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Reconstruction of the phylogeny of Anopheles sp. Based on the Cytochrome Oxidase Sub Unit 1 (CO1) gene in the Minahasa Peninsula, North Sulawesi

Marthy Lingkan Stella Taulu[1], Christina Salaki[2], Juliet E. Mamahit[2], Arthur G. Pinaria[3]

[1]Doctoral Student, Department of Entomology, Postgraduate Program, Sam Ratulangi University, Manado, North Sulawesi Indonesia
[2]Department of Plant Pests and Diseases, Faculty of Agriculture, Sam Ratulangi University, North Sulawesi Indonesia
[3]Department of Plant Pathology, Faculty of Agriculture, Sam Ratulangi University, North Sulawesi Indonesia
Corresponding author : stellataulu16260@gmail.com

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Abstract— *Indonesia is a country with the highest malaria cases in the world. North Sulawesi is known as one of the malaria endemic areas in Indonesia. Malaria can only be transmitted through the bite of the Anopheles sp. Thus, the high case of malaria infection in an area is linear with the high population of Anopheles sp. The identification method to the species level that has high accuracy is by molecular identification using the cytochrome oxidase sub unit 1 (CO1) gene. Based on the CO1 gene, the mitochondrial DNA of Anopheles sp from Tombatu was 92% similar to Anopheles maculatus [KT382822.1] from China. Anopheles sp from Ratahan based on the CO1 gene has a similarity level of 80% with Anopheles barbirostris [KM610029.1] from China. Anopheles sp from Pineleng has a 77% similarity with Anopheles aquasalis [AF417697.1] from Brazil. The CO1 gene sequences of Anopheles sp from Southeast Minahasa (Tombatu and Ratahan), and Anopheles sp from Minahasa (Pineleng) had a nitrogen base size difference of more than 6%. Thus, the variation of the Anopheles sp CO1 gene is relatively high compared to similar sequences that have been recorded on the NCBI gene bank site.*

Keywords— ***Reconstruction of Phylogeny, Anopheles sp. Cytochrome Oxidase Sub Unit 1 (CO1) gene, Minahasa***

I. INTRODUCTION

Malaria is still a major health consideration, especially in tropical countries. Malaria is the world's most dangerous parasitic infection, causing more than a million death and 500 million cases annualy (Penet et al.,2007; Ravichandran et.al. 2007). Malaria may decrease the productivity of individuals, families and the whole due morbidity and mortality (Ravichandran et.al. 2007, Namdeo, et.al., 2006). Malaria remains a leading cause of morbidity and mortality worldwide with an estimated 500 million cases and 2.5 million deaths annually (Stauffer & Kamat 2003). Malaria is a reemerging disease, which is a disease that is re-infected en masse (Arsin, 2012).

Indonesia is a country with the highest malaria cases in the world. North Sulawesi is known as one of the malaria endemic areas in Indonesia. Some malaria endemic areas in North Sulawesi are Minahasa, Southeast Minahasa and North Minahasa. Malaria is an infectious disease caused by a protozoan parasite of the genus Plasmodium, which is transmitted through the bite of the Anopheles mosquito. Malaria can only be transmitted through the bite of the Anopheles sp. Thus, the high case of malaria infection in an area is linear with the high population of Anopheles sp. In Indonesia, vector confirmation has been carried out from 1919 to 2009, and during that period 25 species were found to be positive for the malaria parasite. As a tropical rain forest area, the Minahasa area is separated by forests and mountains. Based on a survey from the Ministry of Health of the Republic of Indonesia in 2009, in North Sulawesi, three main species of Plasmodium vector were

found in humans, namely Anopheles subpictus, Anopheles vagus and Anopheles annullaris (Ministry of Health RI, 2009). Identification is based on morphological characteristics.

However, identification was carried out based on morphological characteristics. Identification of mosquitoes by morphological analysis method has many limitations. The observed specimens often have undergone morphological changes due to immersion with alcohol or formalin from the sampling location. This greatly affects the stage of species identification in the laboratory.

The identification method to the species level that has high accuracy is by molecular identification. Molecular identification using the cytochrome oxidase sub unit 1 (CO1) gene has been widely carried out. CO1 is one of the genes in mitochondrial DNA that has been designated as a molecular barcode. The cytochrome C oxidase sub unit I (COI) gene has special characteristics that are suitable as a tool in evolutionary studies, namely (1) as a final catalyzer in the respiratory chain in mitochondria, so COI is widely studied at the biochemical level, and shows that the structure and size of the COI gene conserved in all aerobic organisms (Rivera et al. 2009). (2) The amino acid sequence correlates with the function of each part of the COI, thus showing the characteristics of the species that possess it (Rivera et al. 2009 Roe & Sperling 2007). (3) A sequence of 658 basepair (bp) at the 5' end was proposed as an animal barcode (Hebert et al. 2003 a,b). These barcodes have been successfully demonstrated to be able to differentiate between species in Lepidoptera (Hebert et al. 2003a; Hajibabaei et al. 2005), beetles (Funk et al. 1995), several insect pests (Toda & Murai 2007) moth Hamona mermerodes (Hulrc et al. 2007). al. 2007), mosquitoes (Cywinska et al. 2006).

