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Morphometry and Phylogeny reconstruction *Aedes* sp. based DNA Mitochondrial cytochrome oxidase gene sub unit 1 (CO1) in North Sulawesi

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Abstract
Aedes sp. known as the mosquito vector of dengue viruses that cause dengue fever disease. North Sulawesi is one of the areas with high cases of dengue fever in Indonesia. Present research was conducted to get the morphological characteristics of *Aedes* sp. in North Sulawesi and to construct phylogenies *Aedes* sp. of North Sulawesi based on partial CO1 gene. Samples *Aedes* sp. obtained from Bitung, North Minahasa, Minahasa, South Minahasa, Kotamobagu, Bolaang Mongondow and Sanger. Location mosquito samples, determined by random purposive sampling method. A total of 15 individual samples of adult mosquitoes every origin, used for the analysis of 16 morphological characters and DNA analysis of CO1 gene. Morphometric measurements performed using Stereomicroscope KH8700 mosquito digital 3-D, equipped with a camera and software measurement. Measurement data, subsequent cluster analysis method of principle component analysis, to get a cluster or group *Aedes*, which has the highest degree of similarity. Molecular characteristics *Aedes* sp., is determined using CO1 gene. DNA extraction, then used as a template for CO1 gene amplification by PCR. The results of this research showed, that has been found morphometry variation of *Aedes* sp. from various regions in North Sulawesi. From phylogeny tree is formed, showed that the four samples of mosquitoes (BM = Bolaang Mongondow, Bt = Bitung, KTG = Kotamobagu and Sa = Sanger) based CO1 gene sequences, have the closest relationship with the phylogeny of *Aedes albopictus.*

Keywords: Morphometry Analysis, CO1 gene, *Aedes* sp, North Sulawesi

1. Introduction
Mosquitoes are members of the order diptera, which are becoming a vector of human disease. Diseases that can be transmitted by mosquitoes include dengue fever (*Aedes* sp.), chikungunya (*Aedes* sp. and *Mansonia* sp.), malaria (*Anopheles* sp.), filariasis (*Culex* sp.) and others. Most of the mosquito transmitted diseases in humans can cause deadly outbreaks in the region. *Aedes aegypti* and *Aedes albopictus* are two species of mosquitoes, which plays an important role in public health in the tropics and subtropics. *Aedes* sp. act as vectors of disease yellow fever (Yellow Fever/YF), dengue fever (Dengue Fever/DF), dengue hemorrhagic fever (Dengue Hemorrhagic Fever/DHF) and Chikungunya. There is still much debate about the dominant vector, Dengue virus in North Sulawesi, whether *Aedes aegypti* or *Aedes albopictus* [1, 2].

Mosquitoes have a very large diversity of species. Besides, the mosquito is one insect species, capable of progressive evolution. Mosquito eradication efforts lasted only a few years, and then will be presenting so more adaptive variants mosquitoes to insecticides. These favorable conditions for the dengue virus that does coevolution with *Aedes* sp [3]. Tropical areas like Indonesia, giving a very broad space for the development of mosquitoes. Identification of mosquitoes is important, because the speed of genetic modification of mosquitoes is very high [4, 5].

Morphometric analysis widely used by entomologists, in order to study the variation in the morphology of insects. Morphometry is a description of the method of morphological characters through measurement, calculation and scoring [6]. Quantification is intended for statistical analysis, so that kinship or relationship between the morphological characters can be validated statistically. Morphometry can be applied to determine the kinship of a particular species, the differentiation of the various species, to determine variations in species and for species identification [7, 8, 9].

High adaptability of mosquitoes, causing mosquitoes can live in almost any type of habitat. In Indonesia, North Sulawesi is one of the endemic areas of malaria and dengue fever. Mosquito vector of dengue fever is *Aedes* sp. In the city of Manado, *Aedes* sp. has been modified morphology [1]. Some areas with high dengue cases in North Sulawesi such, Bolaang Mongondow, Southeast Minahasa Regency and Regency Minahasa are geographically separated. It has been found morphological variations *Aedes* sp. in residential areas and marginal areas (rain forest) in North Sulawesi [10]. It takes a mix of identification and classification techniques, namely the identification of phenotypically plastic or morphology and identification of molecular genetics in identifying *Aedes* sp [11, 12]. Gene cytochrome oxidase subunit 1 (CO1) has been successfully used to identify and reconstruct the insects in North Sulawesi [8, 13, 1].

2. Materials and Methods

Samples

Mosquitoes collected from various regions in North Sulawesi, among other Bitung, North Minahasa, Minahasa, South Minahasa, City Kotamobagu, Bolaang Mongondow and Sanger. Imago *Aedes* sp. was caught by the feeding people method and preserved in 70% alcohol.

