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Phylogeny reconstruction of Gerridae from River Ratatotok and River Talawaan North Sulawesi Based on Mitochondrial DNA 16 S RNA Gene

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Abstract
Has conducted research that aims to get the position species based Gen 16 S RNA Mitochondrial DNA of Water Striders in Talawaan River and Ratatotok River, North Sulawesi. Extraction of total DNA using DNA Innuprep Micro KIT (Analytic Jena, Germany). 16 S RNA gene [16sA (CTG 5'CGC AAA TTT AAC AAC AT 3'); 16sB2 (5'TTT AAT ACA TCG AGG CCA 3')]. 16 S RNA gene amplification using Master Cyler with Rotor Gene Q Series Software 2.0.3. PCR conditions that denaturation initiation stage 94 ^{0}C for 3 minutes, denaturation stage 94 ^{0}C for 30 seconds, annealing step 52 ^{0}C for 50 seconds, extension stage 72 ^{0}C for 1 min and final extension 72 ^{0}C for 5 minutes. Visualization amplicon using Automatic Electrophoresis Qiaxel. Sequencing was performed using the ABI PRISM 3730xl Genetic Analyzer Develop by Applied Biosystems, USA. Construction phylogeny using MEGA 6.0 program. Based on the phylogeny tree formed from Talawaan Water striders have closest kinship with *Limnoporus esakii* while Water striders of Ratatotok has the closest kinship with *Cylindrostethus costalis.*

Keywords: Water Striders, Ratatotok, Talawaan, 16 S RNA

1. Introduction
Until now it has known 67 genera and 751 species of water striders (Hemiptera: Gerridae), 62 genera and 700 species of which live in freshwater ((Polhemus and Polhemus 2008) [10]. In Peninsular Malaysia and Singapore reported 18 genera and 43 species anggang- anggang (Cheng *et al*., 2001) [4], while on the island of Sulawesi and Buton reported there are 20 species of gerridae, with seven new species (Chen and Nieser 1992) [3]. In Java, there are eight species gerridae (Breddin 1905) [16].
Some species gerridae been used as bio-indicators in aquatic ecosystem.Gerris spinolae is one effective insect bio-indicators for monitoring anthropogenic pollution at several pools (Pal *et al*. 2012) [11]. Gerris argentatus, G. odontogaster, G. lateralis, and G. thoracicus act as bio-indicators of heavy metals (Nummelin *et al*. 1998) [8]. Besides gerridae have been reported, allegedly gerridae other species can also be used as bio-indicators in accordance to insect habitat, water has a vital role in aquatic ecosystems, ecological role of these caused some species of insects can be used as bio-indicators of water contamination (Jana *et al*., 2009; Popoola and Otalekor 2011; Setiawan, 2015) [9, 18, 19]. In Minahasa, North Sulawesi, the most species found are Gerris remigis (Lee and Chang, 1974) [20].
Water Striders found in the River melimpiah Talawaan and Raratotok River. Talawaan river is located in North Minahasa Regency Ratatotok while the river is located in Southeast Minahasa Regency. Although far apart geographically, both rivers have in common. Both the river becomes a dumping ground for the rest of the community gold mining processing. Processing artisanal mining using mercury and cyanide. Aquatic insects have a very high sensitivity to the content of heavy metals in the water. Insects tend to adapt well to their environment to survive. Previous research that has been conducted, found differences in morphology water striders Talawaan coming from the river and from the river Ratatotok. Nevertheless morphological differences are not strong enough to ensure water striders that comes from the river and the river Ratatotok Talawaan has different species.
Molecular identification using cytochromec oxidase subunit I gene (COI) has been widely used as a molecular barcode (Hebert *et al*. 2003) [21]. CO1 is represented if of all protein-coding genes of mitochondrial DNA. COI proven intraspecific variation is low, but the interspecific divergence is high among taxa adjacent (closely allied taxa (Ward *et al*., 2005; Hajibabaei *et al*. 2006) [22, 28].

