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Caroline Manuahe
Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia

Mokosuli Yermia Semuel
(1). Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia
(2). Laboratory of Bioactivity and Molecular Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia

Verawati Ida Yani Roring
Laboratory of Bioactivity and Molecular Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia

Correspondence
Mokosuli Yermia Semuel
(1). Department of Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia
(2). Laboratory of Bioactivity and Molecular Biology, Faculty of Mathematics and Natural Sciences, State University of Manado, Tondano, North Sulawesi, Indonesia

Optimization of DNA extraction and the position of mosquito Species from southeast minahasa in North sulawesi using NADH dehydrogenase Gene and Cytochrome oxidase Sub Unit 1 Gene

Caroline Manuahe, Mokosuli Yermia Semuel and Verawati Ida Yani Roring

Abstract
A research has been done that aimed to optimize the extraction of mosquitoes' DNA and to find out the characteristic of NADH dehydrogenase as well as cytochrome oxidase sub unit 1 (CO1) of mitochondrial genes to reveal the position of mosquito species from Southeast Minahasa. Mosquito samples retrieved from Southeast Minahasa on four villages in two subdistricts, namely Tombatu and Toluaan. The reserach was conducted in tree stages. The first stages was extraction of DNA, followed by NADH dehydrogenase gene and cytochrome oxidase sub unit 1 gene amplification and the last stage was electrophoresis and sequencing. The results showed that extraction of mitochondrial DNA from the mosquito using a fore leg organ with modified proteinase-K immersion for 24 hours was the best treatment. Mitochondrial DNA gene amplification N5 (NADH dehydrogenase) did not work while CO1 gene was successfully amplified on modified PCR condition. Mosquitoes' CO1 gene amplified with a length of 702 bp. The frequency GC is 228 or 33.4%. The most numerous amino acid codified are asparagine and serine. The posisition of mosquito samples based on CO1was nearest to *Armigeres subalbatus* [KJ 410334.1] with 89% similarity level, 11% differences of nitrogen base of CO1 gene, therefore the mosquito from Southeast Minahasa conctructed a new node at phylogeny trees.

Keywords: mosquito from Southeast Minahasa, NADH dehydrogenase gene (N4N, N5J), cytochrome oxidase sub unit 1 gene (CO1)

1. Introduction
Mosquitoes are the insect disease vector that is feared in tropical region. Mosquitoes belong to the order Diptera family Culicinae. The types of mosquitoes that can be vectors of diseases, among others: *Aedes* spp, *Culex* spp, *Anopheles* spp, *Mansonia* spp *and Armigeres* spp. *Anopheles* spp is a vector of malaria desease. Research conducted by Sembel *et al* (2009) [20] had found 23 species of *Anopheles* in North Sulawesi but only nine species were known to be active vectors of malaria disease. Malaria deasese caused by *Plasmodium spp* which transmited to human body by the *Anopheles.* Nevertheless, Sembel and his friends have not been doing research of *Anopheles* species residing in the sub-district Toluaan and sub district Tombatu, Southeast Minahasa, North Sulawesi. From the interviews with the local population, it has been revealed that during 1965 to 1980 malarial cases was very high in both areas.
The mosquito has high speed of genetic modification (Sembel, *et al.* 2009; Kaunang, 2014) [20, 14]. It caused mosquitoes' adaptation against various environmental pressure, to name, climate change and the use of insecticides. It also caused genetic variation of mosquitoes in an area (Kaunang, 2014) [14]. Genetic variation in the long run can lead to the emergence of a new mosquito species that are more adaptive. As a vector of disease, then it is more adaptive species require different methods for tackling the population with conventional methods. Thus, the research required the identification of mosquitoes species, to develop methods for tackling the mosquito in the future. The identification of mosquito species as malarial vectors would not give a significant effect on the efforts of mosquito population control methods, therefore the taxonomic status, ecology and behavior must be known accurately (Loaiza *et al.* 2013) [12].
Mosquito identification using morphological data requires taxonomic experts who are experienced or have a lot of mosquito identification studies based on morphological characters. Besides the identification of mosquito morphology requires specimens with

complete organs whereas organs, especially wings, proboscis and the antenna are very fragile and easily damaged when doing sample preparation. Other weaknesses of the mosquito samples generally preservation in ethanol or formalin which can change the color of mosquitoes' body so that it interfere the identification phase based on morphology (Jinbo *et al.* 2011) [10]. Another obstacle faced was the stage of mosquitoes metamorphosis that restrict male or female sample determinations and morphological characteristics of each stages.(Sembel, 2009) [20].

The determination of mosquitoes key organs to distinguish *Aedes, Culex, Anopheles, Armigeres* etc i.e. palpus, antennae, proboscis and the genitalia often cannot be used because of the specimens or samples preparation (Sirivanakarn, 1977; Sembel, 2009) [21, 20]. Therefore it takes accurate, acceptable and universal identification technique. The taxonomic status of the mosquitoes or the position of mosquito species can be ensured using genetic barcoding i.e. using gene markers on mitochondrial DNA. Marker genes or genetic barcoding widely used on animals are cytochrome oxidase sub unit 1 (CO1) and NADH dehydrogenase (NH) genes (Hebert *et al.* 2003) [9]. Species criptik genus *Anopheles* increases rapidly, causing a less accurate morphology based identification to determine the species of mosquitoes (Paredes-Esquivel *et al.* 2009; Bourke *et al*., 2010; Cienfuegos *et al*. 2011) [19, 3, 5]. Nevertheless, in previous study, DNA extraction of mosquitoes is not simple, high protein and RNA contamination contained in exoskeleton can damage DNA molecule, causing low purity and concentration of extracted DNA (Kaunang, 2014; Mokosuli, 2013) [14, 16].

Until 2010, the Southeast Minahasa Regency, still designated as region of endemic malaria in North Sulawesi (Depkes, 2012) [6]. The high incidence of clinical malaria cases in this area was certainly influenced by the abundance and diversity of mosquitoes species. Still a very little scientific data about the species of malarial vectors mosquitoes and other mosquito species in Southeast Minahasa. Thus the research required the identification of mosquitoes species, which are needed in the development of methods for handing the mosquitoes in the future. This study aimed to optimize mosquitoes DNA extraction, to reveal the characteristics of NADH dehydrogenase and cytochrome oxidase sub unit 1 genes and to find out the position of mosquito species in phylogeny trees.

2. Materials and Methods

Sample Collection

Mosquito samples retrieved from Southeast Minahasa, with the help of professional personnel (Figure 1). Samples taken is mosquito anthropogenic or attack humans. Samples were taken using nets or lamit. The samples obtained in the field with nets and direct preservation in ethanol 95% for 2 x 24 hours.