Identification of insects from North Sulawesi using the CO1 gene has been successfully carried out on Aedes sp (Kaunang et.al. 2013; Timah et.al. 2016), Apis dorsata Binghami (Mokosuli et.al. 2013), subterranean termites (Ngangi et.al. 2014), Demselfly (Rantung et.al. 2015) and Marine Gerridae (Warouw et.al. 2015). Molecular identification using the CO1 gene as the basis for reconstructing the phylogeny of Aedes sp. in North Sulawesi as a malaria endemic area. Reconstruction of phylogeny will break the distribution of Anopheles sp. in Minahasa. The results of the phylogeny reconstruction will be very useful for the prevention of malaria vector mosquitoes.

II. MATERIALS AND MEHODS

Sample

Adult mosquito collected used modified method Cheng et. al. (2010). Collection on the fields area randomly. Insects that have been collected will insert in a bottle sample that has been labeled with place and time of data sampling. The bottle was filled with 70% alcohol for identification and preservation.

DNA Extraction, PCR Amplification and Sequencing

Total genomic DNA was extracted larva and adults mosquito using Qiagen DNA Blood and Tissue, according to the manufacturer's protocol. PCR was performed in a total volume of 25 µL containing 1 × reaction buffer, 3 mM MgCl2, 0.24 mM dNTPs, 1.4 µM of each primer LCO1490 : 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO2198 : 5'-TAAACTTCAGGGTGACCAAAAAATCA-3' (Folmer et. al., 1994), 1U Go Taq Flexi DNA polymerase (Promega Corp.) and 2.5 µL of DNA (a 100 time dilution of the original DNA). The PCR program was as follows: 94 °C for 5 min, followed by 40 cycles of 94 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min and a final extension at 72 °C for 5 min. PCR products were purified using Wizard SV Gel and PCR Clean-Up System (Promega Corp). Purified PCR products were analyzed by electrophoresis in 1% agarose gel. The molecular size of the amplified products was estimated using 1 kbp DNA ladder (Biometra). PCR products were sequenced using AB1 PRISM Dye Terminator Cycle Sequencing Ready Reaction System, version 1.1. (Applied Biosystems) in FIRST BASE Singapura

Sequences Analyses and Phylogeny trees reconstructed

Obtained sequences were aligned using MEGA 6.0 and Geneous 6.0 software. Sequences were subjected to Basic Local Alignment Search Tool (BLAST) in order to perform sequence similarity searches (www.ncbi.nih.gov.com). Nucleotide frequencies were calculated using MEGA 6.0 software (Tamura et. al. 2013). The genetic distances (number of nucleotide substitutions per site) among sequences were calculated using the Maximum Composite Likelihood model in Geneous 6.0 software. Phylogenetic trees were reconstructed using two different reconstruction methods: (1) neighbor joining (NJ) and (2) maximum parsimony (MP). The NJ tree was reconstructed using the Maximum Composite Likelihood method. Phylogenetic analyses were conducted in MEGA 6.0 software. Bootstrap support values were obtained by 1,000 replications using both methods (Tamura et. al. 2013).

III. RESULTS AND DISCUSSION

Total DNA Extraction and Purification

Tissue in adult insects is found in the exoskeleton. Thus, to obtain good DNA purity and concentration, the selection of the right organ will determine the success of DNA extraction. In this study, a series of trials were carried out using mosquito organs to obtain total dsDNA with standard purity and concentration. The total DNA in this study was nuclear DNA and mitochondrial DNA isolated from mosquito organ cells which were extracted using the DNA blood and tissue kit. The organs used are the head, thorax, legs and abdomen. From the extraction carried out, it was found that the use of the thoracic organ resulted in the best concentration and purity (table 1). Proteinase K is a key enzyme in DNA extraction. Proteinase-K functions to damage membrane proteins and other proteins in the cytoplasm and nucleoplasm as well as other cell compartments to isolate DNA. The results showed that proteinase K immersion time affected the total DNA purity but had no effect on the total DNA concentration obtained (Table 1).