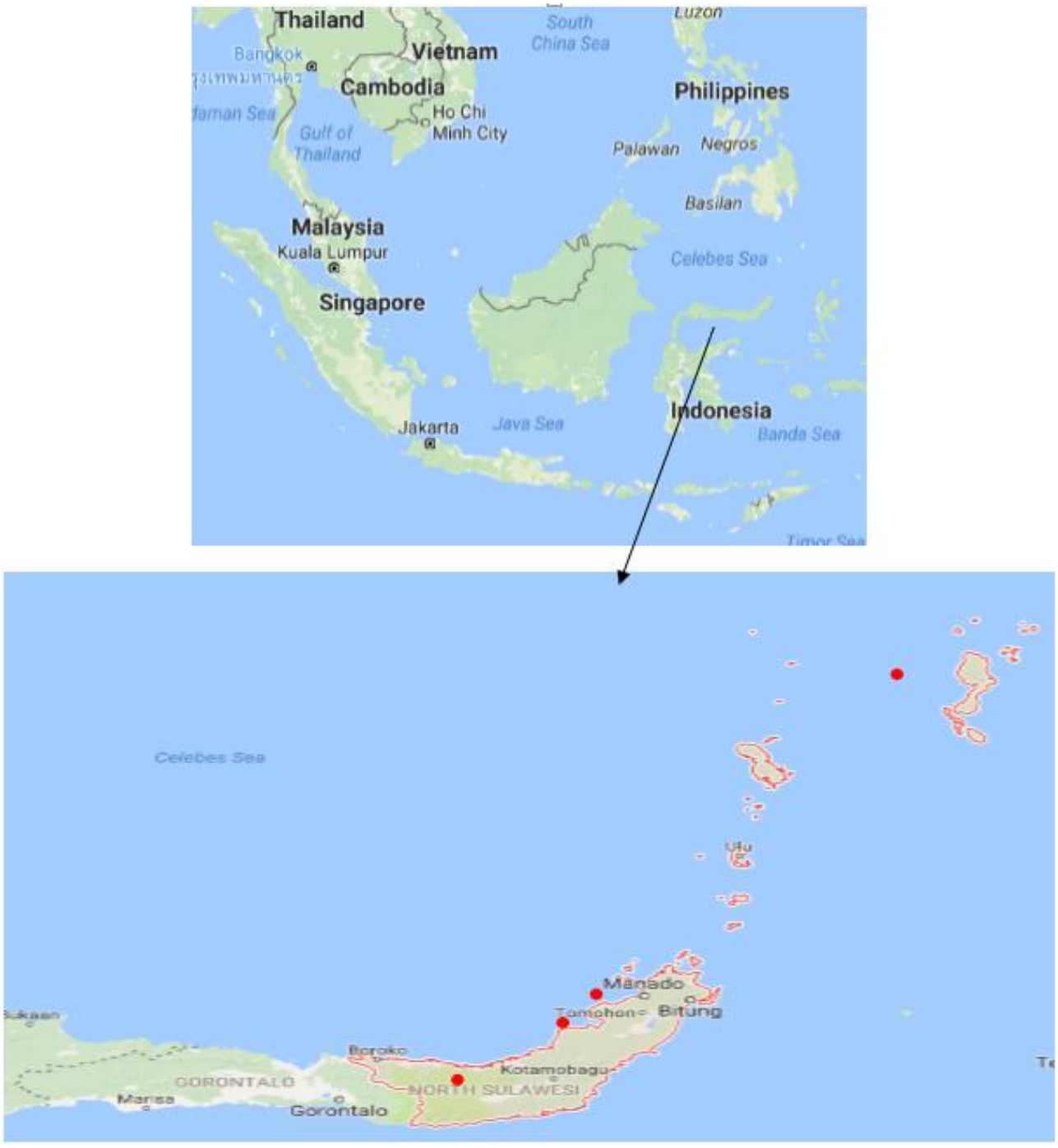


Fig 1: The samples are marked with red dots

(**Source map:** /www.google.com/maps/@0.741694, 123.9825402,10z)

Mitochondrial DNA analysis of gene CO1

A. Extraction and Purification dsDNA Total

Extraction and purification of dsDNA total of mosquitoes, using the procedure on DNeasy Blood & Tissue Kit, Analytik Jena (Germany). Modifications done at several stages of extraction, to get the maximum total DNA extraction. Thoracic used as a source of tissue for DNA extraction. Total DNA extraction, then analyzed the concentration and purity using implen NanoPhotometer. Purity of DNA can be seen with the value of the ratio of A260 / A280 nm, between 1.8 to 2.0. When <1.8, meaning contaminated with protein or protein derivative component contamination, which affects the DNA molecule. However, if> 2.0 means contaminated with RNA (Protocol Kit).