Research methods used in this research is descriptive method. Research phases shown on the flowchart of research (Figure 2).

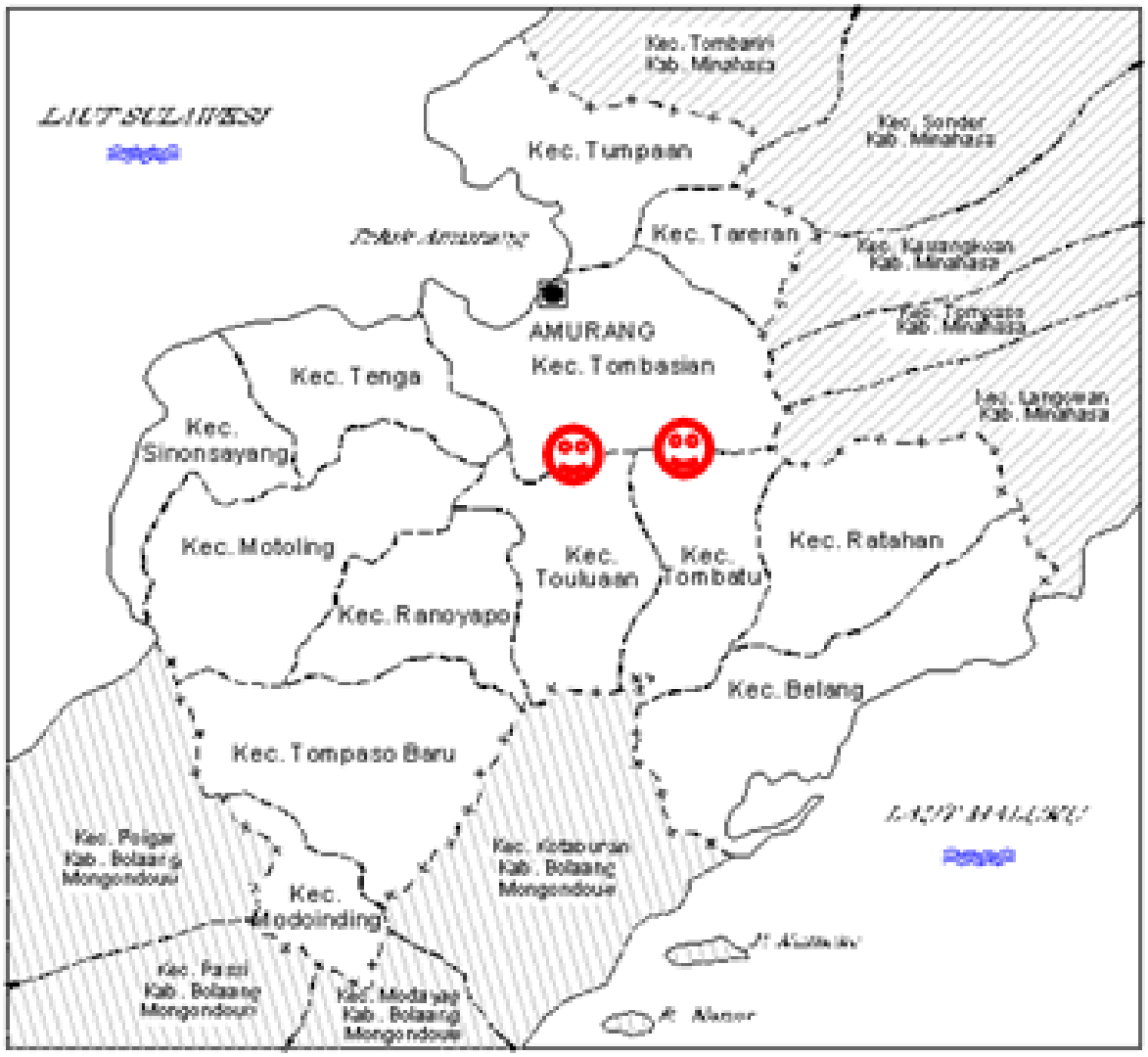


Fig 1: Red mark showed location of Sampling of Mosquito (Source of map : www.sulut.go.id)