Table 1. Purity and Concentration of total Mosquito DNA

No	Organ	Sample weight (mg)	Modified Immersion Proteinase-K (hours)	Purity (A260/A280)	Concentration µg/ml
1	Head	35	12	1,10	25,80
		35	24	1,23	25,84
2	Thorax	35	12	1,72	37,50
		35	24	1,87	36,24
3	Limbs	35	12	1,35	32,10
		35	24	1,45	33,40
4	Abdomen	35	12	1,42	34,50
		35	24	1,52	36,20

Amplification and Visualization of Gene CO1

The results of the CO1 gene amplification were shown by electrophoresis electrogram. Based on the formed band, it shows a high concentration of amplicon in both samples AR1, KL1 and SG1 (Figure 1)

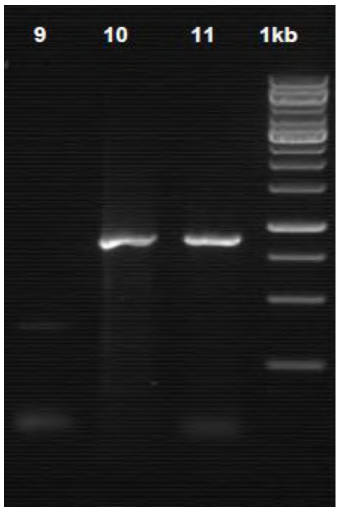

Fig.1 Visualization of the PCR product of Anopheles sp CO1 gene amplicons through 0.8% agarose gel electrophoresis. Anopheles sp from Pineleng/PSA (no. 9), Anopheles sp from Ratahan/RTA (no. 10) and Anopheles sp from Tombatu/TLA (no. 11).

The results of partial sequencing of Gen CO1 in the form of an ABI file, interpreted using Geneous 6.0 software. The sequence lengths of RTA (Ratahan), TLA (Tombatu) and PSA (Pineleng) were 870 bp, 774 bp and 862 bp, respectively. Based on the sequencing chromatogram, it showed that the CO1 gene sequence formed was good (Figure 2).

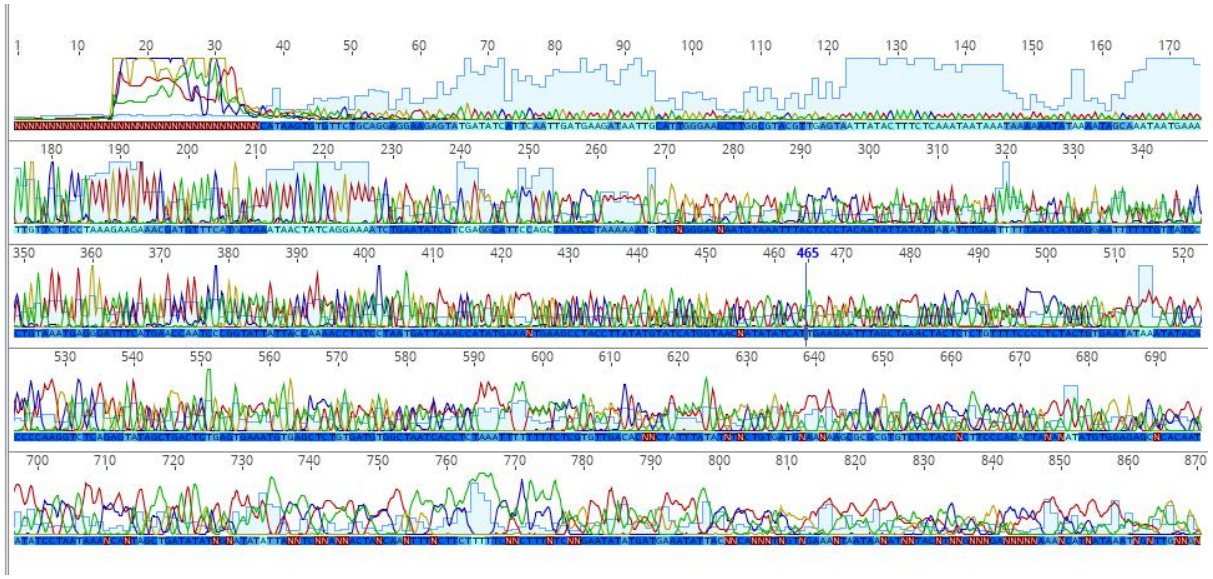

Fig.2a. The nitrogen base sequence of the CO1 RA gene was read with the Geneous Program 6.0