B. Target gene amplification by PCR

Aedes sp CO1 gene amplification using the Top Tag Master Mix, Qiagen and primary CO1 universeal, LCO 1490: 5'GGTCAACAAATCATAAAGATATTGG3 'and HCO 2198: 'TAAACTTCAGGGTGACCAAAAAATCA3' [14]. PCR conditions of initial denaturation 72 °C for 50 seconds and then following denaturation 94 °C for 30 seconds. Annealing 49 °C for 40 seconds, extension 72 °C for 50 seconds, the final extension of 72 °C for 5 minutes. The number of cycles for 35 times.

C. Visualization of PCR products by electrophoresis automatic qiaexel

The resulting amplicons read using Qiagen gel screening DNA Kit (Qiaxel). Working procedures carried out based on a protocol on Kit.

D. Sequencing and Sequence Analysis

Sequencing using the ABI PRISM 3730xl Genetic Analyzer Develop by Applied Biosystems, USA. Output sequencing in the form of file 1st_BASE_1706615_An_LCO.seq and 1st_BASE_1706616_Ae_LCO.seq processed into FASTA format with geneous Software 5.4.3. Files in FASTA format and analyzed alignment, using BLAST (www.ncbi.com), to compare and obtain similarity (percent Identics) with similar gene sequences, which were recorded in NCBI gene bank. Construction phylogeny tree, using the MEGA 7.0 program [15]. Model phylogeny tree was determined through the analysis of substitution, using CO1 gene sequences *Aedes* sp.

Results and Discussion

A. Analysis of Morphometry *Aedes* sp.

A total of 1038 mosquitoes have been collected from six regions in North Sulawesi (Table 1). Mosquitoes were used for morphometric analysis are mosquitoes that still had a complete organ. Mosquito samples were preserved with 70% alcohol, showing no change in color and morphology, after 48 hours of preserved. While the sample is preserved with alcohol 96%, shows morphological changes color and size of mosquitoes. Alcohol as a polar solvent, will draw water present in the mosquito's body, even extracting secondary metabolites are polar in the mosquito's body so as to alter the morphology of mosquitoes, especially the color and shape of the body surface [8].

Fig 2: Aedes sp, observed using Digital Stereomicroscope hirox KH8700. (Note: 1 = Bolaang Mongondow, 2 = Bitung, 3 = Kotamobagu, 4 = Sanger)

Table 1: Origin and the number of mosquito samples

No.	Origin of the samples*	The number of adult mosquito	Mosquitoes were used for morphometry
1	Bitung	155	15
2	Minahasa Utara	163	15
3	Minahasa	173	15
4	Minahasa Selatan	127	15
5	Kotamobagu	205	15
6	Bolaang Mongondow	215	15
	Jumlah	1038	90

* The location of samples is an area that tested positive for dengue fever cases.

Measuring 16 morphological characters was performed on 15 individuals based on the original sample mosquitoes. Each individual mosquitoes were placed in glass objects that have been cleaned and then observed with a stereomicroscope calibrated 3-D hirox KH8700. The results showed size variation of morphological characters obtained Aedes sp in North Sulawesi (Table 2).

Table 2: The average size and standard deviation of the morphological characters *Aedes* sp. (mm)

Morphological Characters	Minut		Bitung		Bolmong		Kotamobagu		Sanger		Amurang	
	Ave.	Stdv	Ave.	Stdv	Ave.	Stdv	Ave.	Stdv	Ave.	Stdv	Ave.	Stdv
PK	0.53	0.0215	0.57	0.0114	0.54	0.0148	0.53	0.0147	0.51	0.0136	0.57	0.0147
LK	0.82	0.0219	0.75	0.0085	0.68	0.0186	0.67	0.0162	0.67	0.0098	0.72	0.0132
PThr	0.42	0.0247	0.54	0.0206	0.5	0.0188	0.49	0.0097	0.48	0.0112	0.53	0.0099
LThr	0.29	0.0149	0.35	0.0126	0.3	0.118	0.32	0.0111	0.31	0.0112	0.37	0.0099
PS	2.29	0.0086	3.14	0.2346	2.98	0.0321	2.97	0.0317	2.95	0.0407	3.03	0.0124
LS	0.66	0.0133	0.74	0.0131	0.68	0.0632	0.67	0.0677	0.66	0.0263	0.74	0.0145
Pab	3.03	0.0124	2.97	0.0109	2.89	0.0333	2.88	0.0352	2.77	0.0139	3,33	0.2533
Lab	0.54	0.0146	0.43	0.0127	0.4	0.0095	0.39	0.0116	0.38	0.0102	0.43	0.0141
PPbs	0.74	0.0577	0.67	0.0183	0.64	0.0120	0.63	0.0127	0.63	0.0147	0.66	0.0177
Pant	0.54	0.0162	0.54	0.0153	0.53	0.0124	0.53	0.0137	0.49	0.0175	0.54	0.0112
PFTd	0.81	0.0162	1.85	0.0101	1.85	0.017	1.83	0.0213	1.8	0.0144	1.93	0.0115
PFTt	1.84	0.0202	2.23	0.0196	2.17	0.090	2.15	0.0914	2.09	0.0288	2.32	0.0315
PFtb	1.75	0.0142	1.74	0.0147	1.72	0.0518	1.71	0.0694	1.73	0.0141	1.83	0.0103
PTTd	1.85	0.0146	1.78	0.0148	1.75	0.0628	1.74	0.0616	1.73	0.0161	1.77	0.0205
PTTt	2.07	0.0206	1.88	0.0188	1.76	0.0835	1.75	0.0687	1.79	0.0198	1.85	0.0178
PTTb	1.77	0.0605	2.54	0.0152	2.41	0.1915	2.4	0.0194	2.28	0.0148	2.34	0.0199