The identification of molecular insect uses gene CO1 in North Sulawesi has been conducted on Gerridae Sea (Warouw *et al*. 2016 [23]; Aedes sp (Kaunang *et al*. 2014) [24], Anopheles (Manuahe *et al*. 2016) [25], Endemic Honey Bee *Apis dorsata* Binghami (Mokosuli *et al*. 2013) [26], Flies (Assa *et al*. 2015) [27]. Identification of molecular water striders in both these rivers besides ensuring notch species also provide scientific data in response to the waste water striders gold processing.

2. Materials and method

2.1 Sample

Imago Water Striders collected Talawaan River and the River Ratatotok. Samples were obtained from the river that has been contaminated by waste processing traditional gold. All samples were preserved with 95% ethanol in a sample bottle. After 24 hours were transferred to the new sample bottles that contain 95% ethanol by the same volume. Prior to the extraction of DNA, each sample is analyzed using a microscope morphology hirox KH8700. Photos morphology confirmed the experts and compared online at data centers tropical insects (http://www.insectidentification.org/).

2.2 Research Procedure

2.2.1 DNA Extraction

Water Striders DNA extraction was performed according to protocol InnuPREP DNA Micro Kit (analytikjena) with some modification. As many as 5 mg hind legs crushed Water Striders using tissue ruptor apparatus in tube 5 ml. The tissue from the legs in the tube then was placed in a termoblock with a temperature of 56 °C for 30 for minutes. The lysis stage : added 200 µ l of Lysis buffer 20 µ l TLS and proteinase K into the tube containing the leg chain Water Striders. 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 centrifused, the tube replaced with new tube, whereas spin filter fixed and go on next stage. Stage of washing: washing solution added HS 400 µ l (in new tube), centrifused at 10,000 g (12,000 rpm) for 30 seconds. The tube replaced spin the filter anyway. Add washing solution MS 750 µ l, centrifuged at 10,000 g (12,000 rpm) for 1 minute. Move on a new tube, spin filter then centrifused at 10,000 g (12,000 rpm) for 30 seconds. The final stage, Elution phase move the washing step results in a new tube while the spin filter fixed. Add 100 mL of elution buffer, then incubated for 5 minutes followed by centrifuged at 6000 g (800 rpm) for 1 minute. Furthermore, the spin filter is issued while elutionnya tube can be stored in a state suu -20 ºC, do the analysis of the purity and concentration of extracted DNA using nano spectrofotometer.

2.2.2 PCR Amplification

Gen 16 S RNA primer used is C1-J-2183 (5'CAA CAT TTA TTT TTT TTT TGA GG 3'), TL2-N-3014 (5'AAT TCC GCA CAT CTA ATC TGC ATT A 3') (Simon *et al*., 1994) [29]. 16 S RNA gene amplification by PCR (polymerize Chain Reaction) using Qiagen Rotor Gene Q Series. DNA extraction ditambahkanTop Qiagen Taq Master MixKit 50 mL (Table 1). Then the sample is inserted in the Rotor Gene Q Series cube, and run with the Rotor Gene Q Series Software 2.0.3. PCR conditions are: Initiation Phase denaturation 94 ^{0}C for 3 minutes, denaturation stage 94 ^{0}C for 30 seconds, annealing step 52 ^{0}C for 50 seconds, tahapextension 72 ^{0}C for 1 min and final extension 72 ^{0}C for 5 minutes. Number of cycles 35 times.

Tabel 1: Formula Top Taq Master Mix Kit (Qiagen)

Bahan	Jumlah
Enzim *Tag polymerase*	25 µl
Primer LCO 1490	2 µl
Primer HCO 2198	2 µl
ddH_20	19 µl
DNA template	1 µl

2.2.3 Electrophoresis

Visualization of PCR products is carried out using automatic electrophoresis Qiaexel Qiagen. 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).