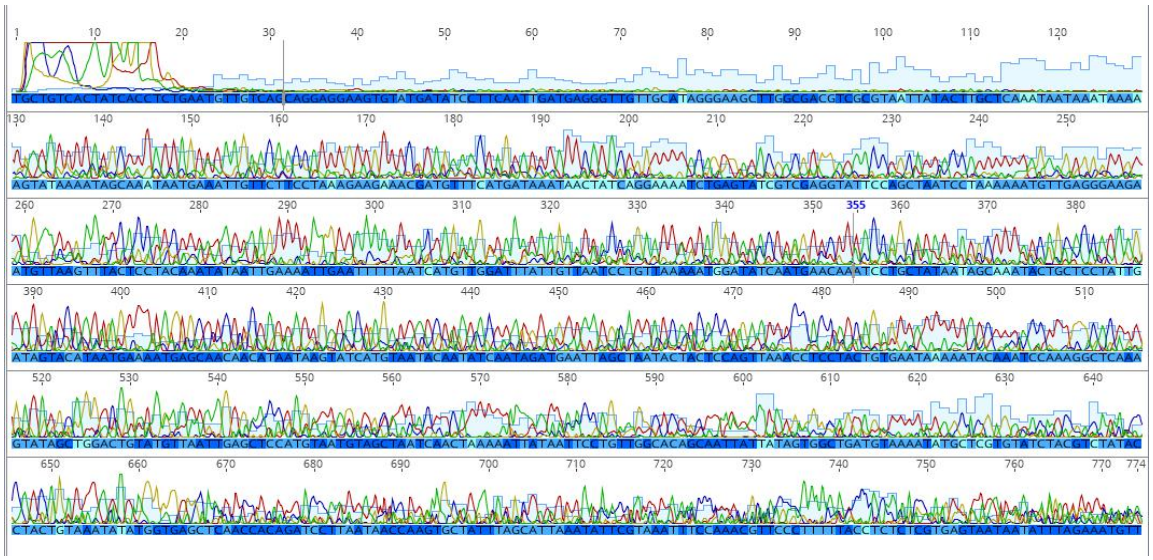

Fig.2b. The nitrogen base sequence of the CO1 gene HAS been read with the Geneous Program 6.0

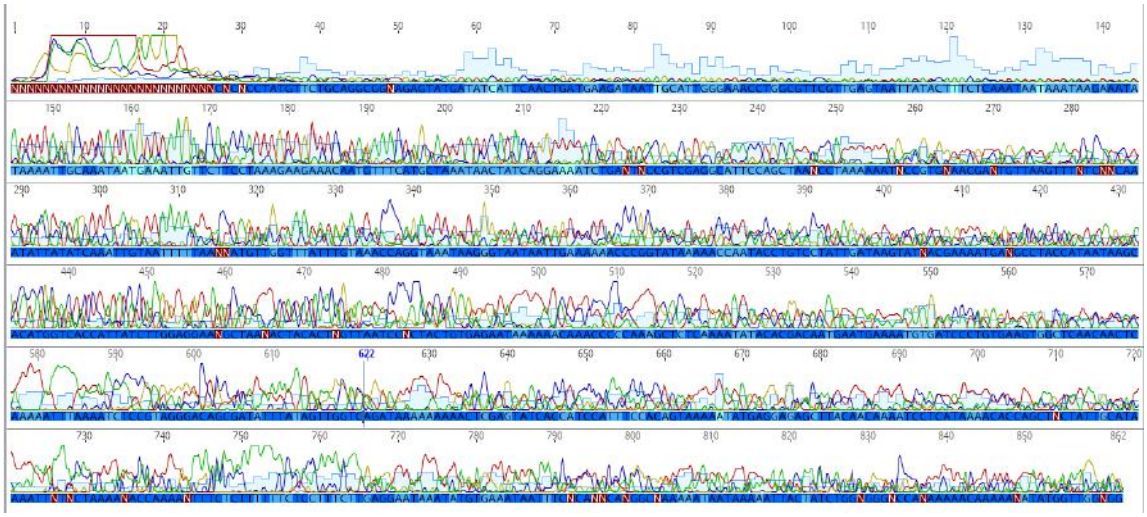

Fig.2c. The nitrogen base sequence of the PSA CO1 gene was read with the Geneous Program 6.0

Table 2. Percentage similarity of TLA CO1 gene sequences compared with the top ten Sequences recorded in the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

No	Description	E value	Identic (%)	Accession
1	Anopheles maculatus voucher AMAC20150811V4 mitochondrion, complete genome	0,00	92	KT382822.1
2	Anopheles albitarsis cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	0,00	89	AF417696.1
3	Anopheles deaneorum isolate D6 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	0,00	89	DQ076230.1
4	Anopheles deaneorum mitochondrion, complete genome	0,00	88	HQ335347.1
5	Anopheles marajoara cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	0,00	88	AF417699.1
6	Anopheles albitarsis isolate A3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	0,00	88	DQ076206.1
7	Anopheles aquasalis isolate GUA109012 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	0,00	88	KC354821.1
8	Anopheles oswaldoi haplotype H11 cytochrome oxidase subunit I gene, partial cds; mitochondrial	0,00	88	DQ784837.1
9	Anopheles oswaldoi haplotype H10 cytochrome oxidase subunit I gene, partial cds; mitochondrial	0,00	88	DQ784836.1
10	Anopheles marajoara isolate C8 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	0,00	88	DQ076223.1

Table 3. Percentage similarity of CO1 RTA gene sequences compared with the top ten Sequences recorded in the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

