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Dna barcoding for identification fly species from differents habitats in Luwuk, Sulawesi Tengah

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Abstract
Flies are members of the Diptera order that many act as vectors of disease, in humans and animals. Fighting of the fly population starts from knowing the position of the species. Research has been conducted to know the position of flies species originating from several habitats in Luwuk City, Central Sulawesi. Three locations as source of fly sample are traditional market (PSR), settlement (RS1) and hospital (RS2). The thorax and back legs are used for DNA extraction and purification. Amplification of CO1 gene was done by PCR method. Visualiasi amplicon of CO1 gene is done by electrophoresis method. Nucleotide sequencing is done by sequencing method. The sequence of CO1 gene obtained in the BLAST analysis on the NCBI website and followed by phylogenetic reconstruction using the Geneous 10.1.1 Program and the MEGA 7.0 Program. The results showed that: PSR fly gene sequences have the highest similarity percentage with *Chrysomya megacephala* SCAU-DE-LD-C26 [KY020769] (99%). While the gene sequences of RS1 and RS2 CO1 each show the highest similarity with *Chrysomya megacephala* CSU160701CB14 [KY001899.1] (99%) and *Hermetia illucens* [HQ541186.1]. However, the reconstruction of the phylogenetic tree formed by RS1 and RS2 flies has an evolutionary relationship or similarity to *Chrysomya megacephala* Voucher CM1, whereas PSR has an evolutionary relationship or similarity to *Chrysomya megacephala* Haplotype SCAU-DE-LD-C26.

Keywords: Fly, DNA barcoding, cytochrome oxydase sub unit 1 (CO1), Luwuk

Introduction
Flies can act as mechanical vectors of transmission of microbes and parasites that cause gastrointestinal diseases such as cholera, typhoid and dysentery (Sembel, 2010) [13]. The disease transmitted by flies depends on the species. Adult flies can carry intestinal worm eggs (*Ascaris* sp., Hookworm, *Trichuris trichiura*, *Oxyiuris vermicularis*, *Taenia solium*, *Taenia saginata*), Protozoa (*Entamoeba histolytica*), intestinal bacteria (*Salmonella* sp., *Shigella* sp. and *Escherichia coli*), virus polio, *Treponema pertenue* (cause of frambusia) and *Mycobacterium tuberculosis* (Preativatanyou *et al*. 2010; Sembel, 2010) [12, 13]. Small flies (Fannia) can transmit different types of Myasis (Gastric, Intestinal and Genitourinary). Cage fly (*Stomoxys calcitrans*) is a vector of anthrax, tetanus, yellow fever and traumatic myasis and entric pseudomiasis (although rare) diseases. Green fly (Phaenicia) can transmit myasis of eyes, bones and organs through wounds. Meat fly (*Sarcophaga*) can transmit skin myasis, nose, tissue, vagina and intestines. Houseflies can transmit more than 64 types of microbes and parasites that cause disease in humans (Sukontason *et al*. 2005; Sembel, 2010) [13].
Number of species of flies is estimated between 60,000 - 100,000 species. Almost all parts of the fly body can transmit disease, such as: body hair, feathers on the limbs, vomit and facesnya. In general the life cycle flies through 4 stages namely: egg -> larva -> pupa-> adult fly (Boror *et al*. 1996) [3] In some species of flies, the eggs remain in the body of adult flies until settled and then born larvae. The duration of the life cycle and the habit of laying places may vary between different types of flies. Similarly, there are differences in the temperature and place of life that are usually for each type of fly (Borror *et al*. 1996, Sembel, 2010) [3, 13].
Flies are able to live in various places on earth, not limited by climate, can even live as a parasite in humans and animals. The capability of this adaptation is influenced by the ability of phenotific or morphological modification initiated by genetic modification (Kaunang, 2015; Preativatanyou, *et al*. 2010) [12] This results in high intraspecies variation (Rotty, 2017; Sukontason, 2005) [14]. The flies are considered vectors of the disease, trying to be eradicated by humans. The use of insecticides, habitats, types of food sources, microbial symbionts, among others, affect the characteristics of phenotypic and genotypic of flies. Phenotypic and