Description: PK (long darikepala), LK (width of the head), THR (the length of the thorax), Lthr (width of the thorax), PS (the length of the wing), LS (width of the wing), Pab (length of the abdomen), Lab (width of the abdomen), Pant (length of antenna), FTD (the length of the femur forelimb), FTT (the length of the femur leg middle), FTB (the length of the femur hind limb), TTD (length of Tibia forelimb), TTT (the length of the middle leg tibia), Lp (length of the rear leg tibia).

A total of 15 individual mosquitoes from each site, used for morphometric analysis. A total of 16 characters morphometry was used to analyze the morphology of *Aedes* sp. Morphometric measurement data *Aedes* sp. has been used to analyze the morphological similarity of *Aedes* sp. in North Sulawesi. Cluster analysis is used to classify *Aedes* sp. from various locations in North Sulawesi based on the degree of similarity morphometry. Dendogram to clarify the tools that form clusters. The result of cluster analysis, obtained two key clusters, namely cluster/group 1 (1: Bolaang Mongondow and 2: South Minahasa) and cluster or group II (6: Kotamobagu, 4: Sanger, 5: Bitung, 3: South Minahasa and 2: Minahasa Utara) (Table 3).

Morphometric analysis studies showed that the geographic location close resemblance does not determine morphometric characters mosquitoes. Based on dendogram in figure 3, the morphological characteristics of *Aedes* sp. of Bolaang Mongondow, it has similarities with *Aedes* sp. of Sanger. In fact, Sanger is located in the Islands while Bolaang Mongondow, located on the mainland of Sulawesi Island. *Aedes* sp. obtained from North Minahasa, showed the greatest morphometric differences, compared to other regions (Figure 3). Thus, based on morphometric characteristics, found many variations of morphometry *Aedes* sp. in North Sulawesi.

Table 3: Results of the Cluster Analysis, Morphometry character of *Aedes* sp.

Agglomeration Schedule						
Stage	Cluster Combined		Coefficients	Stage Cluster First Appears		Next Stage
	Cluster 1	Cluster 2		Cluster 1	Cluster 2	
1	1	6	.002	0	0	2
2	1	4	.041	1	0	4
3	3	5	.078	0	0	4
4	1	3	.124	2	3	5
5	1	2	2.308	4	0	0