No	Description	E value	Identic (%)	Accession
1	Anopheles aquasalis isolate aqua28 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	4e137	85	AF548901.1
2	Anopheles aquasalis isolate aqua10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	4e137	84	AF548894.1
3	Anopheles barbirostris subgroup clade III isolate SMMULZ3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	5e136	83	KM610037.1
4	Anopheles barbirostris subgroup clade III isolate SMMUPR3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	5e136	83	KM610022.1
5	Anopheles oswaldoi haplotype H09 cytochrome oxidase subunit I gene, partial cds; mitochondrial	5e136	83	DQ784835.1
6	Anopheles oswaldoi haplotype H08 cytochrome oxidase subunit I gene, partial cds; mitochondrial	5e136	83	DQ784834.1
7	Anopheles barbirostris subgroup clade III isolate SMMUPR10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	4e142	80	KM610029.1
8	Anopheles barbirostris subgroup clade III isolate th1.10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	6e140	80	EU797223.1
9	Anopheles barbirostris subgroup clade III isolate kh3 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1e136	79	EU797224.1
10	Anopheles barbirostris subgroup clade III	1e136	79	EU797218.1

	isolate th1.9 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial			

Table 4. Percentage similarity of PSA CO1 gene sequences compared with the top ten sequences recorded in the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

No	Description	E value	Identic (%)	Accession
1	Anopheles aquasalis isolate aqua10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	2e100	78	AF548894.1
2	Anopheles aquasalis isolate aqua21 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	7e100	78	AF548900.1
3	Anopheles aquasalis cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	1e121	77	AF417697.1
4	Anopheles oswaldoi haplotype H05 cytochrome oxidase subunit I gene, partial cds; mitochondrial	2e119	77	DQ784831.1
5	Anopheles oswaldoi haplotype H01 cytochrome oxidase subunit I gene, partial cds; mitochondrial	2e119	77	DQ784827.1
6	Anopheles oswaldoi haplotype H04 cytochrome oxidase subunit I gene, partial cds; mitochondrial	1e117	77	DQ784830.1
7	Anopheles oswaldoi haplotype H02 cytochrome oxidase subunit I gene, partial cds; mitochondrial	1e117	77	DQ784828.1
8	Anopheles oswaldoi haplotype H03 cytochrome oxidase subunit I gene, partial cds; mitochondrial	5e116	77	DQ784829.1
9	Anopheles marajoara isolate C1 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial	1e111	76	DQ076216.1
10	Anopheles punctipennis cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product	9e104	76	AF417720.1

The results of BLAST NCBI analysis of the CO1 gene sequences of Anopheles sp from Tombatu showed a 92% similarity with Anopheles maculatus [KT382822.1] from China. Anopheles sp from Ratahan based on the CO1 gene has a similarity level of 80% with Anopheles barbirostris [KM610029.1] from China. Anopheles sp from Pineleng has a 77% similarity with Anopheles aquasalis [AF417697.1] from Brazil. (Table 4, Table 5 and Table 6). Alignment results of Anopheles sp from Tombatu showed 6 different nitrogen base sites with sequences similar to Anopheles maculatus [KT382822.1]. Meanwhile, Anopheles sp from Ratahan showed 39 different nitrogen base sites with similar sequences to Anopheles barbirostris [KM610029.1]. Anopheles sp from Pineleng showed 38 different nitrogen base sites with sequences similar to Anopheles aquasalis [AF417697.1] (Table 4, Table 5 and Table 6). The position of the difference in nitrogen bases indicates a mutation that occurs in Anopheles sp from Minahasa.

Table 5. Alignment of Anopheles from Pineleng with Sequences Similar to NCBI Anopheles aquasalis. (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Anopheles aquasalis cytochrome c oxidase subunit I (COI) gene, partial cds; mitochondrial gene for mitochondrial product
Sequence ID: **AF417697.1** Length: 899 Number of Matches: 1
Range 1: 132 to 884

Score	Expect	Identities	Gaps	Strand	Frame
448 bits(242)	1e-121()	595/773(77%)	38/773(4%)	Plus/Minus	

Features:

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Sbjct  884  TGTTCTGCAGGAGGAAGAGTATGATATCATTCAATAGATGAAGATAATTGCATTGGGAAT  825
Query  95   CCTGGCGTTCGTTGAGTAATTATACTTTCTCaaataataaataagaaatataaaattgca  154
Sbjct  824  GCTGGCGTTCGTTGAGTAATTATACTTTCTCAAATAATAAATAAAAAGTATAAAATAGCA  765
Query  155  aataatgaaatTGTTCTTCCTAAAGAAGAAACAATGTTTCATGCTAAATAACTATCAGGA  214
Sbjct  764  AATAATGAAATTGTTCTTCCTAAAGAAGAAACAATATTTCAAGTTAAATAACTATCAGGA  705
Query  215  AAATCTGANTNCCGTCGAGGCATTCCAGCTAANCCTAAAAAATNCCGTGNAACGANTGTT  274
Sbjct  704  AAATCAGAGTATCGTCGAGGTATTCCTGCTAATCCTAAAAAATGTTGAGGGAAAAATGTT  645
Query  275  AAGTTTN-TCNN-CAAATATTATATCAAATTGTAATTTTTAANNATGTTGG-TTTATTTG  331
Sbjct  644  AAATTTACTCCAACAAATATTATAGAAAATTG-AATTTTTAATCAAGTAGGGTTTATTGT  586
Query  332  TAAACCAGGTAAATAAGGGTAATAATTGAAAAAACCCGG-TATAAAAACCAATACCTG-T  389
Sbjct  585  TAATCCTGTTAAAAGAGGGTA-TCAATGAATAAATCCTGCTATAATAGCAAATAC-TGCT  528
Query  390  CCTATTGATAAGT-ATNACGAAAATGANGC--CTACCATAATAAGCA-CATG--GTCACC  443
Sbjct  527  CCTATTGATAATACATAATGGAAATGA-GCTACTAC-ATAATATGTGTCATGTAGT-ACA  471
Query  444  AT-TATCTTGGAGGA-ANGCTAANACTACACTNGTTAATCCNC-TACTGTGAGAATAAAA  500
Sbjct  470  ATGTCAATTGAAGAATTAGCTAAAACTACCCCAGTTAATCCACCTACAGT-A-AATAAAA  413
Query  501  A-ACAAACCCCCAAAGCTCTCAAAATATA-CACGACAATGAATGAAAA-TGTGATCCCTG  557
Sbjct  412  ATACAAATCC-AAATGCTC--AAAGTATAGCTGGGCTAT-A-TGTTAATTGTGTTCCATG  358
Query  558  TGAAGTGGCTCAACAACTCAAAAATTTAAAATCTCC-GTAGGGACAGCGATATTTATAGT  616
Sbjct  357  CAAAGTGGCTAATCAACT-AAAAATCTTAA-T-TCCTGTAGGAACGGCAATAATTATAGT  301
Query  617  TGGTCAGATaaaaaaaaaCTCGAGTATCACCATCCATTTCCACAGTAAAAATATGAGGAG  676
Sbjct  300  AGCTGAAGTAAAATAAG-CTCGAGTATCTACGTCTATTCCAACAGTAAATATATGATGAG  242
Query  677  AGCTTACAACAAAATCCTCATAAAACACCAGCTNCTATTGCATAAAATTNTNCTAAAANA  736
Sbjct  241  CTCAAACAATAAA-TCCTAATAATCCAATTGCTAGTATAGCATAAATTATTCCTAAATTT  183
Query  737  CCAAAANTTTCTCTTTTTTCTCCTTTCTTGAGGAATAAATATGTGAAATAATT  789
Sbjct  182  CCAAAAGTTTC-CTTTTTACCTCTTTCTTGAGTAATAA-TGTGTGAAATTATT  132
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Table 6. Alignment of Anopheles from Tombatu with Similar Sequences on NCBI Anopheles aquasalis. (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Anopheles maculatus voucher AMAC20150811V4 mitochondrion, complete genome

Sequence ID: **KT382822.1** Length: 14850 Number of Matches: 1

Range 1: 2177 to 2919

Score	Expect	Identities	Gaps	Strand	Frame
1044 bits(565)	0.0()	686/745(92%)	6/745(0%)	Plus/Minus	

Features:

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Query  31    GCAGGAGGAAGTGTATGATATCCTTCAATTGATGAGGGTTGTTGCATAGGGAAGCTTGGC  90
Sbjct  2919  GCAGGAGGAAGGGTATGATATCATTCAATTGATGAAGATAGTTGTATAGGAAAACTTGGT  2860

Query  91    G-ACGTCGCGTAATTATACTTGCTCAAATAATAAATAAAAAGTATAAAATAGCAAATAAT  149
Sbjct  2859  GTTCGTTGTGTAATTATACTTTCTCAAATAATAAATAAAAAGTATAAAATAGCAAATAAT  2800

Query  150   GAAATTGTTCTTCCTAAAGAAGAAACGATGTTTCATGATAAATAACTATCAGGAAAATCT  209
Sbjct  2799  GAAATTGTTCTTCCTAAAGAAGAAACGATGTTTCATGATAAATAACTATCAGGAAAATCT  2740

Query  210   GAGTATCGTCGAGGTATTCCAGCTAATCCTAAAAAATGTTGAGGGAAGAATGTTAAGTTT  269
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Query  270   ACTCCTACAAATATAATTGAAAATTGAATTTTTAATCATGTTGGATTTATTGTTAATCCT  329
Sbjct  2679  ACTCCAACAAATATAATTGAAAATTGAATTTTTAATCATGTTGGATTTATTGTTAATCCT  2620

Query  330   GTTAAAAATGGATATCAATGAACAAATCCTGCTATAATAGCAAATACTGCTCCTATTGAT  389
Sbjct  2619  GTTAATAATGGATATCAATGAACAAATCCTGCTATAATAGCAAATACTGCTCCTATTGAT  2560