2.2.4 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) [32]. Furthermore, CO1 genesequence of mosquitoes were alignedwith 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) [30, 31].

3. Results and discussion

3.1 Extraction and Purification of DNA

Hind legs of imago gerridae of the river Talawaan and the river Ratatotok used to extract DNA. DNA extraction comprised of nuclear DNA and mitochondrial DNA therefore called total DNA (dsDNA = double strand DNA).

Table 4: Concentration and purity of DNA extract Gerridae

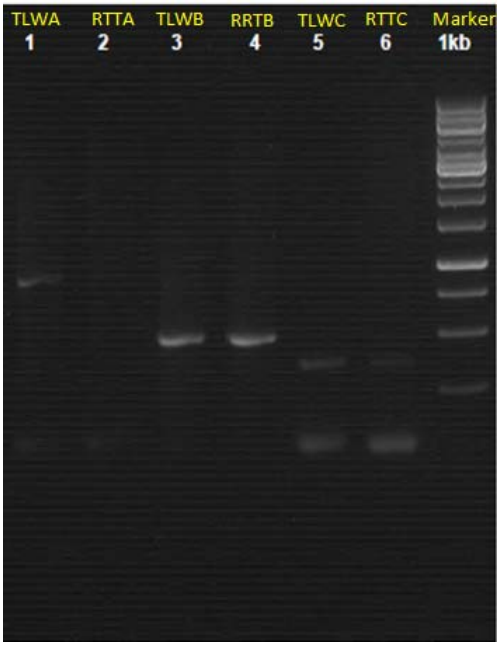
No	Sampel	Kemurnian (A260/A280)	Konsentrasi (µg/ml)
1	TLWA	1,71	32,5
2	RTTA	1,82	40,3
3	TLWB	1,75	42,5
4	TLWC	1,76	32,6
5	RTTC	1,82	33,6

Fig 1: The hind legs that have been used as a source of tissue for DNA extraction Ratatotok

Based on the analysis of the purity of dsDNA has not shown the maximum purity in all samples. Range purity standards based on DNA Extraction Kit is used on A260/A280 is 1.80 to 2.00. From the extraction of the sample only RTTA and RTTC reaching maximum purity (Table 1). The low purity is affected by the contamination of protein, RNA and protein derivative molecules found in tissue and found in the exoskeletons of insects (Mokosuli et.al, 2014; Manuahe *et al*., 2015). Nevertheless, total DNA concentrations are at optimal distribution according to standard DNA extraction kit used is 30 ug/ml - 50 ug/ml. This means immersion applications proteinase-K modified by the researchers provides optimal concentration results. Soaking time proteinase K extraction kit according to the standard protocol is 1 hour whereas in this study is modified to 24 hours soaking time the sample with proteinase-K. The optimal concentration affects gene amplification target of 16 S RNA genes and gene CO1.

3.2 16 S RNA gene amplicons

Results CO1 gene amplification and gene 16 S rRNA evidenced by elektrogram electrophoresis results shown by the formation of ribbon (band) on the well according to the type of sample. 16 S rRNA gene amplified with a length of 400-500 bp. Electrophoresis was performed on the condition of 8% agarose gel, DNA ladder 0.2 mg and 1 mL sample volume of each well. Amplicons TLWB and RRTB establish clear tape when compared with RTTC and TLWA (Figure 8). Thus amplification on samples TLWB and RRTB better than RTTC and TLWA. The thickness of the tape shows the concentration of amplicons formed.



Gambar 13: Elektrogram Visualisasi amplikon gen CO1 dan Gen 16 S rRNA hasil amplifikasi metode RT PCR. (TLWA = Sampel Talawaan gen16 S RNA, RTTA = Sampel Ratatotok gen 16S rRNA, RRTB = sampel ratatotok gen CO1, TLWB = Sampel Talawaan gen CO1, TLWC = sampel Talawaan control gen CO1, RTCC = sampel Ratatotok control gen CO1).