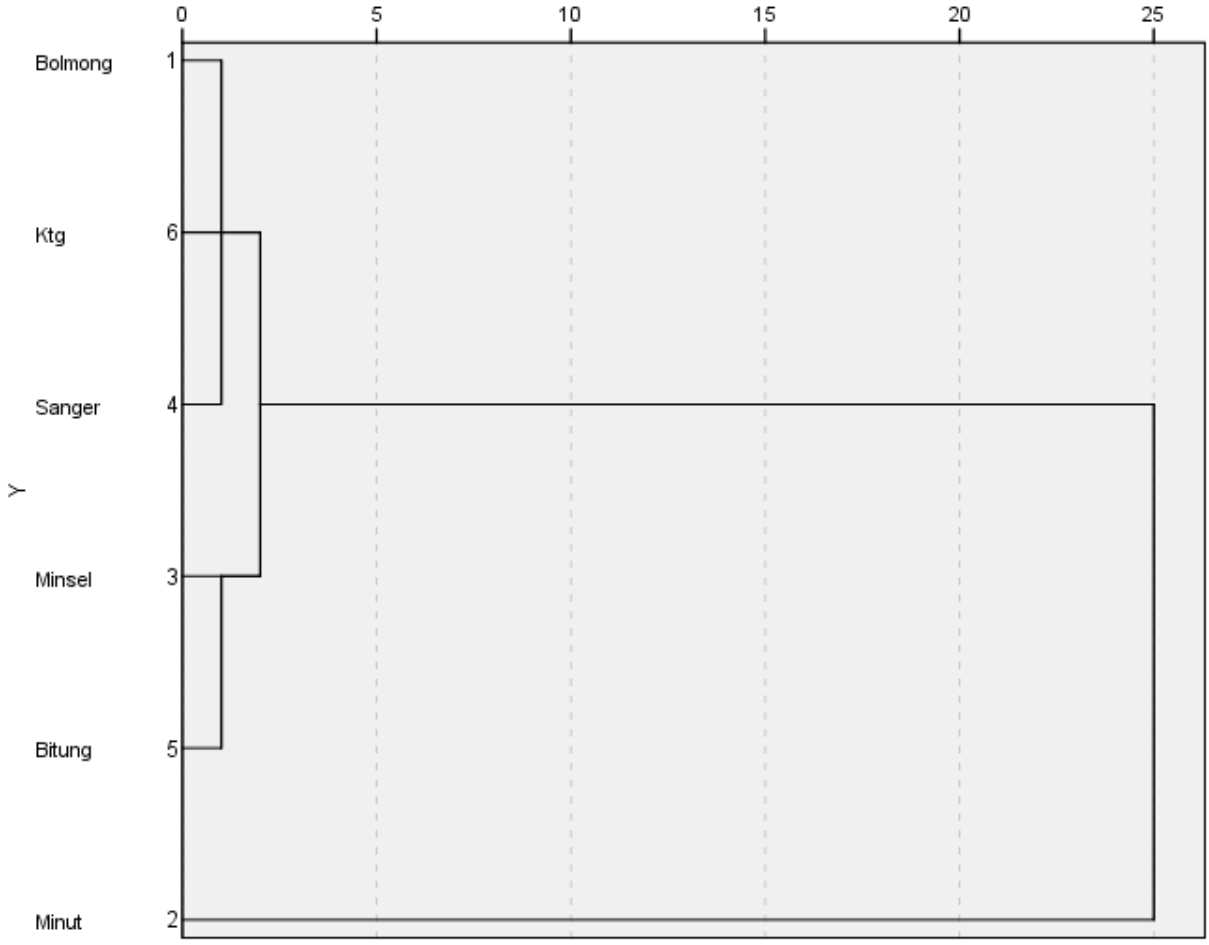


Fig 3: Morphometry Dendogram Aedes sp in North Sulawesi

B. Analysis of DNA gene CO1

1. Extraction and Purification of dsDNA total

The concentration of DNA obtained from tissue of the thorax of the mosquito, is good. Minimal DNA concentration Kit according to the protocol used was 50 pg/ml (Figure 2 and Figure 3). However, the purity of DNA was 1.62, still below the standard that is 1.8 to 2.0 at the absorbance A260/280 (Figure 4 and Figure 5). Thus, ds DNA extracted from the thorax of mosquitoes still contain contaminants RNA and other proteins. DNA variations in a concentration of purity and influenced the DNA extraction phase. Soaking with proteinase K for 24 hours has not been able to degrade the protein that is not expected in the extraction of DNA. Protein and RNA from eksoskleton insects, affects concentration and purity of DNA [8, 12].

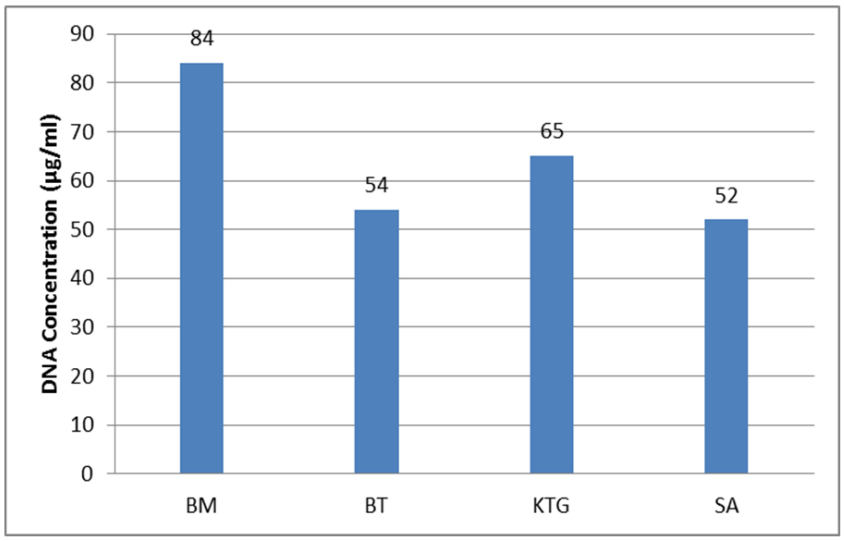


Fig 4: The concentration of DNA extracted using mosquito thorax tissue (BM = Bolaang Mongondowm; BT = Bitung; KTG = Kotamobagu and SA = Sanger)