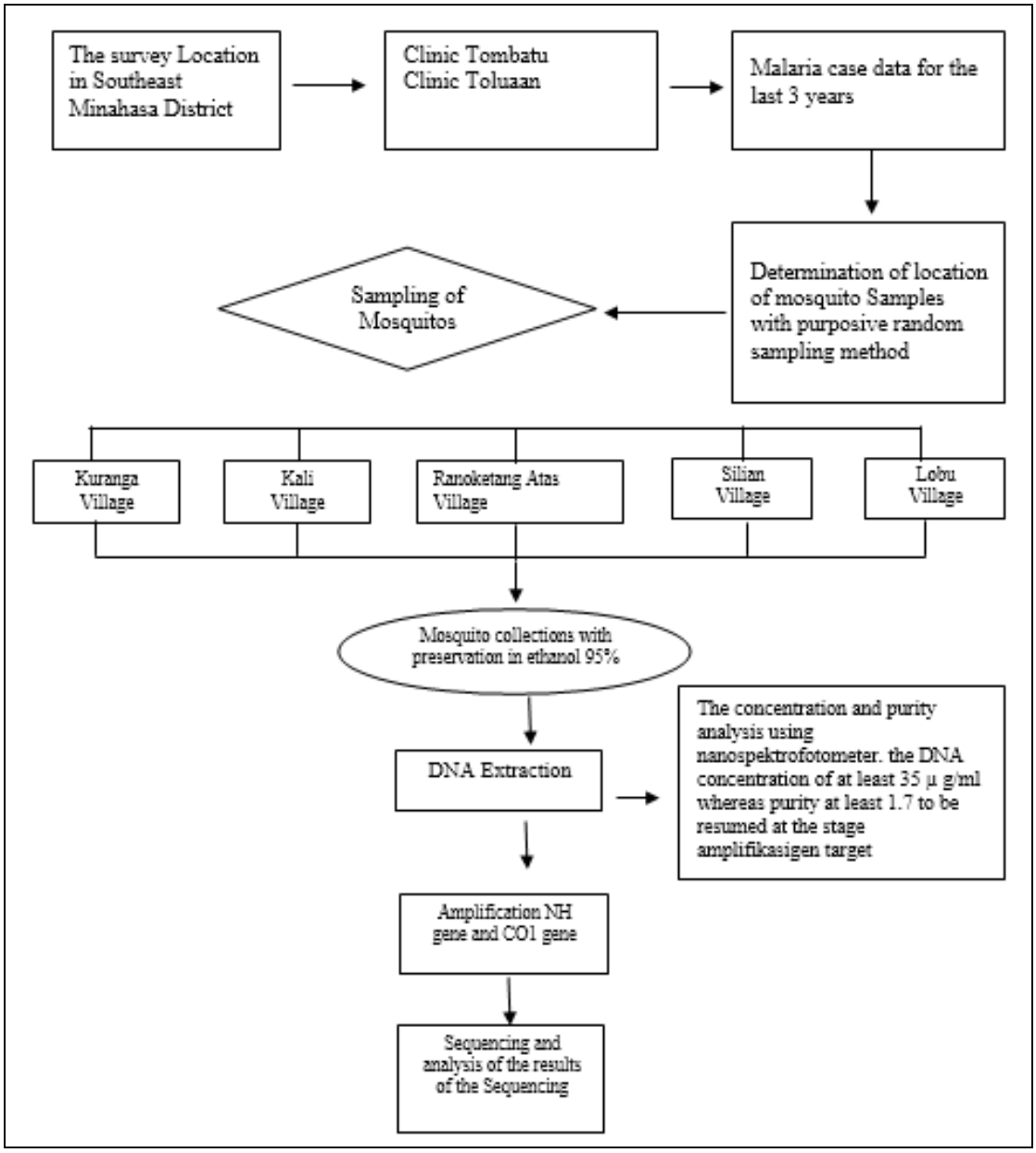


Fig 2: Research Flowchart

3. Research Procedure

3.1 DNA Extraction

Mosquito DNA extraction was performed according to protocol InnuPREP DNA Micro Kit (analytikjena). As many as 5 mg legs crushed mosquito using tissue ruptor apparatus in tube 5 ml. The tissue of mosquito from the legs in the tube then was placed in a termoblock apparatus 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 mosquitoes. 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 binding: 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 centrifuse, 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 (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 rotation per minute) for 1 minute. Furthermore, the spin filter is issued while elutionnya tube can be stored in a state suu -20 ^{o}C, do the analysis of the purity and concentration of extracted DNA using nano spectrofotometer.

3.2 PCR Amplification

Amplification of cytochrome oxidase sub unit 1 gene (CO1) and NADH dehydrogenase (NH) gene be used PCR Kit Solis Byodine (Biometra) with primer NADH dehydrogenase (N4N and N5J) primer cytochrome oxidase dam sub unit 1 (CO1) (Folmer *et al*, 1994) [8]. Primer of NADH dehidrogense consists of forward (N4N 8925) and reverse (N5J 7502). The molecular weight of N4N is 41,66, while N5J is 7502 28,93 (Table 1).

Table 1: Primer NADH dehydrogenase, and Cytochrome oxidase sub unit 1

No	Primers	Sequence	Reference
1	NADH dehydrogenase		
	N4N (forward)	5'GCTCATGTTGAAGCTCC3'	Kambhampati and Smith (1995)
	N5J (reverse)	5'CTAAAGTTGATGAATGAACTAAAG3'	
2	Cytochrome oxidase dam sub unit 1		
	LCO (forward)	5'GGTCAACAAATCATAAAGATATTGG3'	(Folmer *et al*, 1994).
	HCO (reverse)	5'TAAACTTCAGGGTGACCAAAAAATCA3'	

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 ºC for 50 seconds, the extention of 72 ºC for 50 minutes with the number of cycles as much as 35 times and the last stage of the final extention 72 ºC 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, 2 µl template DNA, and as many as 7.5 µ l ddH2O with a total volume of 25 µ l. Time required for PCR method for 1 hour 40 minutes.

3.3 Electrophoresis

Visualization of PCR products is carried out using automatic electrophoresis Qiaexel. Screening DNA Qiagen Kit is used (Qiaxel). This process is used in the alignment of marker 15 bp-5000 bp and size marker 100 bp-1500 bp. Cartridge is inserted as a place of detection of DNA, while the wash buffer required cartridge in detecting DNA. One time running qiaxcel there are 12 samples, one of which is the size marker/marker ladder (at normal electrophoresis).

3.4 The sequencing and sequence analyses

The amplicons of CO1 gene are sent to the service provider sequencing ABI Pro Singapore. Analysis of sequencing products generated were then analyzed using software Geneous 5.6.4 (Drummond *et al.* 2012) [7]. Furthermore, CO1 gene sequence of mosquitoes were aligned with National Center of Biotechnology Information (NCBI) BlastN database by using Basic Local Alignment Search Tools (BLAST) algoritm on http://blast.ncbi.nlm.nih.gov/Blast.cgi, followed by construction phylogeny online (Altschul *et al.* 1997; Waiho *et al*, 2013) [1, 23].

4. Results and Discussion

4.1 Description of sampling

A total of 150 mosquito samples obtained from four villages in the district of Souteast Minahasa, North Sulawesi (Table 1). Geographical conditions of these villages have a lot of flood areas pools or talaga (local language)), the rice fields, puiddle area, a small river and in the village of Kali, there is a small lake called Buleleng. From the data of Pusat Kesehatan Masyarakat (community health centers) sub district of Toluaan found malaria cases up to December 2014 amounted to 6 positive cases of Malaria. Catching mosquitoes is done in the afternoon at 18:30 hourss until 19:00 hours. Mosquitoes were captured, selected to have a complete body structure, especially still has a head, thorax, legs and abdomen then preserved in ethanol 95% (Table 2). After 24 hours, in ethanol, based on morphological observation using a stereo microscope, has not shown any change in body color and shape.

Table 2: Mosquito, Location and habitat Characteristics

No	Villages	Amounts of individual mosquitoes	Habitat
1	Kuranga	30	close to a small river and rice fields
2	Silian	30	close to the rice fields, dense of vegetation
3	Kali	30	close to the small lake, with forest
4	Ranoketang atas	30	close to the rice fields, dense of vegetation
5	Lobu	30	Close to the forest

4.2 DNA Extraction

The extraction of DNA using the organs of the thoracic, abdominal, head and legs of mosquitoes showed different concentrations and purity. Concentration and purity is best obtained from extraction of the mosquito legs i.e. each 1.67 and 55,6 µg/ml. According to the raw purity of DNA extraction results to be able to proceed at amplification stage, at least had 1.7 purity and concentration 35 µl/ml. Thus mosquito DNA extract purity results are still under minimal standard except the DNA extract from hind legs of mosquito (Table 3).