Based elektrogram formed, amplification on samples TLWB and RRTB work well evidenced by the band formed. Samples TLWC and RTTC has not shown that optimal amplification success is characterized by a double band formed and the appearance of amplicons less clear tape. TLWA sample amplification and RRTA, has not managed optimally, only a

thin band formed in the sample while the sample RRTA TLWA not formed ribbon thus concluded amplification was unsuccessful. From this research it is known that the concentration of DNA at the stage of extraction and purification of DNA that is not enough to determine the success optimal amplification of target genes by PCR. Sensitivity primers used greatly affect the success of the target gene amplification. In this study, 16 S RNA gene amplification works well while the CO1 gene has not been successful.

3.3 Sequence

16 S RNA gene sequence reads at 400-600 bp. Sequencing in the form seq file, read the Program Geneious 9.0. From the results of sequencing product reading showed the sequencing process works so well marked with the chromatogram peaks between separate base type is perfect or not coincide (Figure 9). Pita separate chromatogram better show the 16 S rRNA gene sequencing Aggang Aggang successful.

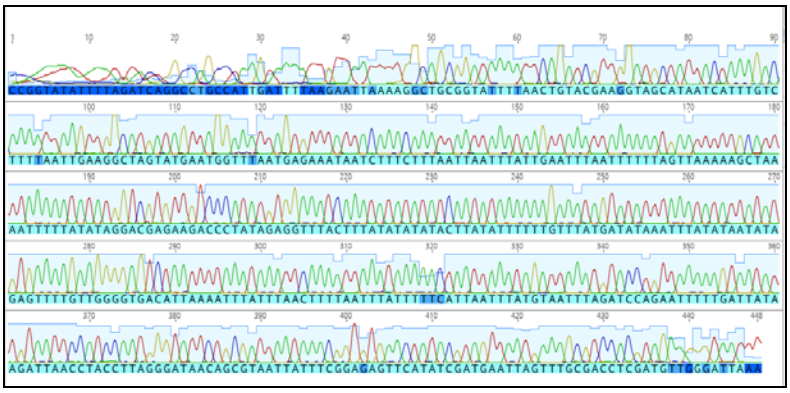


Fig 2: The chromatograms Sequencing products of TLWB (Talawaan River)

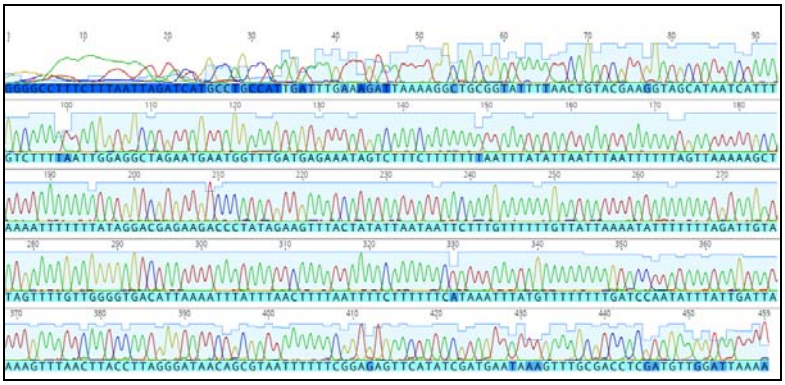


Fig 3: The chromatograms Sequencing products of RRTB (Ratatotok River)

3.4 Characteristic of Sequences

The results of sequence analysis using the Program geneous 9.0; 16 S rRNA gene sequences of samples Talawaan (TLWB) has a length of 448 bp while the sample Ratatotok (RRTB) at 453 bp. These results are consistent with the long-range 16S rRNA gene specifically to the insect that is 415-500 bp (Simon *et al* (1994) [29]. The percentage of nitrogen bases guanine and cytosine (GC%) was 25.7% and 25.2% (Table 4). The substitution of bases were analyzed using a 6.0 Mega Program (Table 5).