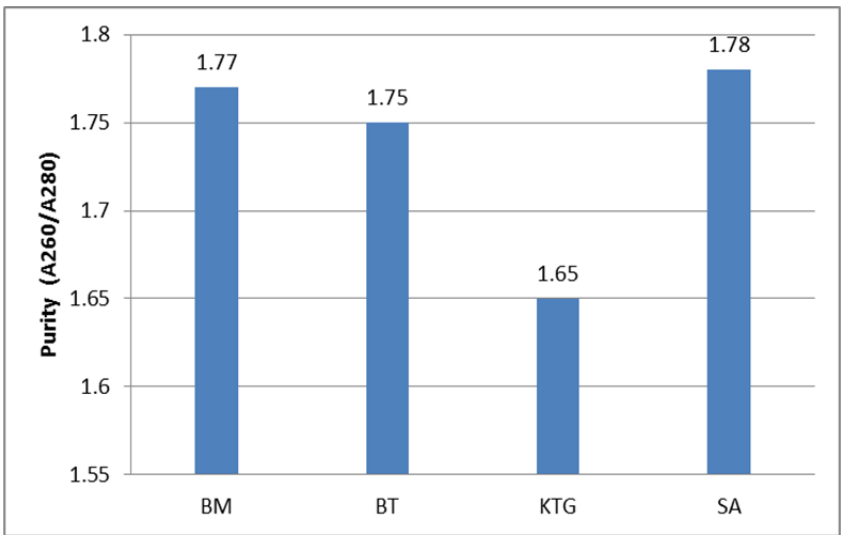


Fig 5: The purity of the DNA extracted using mosquito thorax tissue (BM = Bolaang Mongondowm; BT = Bitung; KTG = Kotamobagu and SA = Sanger)

2. CO1 gene amplification by PCR

CO1 gene amplification was performed in a modified PCR conditions. The initial PCR condition was 94 ^{0}C (50 seconds) to denaturation, 50 ^{0}C (4 minutes) to annealing, 72 ^{0}C (4 minutes) for extension and final extension of 4 minutes at the same temperature. CO1 gene amplification results mosquitoes in the early PCR conditions were not visualized in electrophoresis. Modification of PCR conditions were denaturation temperature 94 ^{0}C for 30 seconds, annealing temperature 49 ^{0}C for 4 minutes, elongation or extension: temperature 72 ^{0}C for 5 minutes and a final extension at the same temperature for 5 minutes. CO1 gene amplicons formed, as shown by the band formed in elketrogram electrophoresis results (Figure 5).

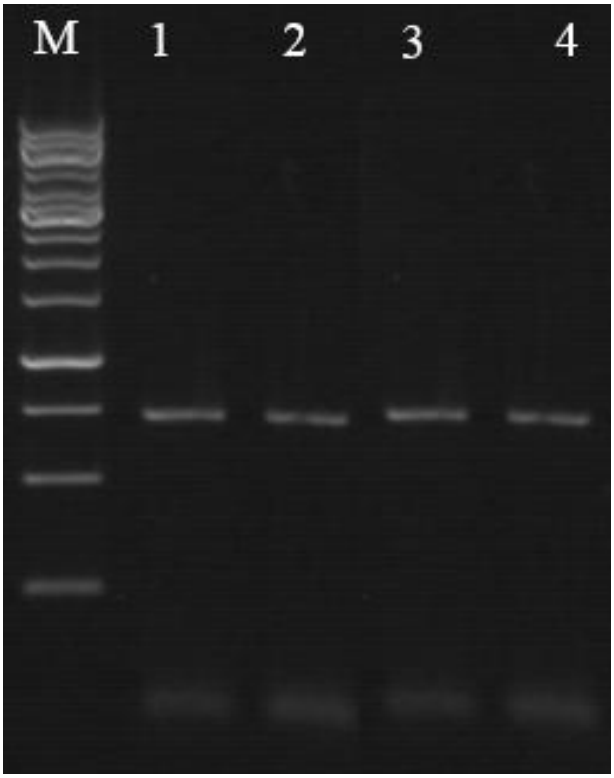


Fig 6. Elektrogram CO1 gene amplification of *Aedes* sp. CO1 gene is read on the 415 bp to 451 bp (M = marker 1000bp, 1 = Bm, 2 = Bt, 3 = Mktg, 4 = Sa)

