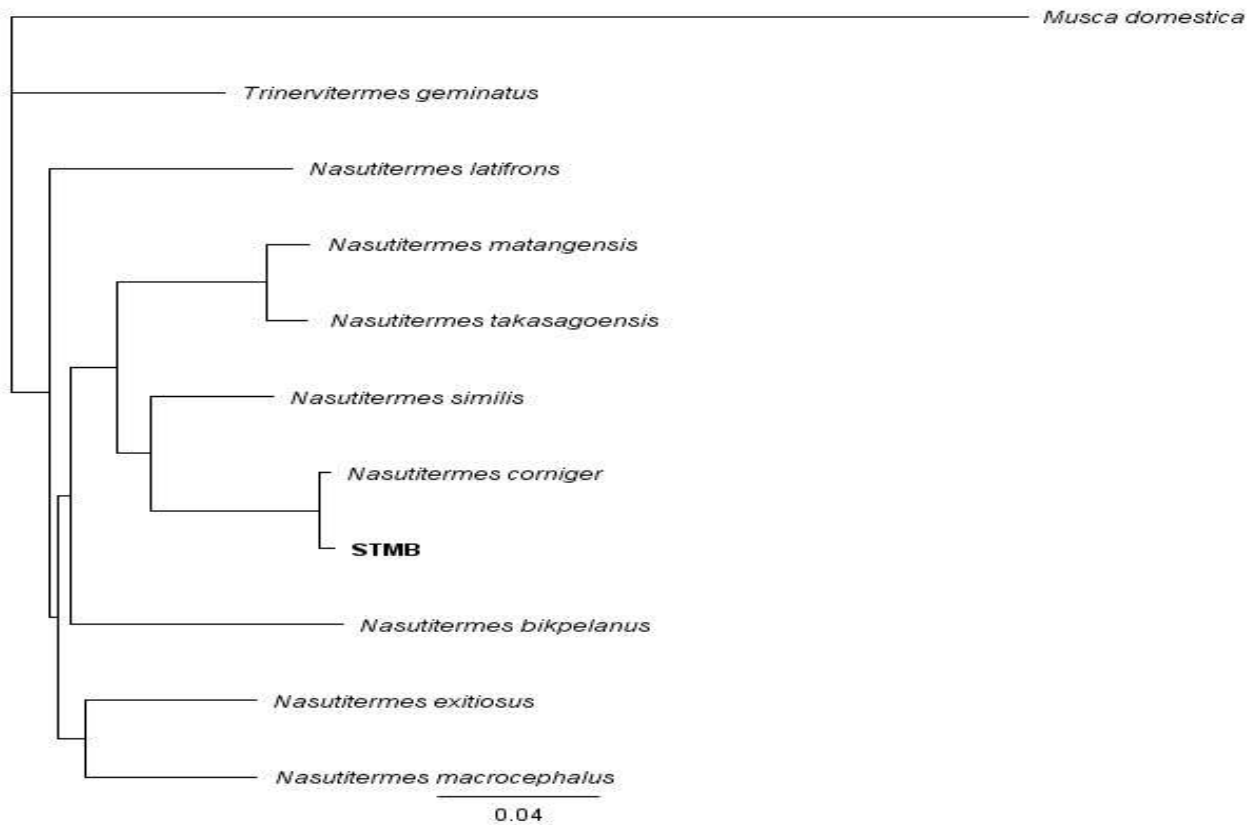

Figure 8. Phylogeny tree of STMB insect (termite) based on National Center for Biotechnology Information (NCBI) data base[9].

Figure 8 showed the presence of termite (STMB) based on DNA sequences, edited using the *ClustaW algorithm.* Identification of sample insects, according to Gen Bank NCBI database using BLAST (*Basic Local Alignment Search Tool*) method (www.ncbi.nlm.nih.gov)[9], showed that the STMB insect (termite) in the phylogeny tree was very close to *Nasutitermes corniger* (*Query ID: lcl | Query_18869*). This termite species has close branching to species of *Nasutitermes similis*, *Nasutitermes matangensis*, *Nasutitermes takasagoensis*, *Nasutitermes bikpelanus*, *Nasutitermes exitiosus*, *Nasutitermes macrocephalus*, *Nasutitermes latifrons*, *Trinervitermes geminatus* and *Musca domestica*. Comparison with other nearby insect species can be seen in Table 3.

Table 3. Distance table of STMB (termite) (*Query ID: lcl | Query_18869*) compare to other insects

No.	Specimen name	1	2	3	4	5	6	7	8	9	10	11
1	*Musca domestica*	100										
2	*Nasutitermes latifrons*	74.3	100									
3	*Trinervitermes geminatus*	75.8	89.7	100								
4	*Nasutitermes matangensis*	74.5	88.4	88.4	100							
5	*Nasutitermestakas agoensis*	74.6	88.3	88.9	98	100						
6	*Nasutiterme ssimilis*	75.2	89.5	88.9	92.2	92.4	100					
7	*Nasutitermes corniger*	74.6	89.8	88	90.6	90.4	93.2	100				
8	**STMB** (termite)	74.2	89.5	88	90.9	90.7	92.9	99.4	100			
9	*Nasutitermes bikpelanus*	74	88.4	88	88.9	89.1	89.8	88	88	100		
10	*Nasutitermes exitiosus*	75.1	89.8	90.1	90	90	90.7	89.8	89.8	89.5	100	
11	*Nasutitermes macrocephalus*	75.5	90.1	89.5	90.6	90.3	91	88.8	88.4	89.2	92.2	100

Table 3 showed that the termite (STMB) (*Query ID: lcl | Query_18869*) has a similarity with the species of *Nasutitermes corniger* of 99.4%, followed by other termite species namely *Nasutitermes similis* (92.9%), *Nasutitermes takasagoensis* (90.7%), *Nasutitermes matangensis* (90.9%), *Nasutitermes litirons* (89.8%), *Nasutitermes latifrons* (89.5%), *Nasutitermesm acrocephalus* (88.4%), *Nasutitermes bikpelanus* (88%), *Trinervitermes geminatus*(88%), and *Musca domestica* (74.2%).

IV. Conclusion

Molecular identification of the Mitochondrial CO1 gene Barcode of several insect species carrying *P. palmivora* disease in cocoa plantations in North Sulawesi Province are as follows:

1. Black ant (STHT) has a single DNA fragment measuring 638 base pairs, very close as the *Dolichoderus rugocapitus* (*Query ID: lcl | Query_98113*), has a 94.8% spacing. Taxonomically, it belongs to the order Hymenoptera, subordo Apocrita, family Formicidae, and genus *Dolichoderus*.
2. Red ant (STMK) have a single DNA fragment measuring 658 base pairs, very close as the *Monomorium destructor* type (*Query ID: lcl | Query_38701*), has a 100% spacing. Taxonomically, it is included in the order Hymenoptera, subordo Apocrita, family Formicidae, and genus *Monomorium*.
3. Termite (STMB) have a single DNA fragment measuring 604 base pairs, very close as the *Nasutitermes corniger* type (*Query ID: lcl | Query_18869*), has a 94.8% spacing. Taxonomically, it belongs to the order Isoptera, family Termitidae, and genus *Nasutitermes*.

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