Table 2: Karakteristik Sekuens

No	Sekuens	Panjang Sekuens	% GC	HQ%	Jumlah dan % Jenis basa nitrogen			
					A	C	G	T
1	TLWB 16 SA	448 bp	25,7 %	87.5%	199 (43, 9%)	71 (15, 7%)	43 (9, 5%)	140 (30, 9%)
2	RRTB 16 B2	453 bp	25,2 %	91.6%	139 (30, 3%)	43 (9, 4%)	75 (16, 3%)	202 (44, 0%)

3.5 Alignment analysis

Alignment analysis can be used to compare two or more sequences. The program used for the analysis of alignment is the BLAST program (Basic Local Allignment Search Tools). This program is accessible through the website of the National Center for Biotechnology Information at the National Library of Medicine in Washington, DC (http://www.ncbi.nlm.nih.gov/BLAST). The results of the analysis of the alignment shown in the following figure. Query shows the samples submitted by the researcher (447 bp). Red line indicates that the two sequences that have very similar sequences of more than 200 nucleotides.

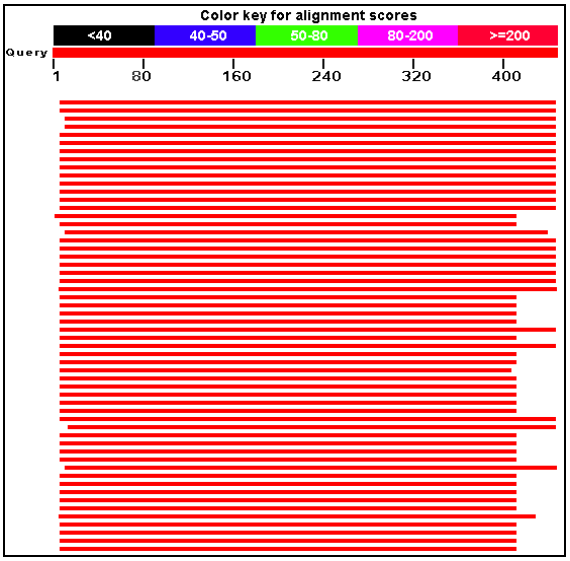


Fig 4: Distribution sequences that are similar to the results of analysis of samples TLWB alignment on NCBI website

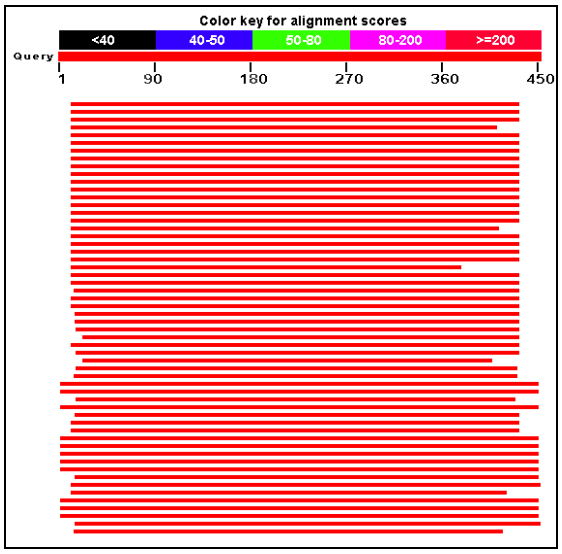


Fig 5: Distribution sequences that are similar to the results of analysis of samples RRTB alignment on NCBI website

TLWB alignment with NCBI BLAST sequence results showed seven sites there are different types of nitrogenous bases. Seven of the site shows the location of the mutation between the two sequences being compared. The degree of similarity between sequences and sequences TLWB Gerris babai was 408/445 (92%) (Figure 6).