Query  390   AGTACATAATGAAAATGAGCAACAACATAATAAGTATCATGTAATACAATATCAATAGAT  449
Sbjct  2559  AATACATAATGAAAATGGGCAACAACATAATAAGTATCGTGTAGTACAATATCAATTGAT  2500

Query  450   GAATTAGCTAATACTACTCCAGTTAAACCTCCTACTGTGAATAAAAATACAAATCCAAAG  509
Sbjct  2499  GAGTTAGCTAATACTACTCCAGTTAATCCTCCTACTGTAAATAAAAAAACAAATCCAAAG  2440

Query  510   GCTCAAAGTATAGCTGGACTGTATGTTAATTGAGCTCCATGTAATGTAGCTAATCAACTA  569
Sbjct  2439  GCTCAAAGTATAGCTGGACTGTACGTTAATTGAGTTCCGTGTAATGTAGCTAGTCAACTA  2380

Query  570   AAAATTATAATTCCTGTTGGCACAGCAATTATTATAGTGGCTGATGTAAAATATGCTCGT  629
Sbjct  2379  AAAATTTTAATTCCTGTAGGTACAGCAATAATTATAGTAGCTGATGTAAAATATGCTCGT  2320

Query  630   GTATCTACGTCTATACCTACTGTAAATATATGGTGAGCTCAACCAC-AGATCCTTAATAA  688
Sbjct  2319  GTATCTACGTCTATTCCTACTGTAAATATATGATGAGCTCAAACAATAAATCCT-AATAA  2261

Query  689   -CCAAGTGCTATT-TAGCATTAAATATTCGTAAATTTCCAAACGTTCCCTTTTTACCTCT  746
Sbjct  2260  TCCAATAGCTAGTATAGCATAAATTATTCCTAAATTTCCAAATGTTTCCTTTTTACCTCT  2201

Query  747   CTCGTGAGTAATAATATTTAGAAAT  771
Sbjct  2200  TTCTTGAGTAATAATATG-AGAAAT  2177
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Table 7.Alignment of Anopheles of Ratahan with Similar Sequences in NCBI Anopheles aquasalis. (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

Anopheles barbirostris subgroup clade III isolate th1.10 cytochrome oxidase subunit I (COI) gene, partial cds; mitochondrial
Sequence ID: **EU797223.1** Length: 756 Number of Matches: 1
Range 1: 62 to 756

Score	Expect	Identities	Gaps	Strand	Frame
508 bits(275)	6e-140()	570/715(80%)	39/715(5%)	Plus/Minus	