3. Sequencing

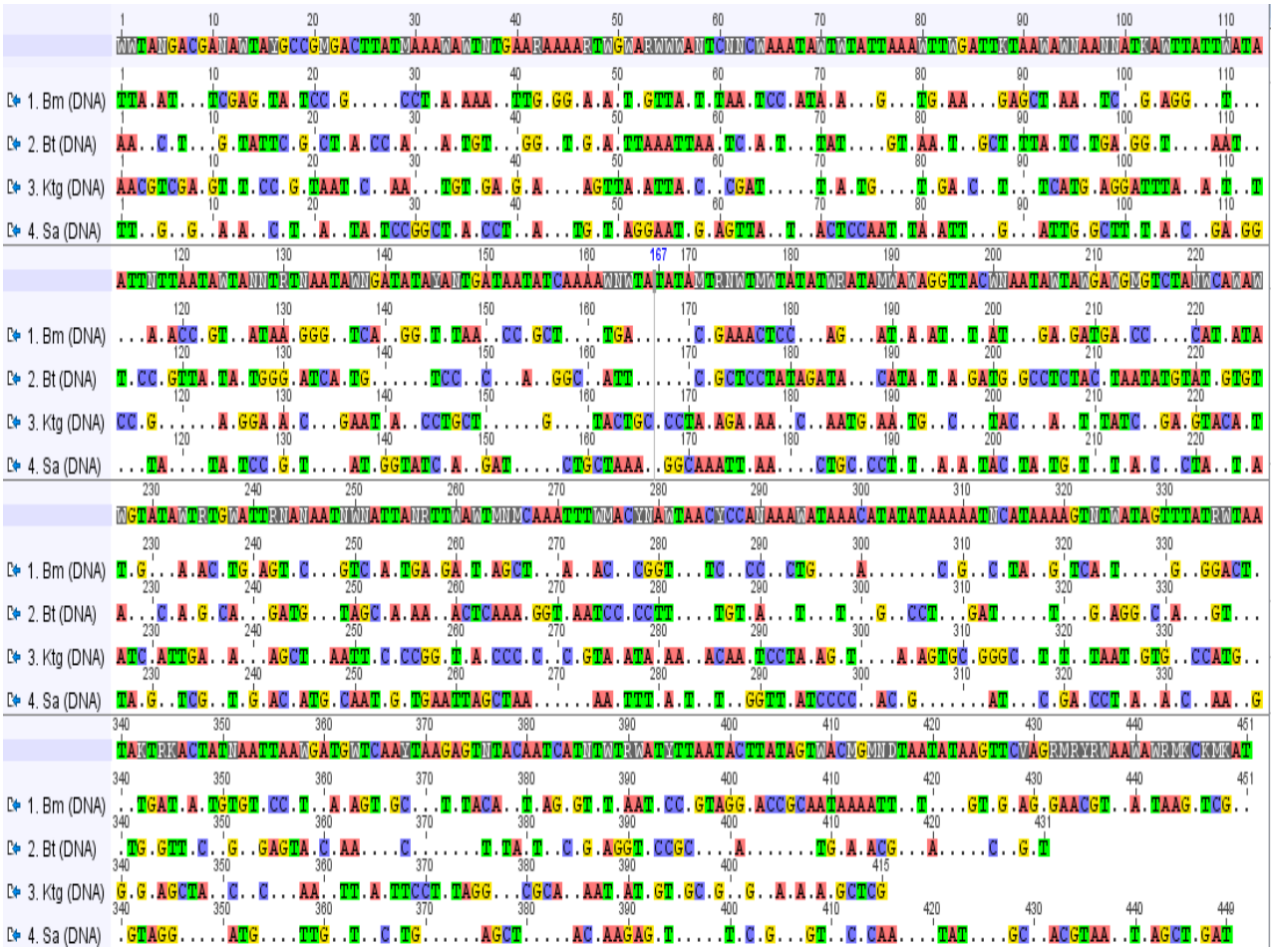
The sequencing results showed that the length of *Aedes* sp. CO1 gene sequences in North Sulawesi have had differences. CO1 gene sequences of Bolaang Mongondow, has a length of 451 bp, while Sanger has a length of 415 bp (Table 4). Thus, there has been a difference in the composition of nitrogen bases *Aedes* sp. CO1 gene from different regions of North Sulawesi. Differences in the composition of nitrogen bases, affect the amino acids encoded by the gene CO1 from each region.

Table 4: Characteristics of CO1 gene *Aedes* sp.

The origin of Samples	The Length of Nucleotide (base pair = bp)	% GC
Bm	451	29
Bt	431	29,7
Ktg	449	29,4
Sa	415	29,6

An alignment with geneous program, showing the location of the different sites, nitrogenous bases of four Aedes sp CO1 sequences of North Sulawesi. Thus, variations of the nitrogenous bases in the gene CO1 mosquito samples derived from Bolaang Mongondow, Bitung, Kotamobagu and Sanger is high. Differences in the composition of nitrogen bases, indicate mutations that have occurred.

Table 5: Alignment four CO1 sequences Aedes in North Sulawesi



Results of BLAST analysis of CO1 gene sequences obtained Bitung, Kotamobagu and Sanger had identical rates highest in *Aedes albopictus* (AY101847.1). CO1 gene sequences identical Bolaang Mongondow had the highest rate of *Aedes albopictus* (KP 896574.1). However, percent identics Aedes CO1 gene sequences of various sample locations in North Sulawesi has a difference (Table 6).