Table 3: The purity and concentration of mosquito DNA extraction with InnuPrepp Analyticjena Kit (Biometra, Germany) with the modifications of protocol by Researchers

No	Mosquito organ	Purity (A260/A230)	Concentration (µl/ml)	Modifications of protocol by Researchers	Conclusion
1	Forelegs	1,20	9,20	According Protocol	Not continue at PCR stage
	Forelegs	1,67	50,00	Submersion in Proteinase-K at 24 hours	Continue at PCR stage
2	Hindlegs	1,00	6,40	According Protocol	Not continue at PCR stage
	Hindlegs	1,34	18,00	Submersion in Proteinase-K at 20 hours	Not continue at PCR stage
3	Thoraks	0,50	5,50	According Protocol	Not continue at PCR stage
	Thoraks	1,30	12,00	Submersion in Proteinase-K at 18 hours	Not continue at PCR stage
4	Abdomen	0,70	43,00	According Protocol	Not continue at PCR stage
	Abdomen	1,20	23,00	Submersion in Proteinase-K at 10 hours	Not continue at PCR stage
5	Head	0,50	2,50	According Protocol	Not continue at PCR stage
	Head	1,40	7,80	Submersion in Proteinase-K at 8 hours	Not continue at PCR stage

4.3 Amplification and visualization of Amplicon

Based on the purity and concentration of DNA, DNA extraction results from the forelegs is continued on the stage of amplification of CO_1 gene and NH gene. The NH gene is not amplificated properly, confirm of visualization with automatic electrophoresis qia exel, the band unreadable or not formed. Amplification using CO_1 gene after visualitation with automatic electrophoresis showed positive results because it formed a stable bands. Thus only the CO_1 gene amplification was successful while NH has not shown positive results to be able to proceed at this stage of the sequencing.

Both of CO1 gene and NH gene amplified with modification PCR conditions. The initial PCR conditions are 94 ^{0}C (50 seconds) for denaturation, 50 ^{0}C (4 minutes) for annealing, 72 ^{0}C (4 minutes) to extension and 4 minute final extension with the same temperature. CO_1 gene amplification results and of the gene N5 mosquitoes on the initial PCR conditions are unreadable on electrophoresis. Modification of the conditions of the PCR denaturation was performed: 94 ^{0}C temperature 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. CO_1 Amplicon formed indicated by the chromatogram of electrophoresis in the columns A2, A3 and A9. CO_1 gene amplification of mosquitoes that is formed has a length of 702 bp while the N5 gene succes to amplified (Figure 3).

Fig 3: Visualizes CO1 gene amplicon results with automatic electrophoresis qia exel. The columns A2, A5 and A9 column is the CO1 gene amplicons of mosquito were amplified. A12 is alignment marker 15 bp-5000 bp (Qiagen, USA).

Concentration amplicons of CO1 gene that is formed or the result of PCR is 2.53 ng/µl (Figure 4). CO1 gene amplicons visualization results with good bands compared to controls showed the success of the target gene amplification, while the concentration of CO_1 gene amplicons were amplified showed a success rate of CO_1 gene amplification.

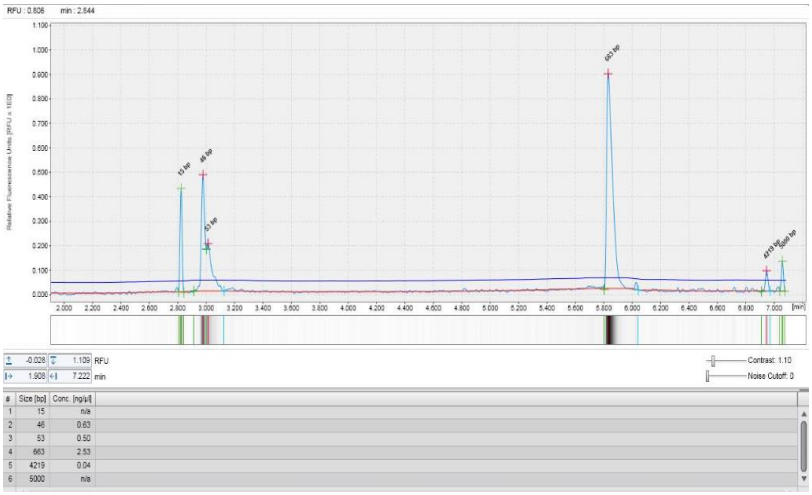


Fig 4: Concentration amplicons of mosquioto CO1 gene results from PCR which is read by automatic electrophoresis qia exel.

4.4 Sequencing

Data sequencing process results in the form of files, *seq*. from First BASE Singapore analyzed using 5.4.3 Geneous Program (Drummond *et al*. 2012) [7], to get the base sequence of the gene CO1 mosquitoes. The base sequence of the mosquito CO1 gene in FASTA format is then used for the analysis of the NCBI BLAST alignment method. Mosquito sample sequence has a length of 683 bp (Figure 5)

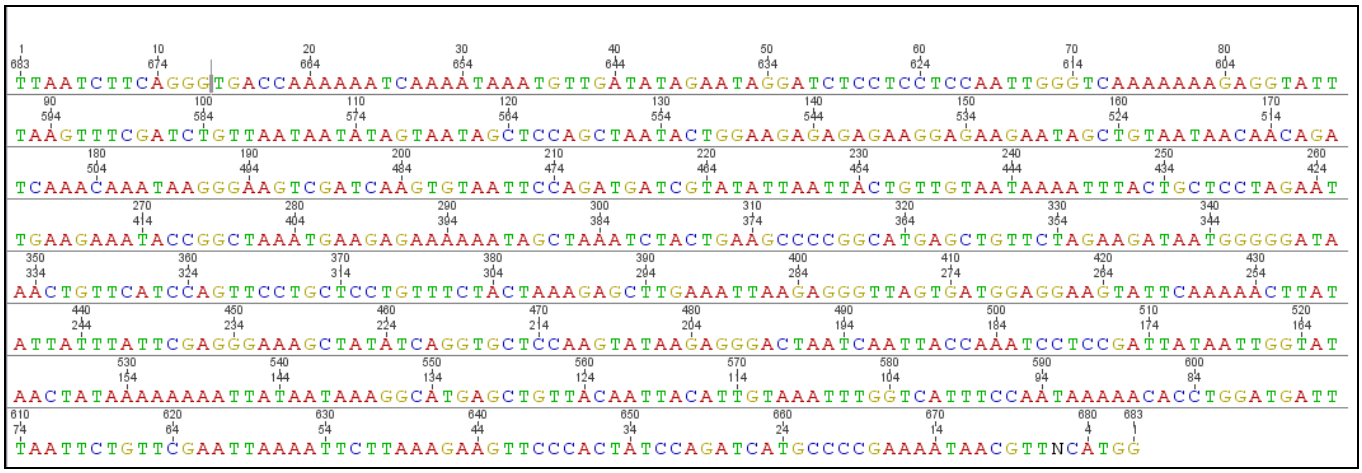
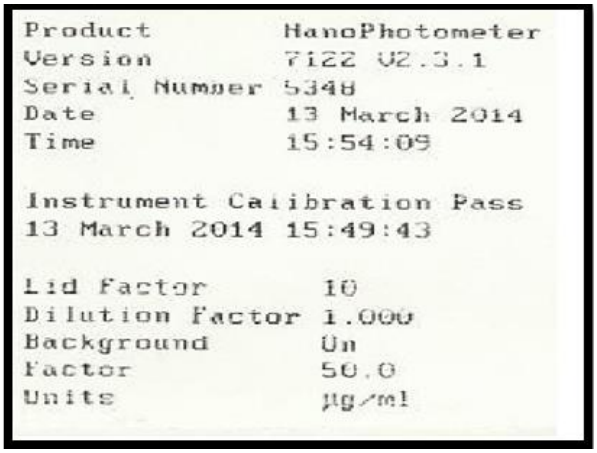