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Sbjct  696  GAAAGC-AGGTGTACGTTGAGTAATTATACTTTCTCAAATAATAAATAAAAAATATAAAA  638
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Sbjct  401  ACAGCTCCTATTGATAAAAC-ATAATGGAAATGA-GCAACTACATAATATGTATCATG-T  345
```

This genetic variation is supported by the results of morphometric analysis which indeed show differences in several morphometric characters, including the shape of the strip on the pronotum and the color of the antennae tip on Rhynchoporus sp. cream-colored sago palm and Rhynchoporus sp. in black sugar palm (Korua et.al. 2015). Polymophism can occur in a population if more than one morphological variation is found at the same location and time (Ford, 1965, Abad et. al. 2014). If random mating occurs and each individual has the potential to mate, then morphological changes can take place in a population (Abad et. al. 2014).

Phylogenetic Analysis and Construction

The substitution matrix between Anopheles sp from Tombatu, Ratahan and Pineleng was compared with 22 BLAST sequences at the NCBI site built using the Maximum Likelihood Model on the MEGA 6.0 program. The form of transitional substitution is indicated by the numbers in bold in table 6. While the transversional substitution is written in italics in table 6. Nucleotide frequency A = 33.70%, T/U = 36.75%, C = 14.11% and G = 15.44 %. The maximum value of the Log Likelihood from the calculation results is 9678,156.

Table 8. Estimation of the Maximum Likelihood Model Substitution Matrix in the MEGA 6.0 . Program

	A	T/U	C	G
A	-	*8.24*	*3.16*	**3.71**
T/U	*7.55*	-	**12.03**	*3.46*
C	*7.55*	**31.33**	-	*3.46*
G	**8.09**	*8.24*	*3.16*	-

Phylogenetic construction

The phylogeny construction was carried out using two models, namely Neighbor Joining and UPGMA. These two models are used because they have similarities, namely the evolutionary approach and to see the position of the Anopheles sp species from Minahasa. The Neighbor Joining model phylogeny construction was built with 22 sequences similar to the NCBI BLAST results. Three monophyletic clades were formed, where Anopheles sp from Tombatu and Ratahan were in the same clade while Anopheles from Pineleng was in its own clade. The phylogenetic tree construction using the UPGMA model also placed Anopheles sp from Pineleng in its own node, while Anopheles sp from Tombatu and Ratahan formed the same node but still in one monophyletic clade. In the UPGMA model, only 2 monophyletic clades were formed.

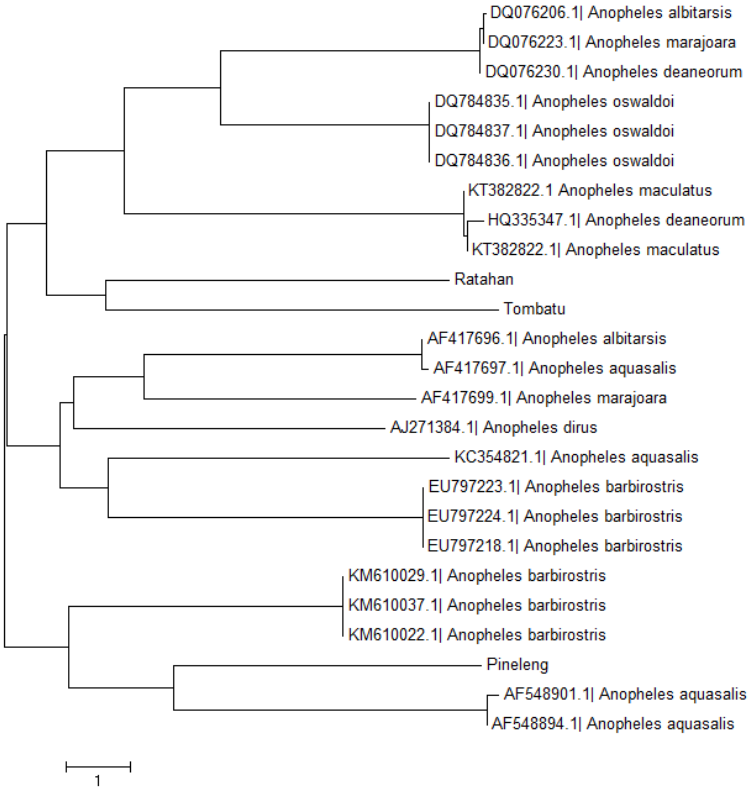

Fig.3: Anopheles sp phylogeny tree from Tombatu, Ratahan and Pineleng compared to 22 BLAST sequences at the NCBI site, built using the Neighbor Joining Model, bootstrap 1000 x.

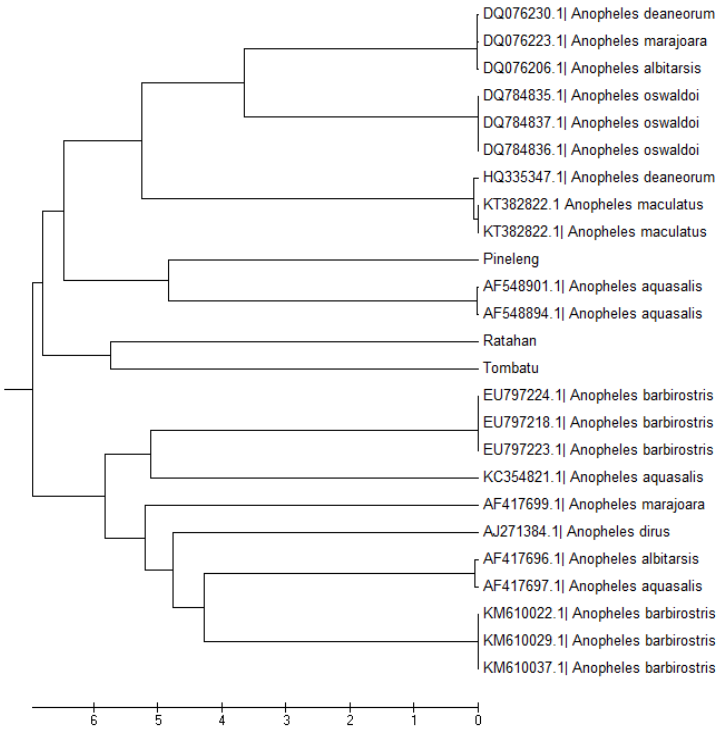

Fig.4: Anopheles sp phylogenetic tree from Tombatu, Ratahan and Pineleng compared to 22 BLAST sequences at the NCBI site, constructed using the UPGMA Model, bootstrap 1000 x.

IV. CONCLUSION

1. Based on the CO1 gene, the mitochondrial DNA of Anopheles sp from Tombatu is 92% similar to Anopheles maculatus [KT382822.1] from China. Anopheles sp from Ratahan based on the CO1 gene has a similarity level of 80% with Anopheles barbirostris [KM610029.1] from China. Anopheles sp from Pineleng has a 77% similarity with Anopheles aquasalis [AF417697.1] from Brazil.

2. The CO1 gene sequences of Anopheles sp from Southeast Minahasa (Tombatu and Ratahan), and Anopheles sp from Minahasa (Pineleng) have nitrogen base size differences of more than 6%. Thus, the variation of the Anopheles sp CO1 gene is relatively high compared to similar sequences that have been recorded on the NCBI gene bank site.

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