Table 6: Results of Analysis of Basic Local Alignment Test (BLAST)

Sekuens Gen CO1 Samples	E_{value}	Percent identical to similar sequences, recorded in NCBI gene bank	Species description recorded in the NCBI gene bank	Accesion Number
Bitung (Bt)	7e-6	92 %	Aedes albopictus	AY101847.1
Bolaang Mongondow (Bm)	0,00	99 %	Aedes albopictus	KP 896574.1
Kotamobagu (Ktg)	2e-7	85 %	Aedes albopictus	AY101847.1
Sanger (Sa)	7e-56	91 %	Aedes albopictus	AY101847.1

Differences percent identical CO1 gene sequences, the mosquitoes from four regions in North Sulawesi with similar sequences BLAST results, indicates the level of the base sequence similarity. Thus Aedes CO1 gene derived from Kotamobagu (identical percentage of 85%), has had a base sequence differences nigrogen by 15%, with most Identics sequences based on the results of BLAST. Reconstruction of the phylogeny tree is done using the Neighbor-Joining method with Genetic Distance Model: Tumura-Nei. Tree phylogeny formed indicates that the sequences CO1 Kotamobagu (KTG) closer to the sequences CO1 derived from Bitung (Bt), but has many many differences with sequences CO1 derived from Sanger (Sa) and Bolaang Mongondow (Bm) (Figure 5).

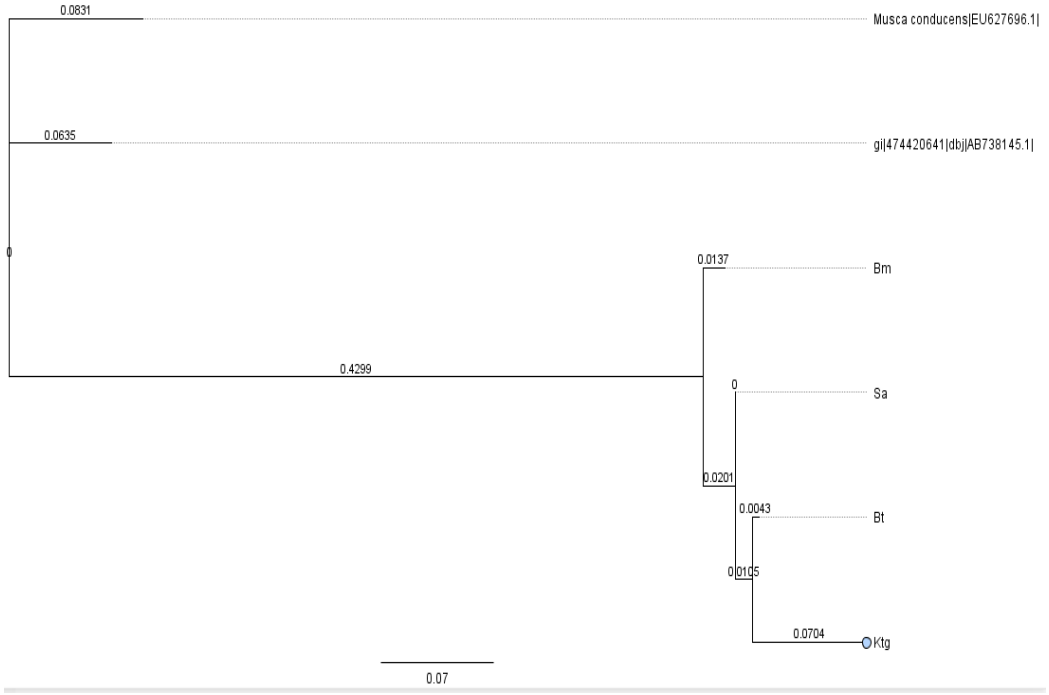


Fig 6:

Reconstruction of the phylogeny tree was also done online at the NCBI web site. Formed of phylogeny tree, put a mosquito of Bolaang Mongondow (query_146385), Bitung (query_198233), Kotamobagu (query_116033) and Sanger (query_175601) more synonymous with Aedes albopictus (Figure 6). Based on the formed phylogeny tree, indicating that the four samples of mosquitoes by gene CO1, has the closest relationship phylogeny, with *Aedes albopictus.*

Conclusion

From the results of this study concluded:

1. Character morphometry mosquitoes from Bolmong and Sanger showed the degree of similarity closest to forming a cluster. Morphometric characters of North Minahasa with Aedes most different from other areas.
2. Based on the phylogeny tree formed using CO1 gene, a sample Kotamobagu (KTG) closer to Bitung (Bt), but has had many differences with Sanger (Sa) and Bolaang Mongondow (Bm).
3. Based on the reconstruction of the phylogeny tree that forms showed that the four samples of mosquitoes that Bm, Bt, KTG and Sa have the closest relationship with *Aedes albopictus* phylogeny.

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