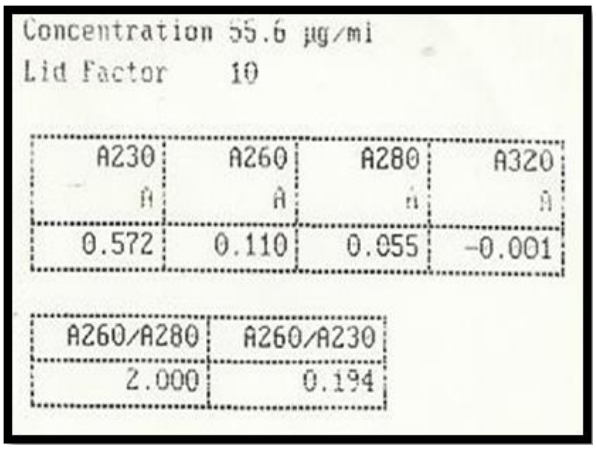
Fig 5: Nitrogen base sequence of CO1 gene of Mosquito samples from Southeast Minahasa analysis results with geneous Program 5.4.3.

Frequency of nitrogen bases adenine 260 (38.1%), Cytosine 103 (15.1%), Guanine 125 (18.3%) and Thymine 194 (28.4%). One nucleotide (0.1%) is not legible type of nitrogenous bases. Guanine and Cytosine frequency of 228 or 33.4% of the total nucleotide CO1 gene of mosquitoes. Amino acids serine and asparagine is the most abundant amino acids encoded (Figure 6 and Appendix).

Appendix 1

Then the samples are tested for purity by Using nano spectofotometer DNA purity is at a concentration of 55.6 mL / ml with a ratio A260 / A280 2.00.





Appendix 2.

CCATGNAACGTTATTTTCGGGGCATGATCTGGATAG
TGGGAACTTCTTTA
AGAATTTTAATTCGAACAGAATTAAATCATCCAGGT
GTTTTTATTGGAAA
TGACCAAATTTACAATGTAATTGTAACAGCTCATGC
CTTTATTATAATTT
TTTTTATAGTTATACCAATTATAATCGGAGGATTTGG
TAATTGATTAGTC
CCTCTTATACTTGGAGCACCTGATATAGCTTTCCCTC
GAATAAATAATAT
AAGTTTTTGAATACTTCCTCCATCACTAACCCTCTTA
ATTTCAAGCTCTT
TAGTAGAAACAGGAGCAGGAACTGGATGAACAGTTT
ATCCCCCATTATCT
TCTAGAACAGCTCATGCCGGGGCTTCAGTAGATTTA
GCTATTTTTTCTCT
TCATTTAGCCGGTATTTCTTCAATTCTAGGAGCAGTA
AATTTTATTACAA
CAGTAATTAATATACGATCATCTGGAATTACACTTG
ATCGACTTCCCTTA
TTTGTTTGATCTGTTGTTATTACAGCTATTCTTCTCCT
TCTCTCTCTTCC
AGTATTAGCTGGAGCTATTACTATATTATTAACAGAT
CGAAACTTAAATA
CCTCTTTTTTTGACCCAATTGGAGGAGGAGATCCTAT
TCTATATCAACAT
TTATTTTGATTTTTTGGTCACCCTGAAGATTAA

Length: 683

	Freq	%	%	non-ambig
A	:	260	38.1%	38.1%
C	:	103	15.1%	15.1%
G	:	125	18.3%	18.3%
T	:	194	28.4%	28.4%
N	:	1	0.1%	
GC	:	228	33.4%	33.4%

Rough Tm: 77.6 °C

Amino Acids & Codons Options
Freq %

A	: 2 0.9%
C	: 13 5.8%
D	: 3 1.3%
E	: 4 1.8%
F	: 3 1.3%
G	: 7 3.1%
H	: 3 1.3%
I	: 18 8.0%
K	: 24 10.6%
L	: 3 1.3%
M	: 5 2.2%
N	: 26 11.5%
P	: 3 1.3%
Q	: 0 0.0%
R	: 16 7.1%
S	: 37 16.4%
T	: 6 2.7%
V	: 5 2.2%
W	: 6 2.7%
Y	: 19 8.4%
*	: 23 10.2%
?	: 1