genotypic modification of house fly has been widely reported (Sukontason, 2005) [14]. Long-term genotypic variation can cause morphological (phenotypical) changes. The presence of genotypic variation and for the identification of the position of species has been widely used genes in mitochondrial DNA as barcodes. The sub-cytochrome oxyphase subtype 1 (CO1) gene is most widely applied as a molecular barcode for the identification of species of animal species with very high accuracy (Herbert *et al*. 2003; Hajibabaei *et al*., 2005; Harmon *et al*., 2006; Hulcr *et al*., 2007).

The evolution of mitochondrial DNA is approximately 2.3% per million years (Bower, 1994). The sequence of genetic information contained in mitochondrial DNA is reported to characterize a population, phylogenetic and possible to reconstruct evolutionary history (Avise *et al*., 1987; Espin, 1992; Lessinger *et al*., 2000; Mokosuli, 2013) [1, 8, 11]. The cytochrome oxidase gene is one of the genes present in the mitochondrial genome and is widely used for molecular identification analysis. The use of the universal CO1 gene for the identification of insects in Sulawesi has been reported on *Apis dorsata* Binghami (Mokosuli, 2013) [11], *Aedes* sp. (Kaunang *et al*. 2015; Manuahe *et al*. 2016) [10], and bed bugs (Kalangi *et al*. 2016), marine Gerridae (Warouw *et al*. 2015), agang agang (Waha *et al*. 2017) [19] and Demselfly (Rantung *et al*. 2015).

Recentlly, there are still few reports of studies of molecular phylogenetic studies, flies from mixed populations in Sulawesi. Identification of fly species is very important for the effort to control flies as disease vectors. Along with the development of the population, poor sanitation, causing flies populations to flourish in the last decade in Luwuk City. Cases of diarrhea and other gastrointestinal infections are high in Luwuk and Bangai regencies, Central Sulawesi, in the last five years (BPS Luwuk, 2017). In previous research, there has been found mixing of species in one population of flies in Luwuk (Kanan *et al*. 2017). Catching flies randomly, found more than one species that perform activities in the same niche. Molecular species identification of flies, necessary for the control of flies as vectors of the disease. Knowing the species of fly accurately, it will make it easier to know the behavior of flies and the type of pathogenic microbes that are bundled by a species of fly.

Materials and Methods

Sample

Flies are obtained by direct capture techniques. Locations of fly catching was done in several places in Luwuk City namely :Traditional Market, Settlement Area and Hospital. The caught flies were preserved in 70% ethanol, then used as samples for DNA analysis. Thorax and legs of house flies, have been used as a tissue source for the extraction and purification of house fly DNA.

Tools and Materials

The tools used in this research include: Tissue ruptor Qiagen, V-1 vortex plus Biosain, orbitals shaker OS-20 Biosain, micropipette eppendorf, mini personal centrifuge Tommy Digital Biology P Class, nanophotometer, centrifuse 5430R eppendorf, master cycles pro s Eppendorf, gel documentation system fire reader UVitec, qiaxel automatic electrophoresis Qiagen, sequence ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA. The materials used was : ethanol p.a. (merck), chloroform p.a. (merck), DNeasy Blood & Tissue Kit (Qiagen), CO1 Primer: LCO1490: GGTCAACAAATCATAAAGATATTGG and HCO2198: AAACTTCAGGGTGACCAAAAAATCA (Folmer *et al*. 1994), Qiagen Top Taq Master Mix Qiagen (USA), Qiaxel DNA Screening gel kit and 2 µl - 100 µl Qiagen (USA).