Gerris babai mitochondrial gene for 16S ribosomal RNA, partial sequence
Sequence ID: **dbj|AB026585.1|** Length: 503 Number of Matches: 1
Range 1: 58 to 500

Score	Expect	Identities	Gaps	Strand	Frame
610 bits(330)	8e-171()	408/445(92%)	7/445(1%)	Plus/Minus	

Features:

```
Query  8    ATTTTA-GATCAGGCCTG-CCATTGA-TTTTAAGAATTAAAAGGCTGCGGTATTTTAACT  64
Sbjct  500  ATTTAACGATCAGACCTGCCCATTGATTTTATGAATTAAAAGCTGCGGTATTTTAACT  441
Query  65   GTACGAAGGTAGCATAATCATTTGTCTTTTAATTGAAGGCTAGTATGAATGGtttaatga  124
Sbjct  440  GTACGAAGGTAGCATAATCATTTGTCTTTTAATTGAAGGCTAGTATGAATGGTTTGATGA  381
Query  125  gaaataatctttctttaatttaatt-tattgaatttaatttttagttaaaaagctaaaat  183
Sbjct  380  AAAATAAACTTTCTTTTTTAATTATTTGAATTTAATCTTTAGTTAAAAAGCTAAAAT  321
Query  184  ttttaTATAGGACGAGAAGACCCTATAGAGGtttactttatatatatactta-tattttt  242
Sbjct  320  TTTTTATAGGACGAGAAGACCCTATAGAAGTTTACTTTATTTAT-TAATTATTATTTTT  262
Query  243  tgtttatgatataaatttatataatataGAGTTTTGTTGGGGTGACattaaaatttattt  302
Sbjct  261  TGTTTATTAAATAATTTTTTAGAATATAAAGTTTTGTTGGGGTGACATTAAAATTTATTT  202
Query  303  aacttttaatttatttttcattaatttatgtaatttagatccagaatttttgattataag  362
Sbjct  201  AACTTTTAATATTTATTCATTAATTTATGTTTTTTGATCCAGAATTTTTGATTATAAG  142
Query  363  attaaCCTACCTTAGGGATAACAGCGTAATTATTTCGGAGAGTTCATATCGATGAATTAG  422
Sbjct  141  ATTAACTTACCTTAGGGATAACAGCGTAATTTTTCGGAGAGTTCATATCGATGAATTAAG  82
Query  423  TTTGCGACCTCGATGTTGGGATTAA  447
Sbjct  81   TTTGCGACCTCGATGTTGA-ATTAA  58
```

Fig 6: Sequence alignment

RRTB sequence alignment with the results of BLAST on NCBI website showed four sites different types of nitrogenous bases. The four sites showing the location of the mutation between the two sequences being compared. The degree of similarity between sequences and sequences Limnogonus TLWB fossarium was 419/423 (99%) (Figure 19).

3.6 Reconstruction phylogeny tree

Reconstruction gerridae of Ratatotok River and the River Talawaan using BLAST results termirip 10 sequences from the gene bank at the NCBI web site. Construction phylogeny using MEGA 6.0 program with two models: Model Neighbor Joining and minimum legal Evolution as a comparison (Figure 12).

Fig 7: Reconstruction of Phylogeny Trees with Minimum Model Evolution Using MEGA Program 6.0.

Fig 8: Reconstruction of Phylogeny Trees with Minimum Model Evolution Using MEGA Program 6.0.

The results of the molecular phylogeny reconstruction model shows anggang angggang Minimum evolution of Talawaan and Ratatotok separately on different nodes. Construction Aggang Aggang phylogenies using two models show Aggang Aggang phylogeny tree of Talawaan and Ratatotok in the same position. Thereby further strengthen the position Aggang Aggang the phylogeny tree based on the 16 S rRNA gene.

4. Conclusion

Based on the phylogeny tree formed from Talawaan Water striders have closest kinship with *Limnoporus esakii* while Water striders of Ratatotok has the closest kinship with *Cylindrostethus costalis.*

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