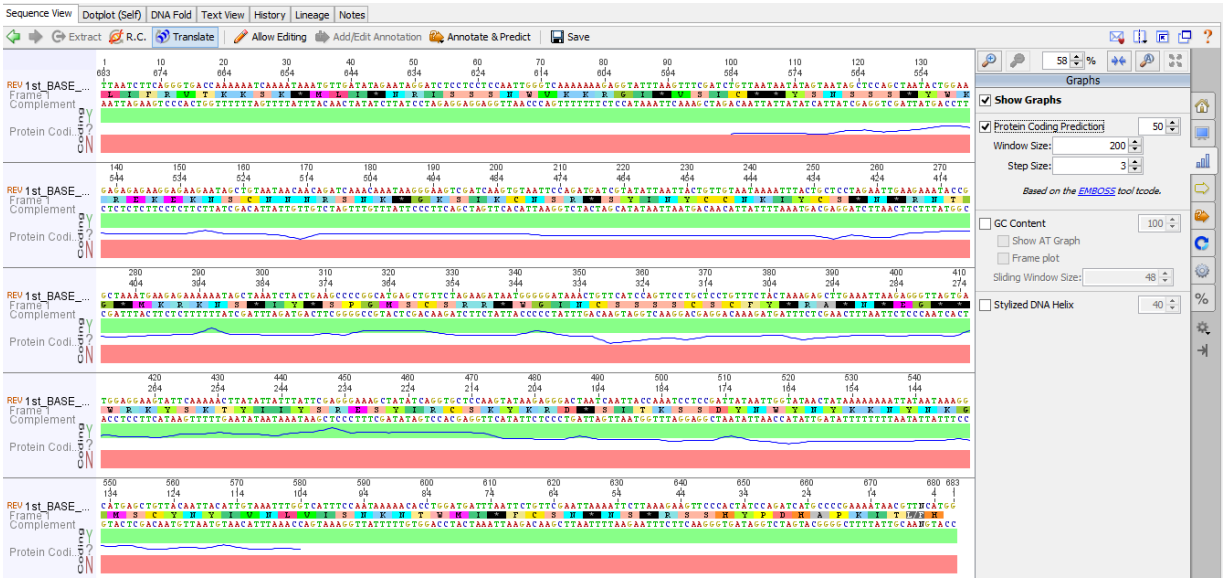
Codon AA % of AA Freq

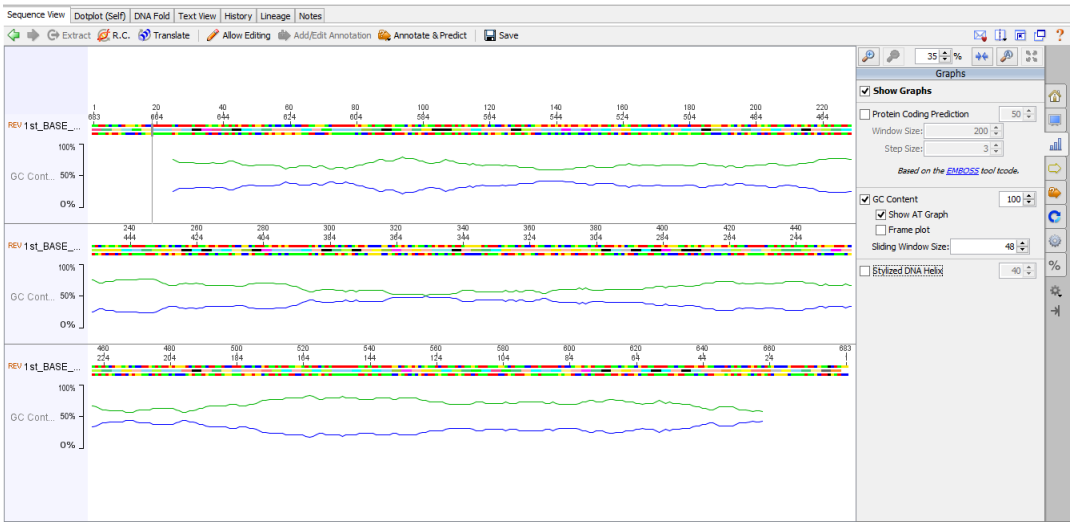
GCA	A	0.0%	0	
GCC	A	50.0%	1	
GCG	A	0.0%	0	
GCT	A	50.0%	1	
TGC	C	23.1%	3	
TGT	C	76.9%	10	
GAC	D	33.3%	1	
GAT	D	66.7%	2	
GAA	E	25.0%	1	
GAG	E	75.0%	3	
TTC	F	100%	3	
TTT	F	0.0%	0	
GGA	G	0.0%	0	
GGC	G	42.9%	3	
GGG	G	28.6%	2	
GGT	G	28.6%	2	
CAC	H	33.3%	1	
CAT	H	66.7%	2	
ATA	I	16.7%	3	
ATC	I	33.3%	6	
ATT	I	50.0%	9	
AAA	K	62.5%	15	
AAG	K	37.5%	9	
CTA	L	0.0%	0	
CTC	L	0.0%	0	
CTG	L	0.0%	0	
CTT	L	0.0%	0	
TTA	L	33.3%	1	
TTG	L	66.7%	2	
ATG	M	100%	5	
AAC	N	23.1%	6	
AAT	N	76.9%	20	
CCA	P	33.3%	1	
CCC	P	33.3%	1	
CCG	P	33.3%	1	
CCT	P	0.0%	0	
CAA	Q	0.0%	0	
CAG	Q	0.0%	0	
AGA	R	62.5%	10	
AGG	R	37.5%	6	
CGA	R	0.0%	0	
CGC	R	0.0%	0	
CGG	R	0.0%	0	
CGT	R	0.0%	0	
AGC	S	21.6%	8	
AGT	S	8.1%	3	
TCA	S	13.5%	5	
TCC	S	37.8%	14	
TCG	S	13.5%	5	
TCT	S	5.4%	2	
ACA	T	0.0%	0	
ACC	T	66.7%	4	
ACG	T	16.7%	1	
ACT	T	16.7%	1	
GTA	V	20.0%	1	
GTC	V	40.0%	2	
GTG	V	20.0%	1	
GTT	V	20.0%	1	
TGG	W	100%	6	
TAC	Y	36.8%	7	
TAT	Y	63.2%	12	
TAA	*	65.2%	15	
TAG	*	13.0%	3	
TGA	*	21.7%	5	

Codons with ambigs 1

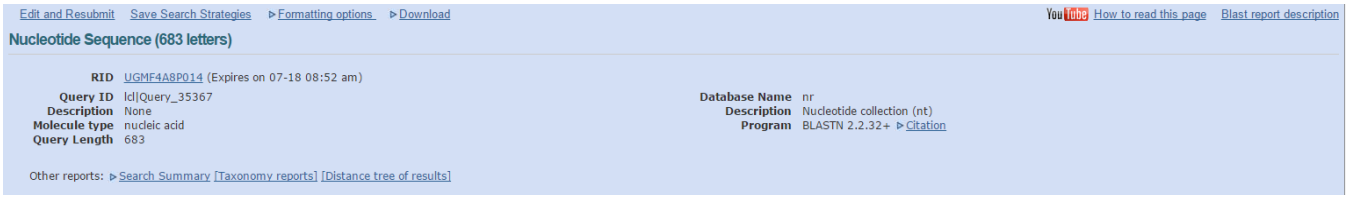
Kode Asam Amino :

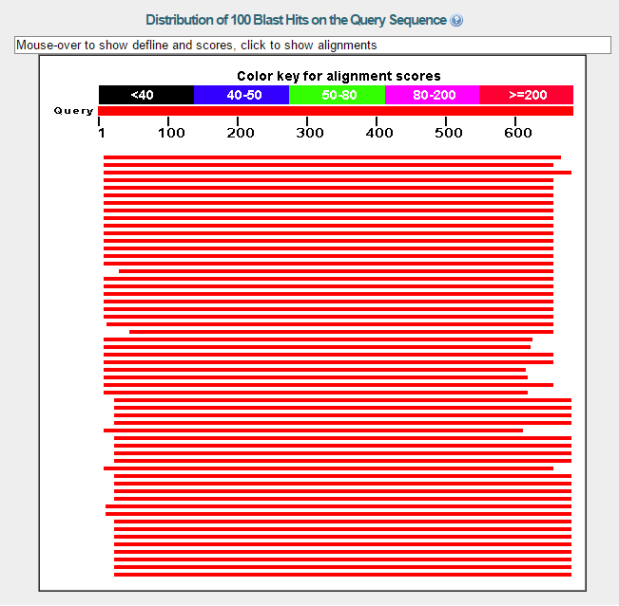
G	-	Glycine (Gly)
P	-	Proline (Pro)
A	-	Alanine (Ala)
V	-	Valine (Val)
L	-	Leucine (Leu)
I	-	Isoleucine (Ile)
M	-	Methionine (Met)
C	-	Cysteine (Cys)
F	-	Phenylalanine (Phe)
Y	-	Tyrosine (Tyr)
W	-	Tryptophan (Trp)
H	-	Histidine (His)
K	-	Lysine (Lys)
R	-	Arginine (Arg)
Q	-	Glutamine (Gln)
N	-	Asparagine (Asn)
E	-	Glutamic Acid (Glu)
D	-	Aspartic Acid (Asp)
S	-	Serine (Ser)
T	-	Threonine (Thr)

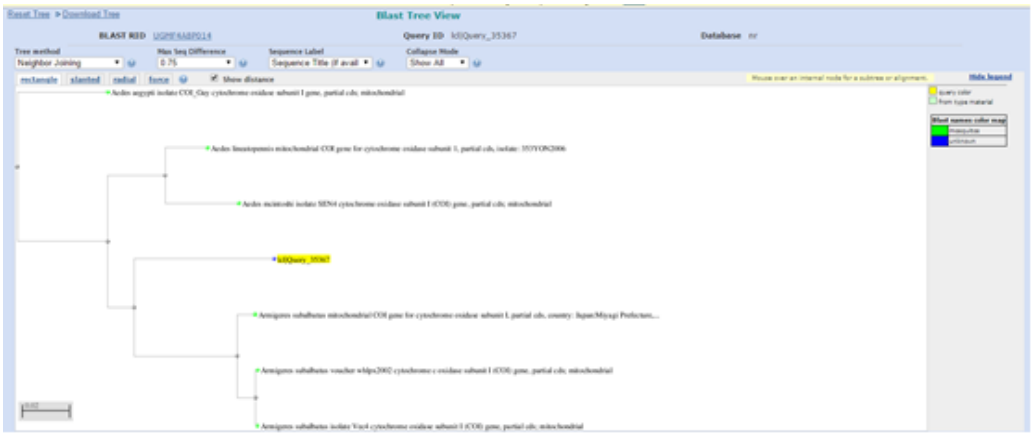




BLAST Analysis and Phylogeny trees







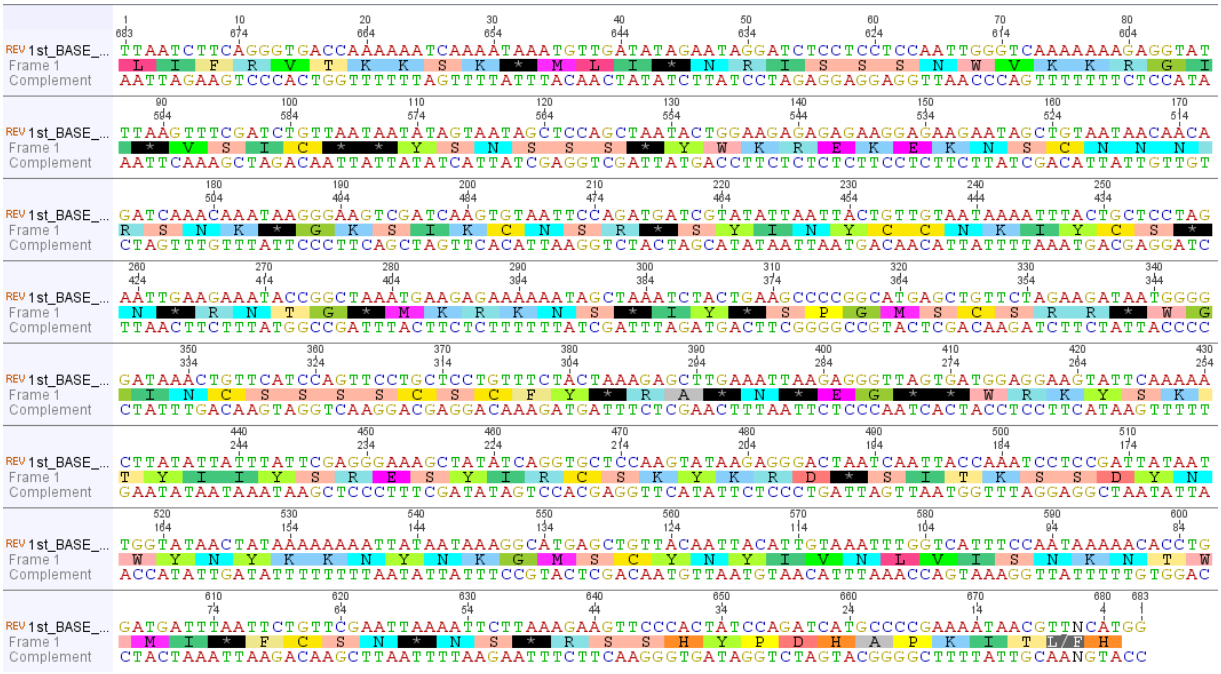


Fig 6: Amino acids encoded in the gene CO1 mosquito from Southeast Minahasa.

5. Discussion

Of all the organs of mosquitoes, DNA from forelegs more extracted using Innu PREP DNA Micro Kit (analytikjena), compared with organs of the head, thorax and abdomen. Therefore N5 and CO1 gene as the target gene amplification and sequencing of the gene is located in the mitochondrial genome then the best organs for extracted should have a lot of muscle tissue. In the muscle cells have many mitochondria organelles (Bruce *et al*. 2014) [12]. Legs are locomotor from mosquitoes that have a lot of muscle tissue. Tissue on insects are inside of exoskeleton. Exoskeleton contains a lot of chitin molecules and another molecule that can damage DNA extraction results. DNA extraction on mosquitoes, flies, honey bee and damselfly previously done requires a lot of protocol modification kit to obtain the expected purity and concentration (Mokosuli, 2013; Ngangi, 2014, Kaunang, 2014) [16, 17, 14]. Soaking proteinase-K modified for longer (24 hours) than that of the Protocol of the kit (30 minutes) turned out to be destructive molecules contamintas which has the structure of a protein which increases concentration and purity of DNA extraction of total yield. In this research, modification of the conditions of the PCR amplification of a gene also increased the success of CO1 gene but not on the of N5 gene.

Species identification using CO1 gene managed to identify mosquito species from 16 species of mosquito that used in Singapore (Chan *et al*. 2014) [4]. CO1 gene rDNA and internal transcribed spacers of second (ITS2) most used to identify the mosquito *Anopheles* genera and *Mansonia* genera (Loaiza *et al*. 2013; Wilkerson *et al*., 2005; Marrelli *et al*., 2006; Li and Wilkerson, 2007; Rueanghiran *et al*. 2011) [12, 23, 15, 13, 18] From this research it is known that mitochondria DNA extraction and amplification of the mosquito cannot be done on the basis of the Protocol guide kit is used. Needed special treatment starting phase sample preparation, target organs for extraction to the amplification of the target gene.

Results of the analysis of alignment with the BLAST method, mosquito CO1 sequences of Southeast Minahasa has the closest similarity level that is 89% (E value 0.0) with Armigeres subalbatus [KJ410334.1]. Construction phylogeny using Neighbor Joining method (1000 x bootstrap) is done online at http://blast.ncbi.nlm.nih.gov/Blast.cgi. A total of 100 sequences with the closest resemblance to the results recorded in the NCBI BLAST, used to construct phylogenetic trees. Sequences used to have the lowest similarity 86% and the highest 90%. CO1 position mosquitoes (query_35367) or samples of mosquitoes from the District Tombatu Southeast Minahasa forming its own node with the closest kinship to the genera of *Armigeres* (Figure 9). This result is in contrast to the notion that a sample of mosquitoes captured by using the bait is of the genera of *Anopheles* (Figure 9).

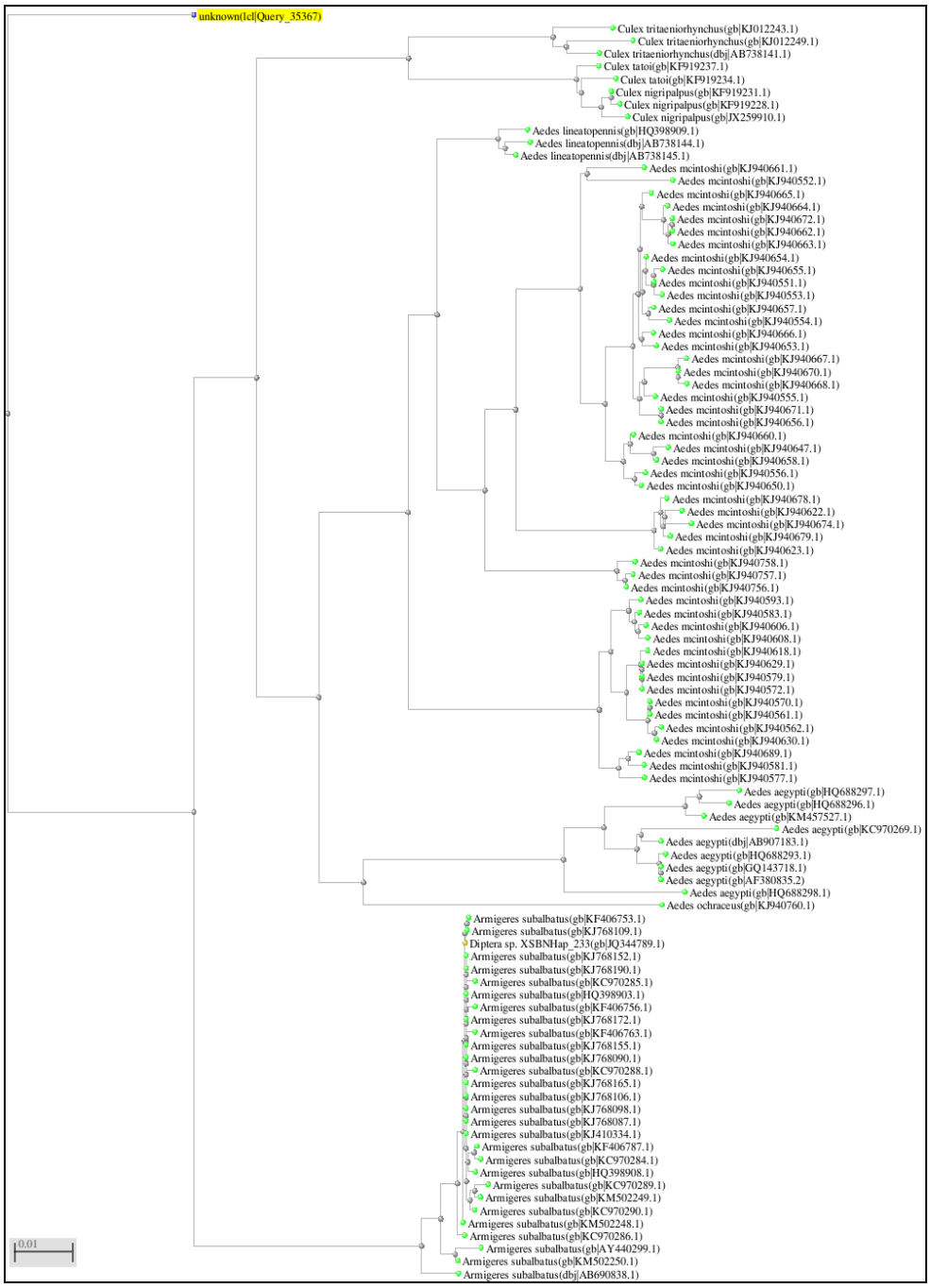


Fig 9: Phylogenetic tree based on CO1 gene sequences (query_35367) of Mosquitoes from Southeast Minahasa.

6. Conclusion

1. Extraction of mitochondrial DNA from mosquitoes with the best concentration and purity, using organ forelimb with immersion proteinase-K for 24 hours.
2. Modification of the PCR conditions in gene amplification process does not succeed at the NH gene, but succeeded in CO1 gene, evidenced by visualization of chromatograms electrophoresis.
3. CO1 gene of mosquitoes from Southeast Minahasa amplified with a length 702 base pair, while GC frequency 228 or 33.4%. Amino acids the most codified are asparagine and serine.
4. Position the mosquito samples based on the CO1 gene phylogeny tree compared to 100 sequences from BLAST results in NCBI, closer to *Armigeres subalbatus* [KJ410334.1] with 89% similarity level. Nevertheless, based on the CO1 gene, mosquito samples from Minahasa southeast to form a new node.

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