DNA Extraction, PCR and Electrophoresis

Total genomic DNA was extracted from RPW samples using DNeasy Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was amplified by PCR method, using Rotor Gene, Qiagen. The composition of the pareax used in the PCR process consisted of: 6.4 µl sterile ddH2O, 1 µl dNTP 2.5 mM, 1.25 µl Mg2 + free buffer, 1.5 µM MgCl2 25 mM, 0.1 µl Taq polymerase 5 mM, 1 µl of extracted DNA, 0.625 µl of CO1 primer. The total volume of reagents used was 12.5 µl. Amplification was done in three stages of predenaturation stage with temperature 940C for 5 minutes. Next multiplication of 35 cycles with denaturation temperature 940C for 30 seconds, annealing 500C for 50 seconds, 720C exetension for 50 seconds and final extension at 720C for 5 minutes.Amplicon of CO1 gene of flies were visualized using Qiaxel Automatic electrophoresis, Qiagen. with a Qiaxel DNA Screning kit (Qiagen).

Sequencing and sequencing analysis

Sequencing using ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA through FIRST BASE Singapore Sequencing Services. The sequenced CO1 genes were analyzed using the Geneous 11.1.0 and MEGA 6.0. Furthermore, each sequence of CO1 gene of flies was analyzed alignment by BLAST method (Basic Local Alignment Search Tool) at http://blast.ncbi.nim.nih.gov/Blast. A total of 31 sequences with a similarity level of more than 90% were used for the reconstruction of phylogeny trees. The phylogeny tree was formed using the MEGA Program 7.0 program. The phylogenetic tree model was determined after sequence substitution analysis.

Results and Discussion

The thorax and legs have been used to extract of the total DNA of flies. The DNA yield from extraction and purification, consisting of nucleid DNA and mitochondrial DNA.

Mitochondria are found in the thorax and legs of insects because there are many muscle tissues (Borror, 1996) [3]. The volume DNA obtained from each flies sample is 100 µl. DNA Extraction results were analyzed for concentration and purity. The highest total DNA concentration was obtained from the PSR sample (42.70 µg / ml) with a purity of 1.75 (Table 1)

Table 1: The concentration and purity of total DNA from flies in Luwuk

No	Sample	Concentration (µg/ml)	Purity
1	PSR	42,70	1,75
2	RS1	64,37	1,72
3	RS2	54,45	1,67

Explenation: PSR : (flies from traditional market), RS1 (flies from hospital), RS2 (flies from human settlements)

Based on the concentration and purity of the extracted DNA, it shows that the Qiagen Blood and Tissue kit, which is used to extract the DNA of flies is effective in extracting the total DNA from the limbs and the thorax of flies. Difficulty in extracting insect DNA, compared to other organism samples are contents of chitin, phenolic compounds and peptides in exoskeleton. The contents, can decrease the effectiveness of buffer and proteinase in the kit (Timah and Mokosuli, 2016; Manuahe *et al.* 2015; Mokosuli, 2013) [10, 11]. In the present

study, sample preservation treatments and modification of protocol kits was done. Modification done on the destruction of thoracic and legs with tissue ruptor and long tissue soaked with *protenase* K in 24 hours at 64^0C. This modification proved to increase the concentration and purity of the total DNA of flies. The total DNA concentration distribution based on the Kit protocol used was 30 µg / ml up to 70 µg / ml. Thus, the total DNA concentration obtained in this study is quite good. While total DNA purity is at the distribution of 1,7 - 2,0 (A260 / A280). Total DNA purity of the results of this study is still quite good. However, the mitochondrial DNA content present in the total new DNA will be known after the amplification of the target gene by using a specific primer (in this study used primer for CO1 gene) by PCR method.

Amplification of CO1 gene of flies used PCR method

DNA total of flies was used as a templete of CO1 gene amplification by PCR method. Of the four stages of PCR, the annealing stage is a crucial stage, therefore, temperature and time modification, greatly influences the amplification of the CO1 gene. In this present study, annealing temperature modification was performed which proved to produce amplicons as expected. Visualization of amplicon content of CO1 gene is done using electrophoresis. The length of the PS1 CO1 gene is 690 bp, RS1 is 694 bp and RS2 is 689 bp (Figure 1).

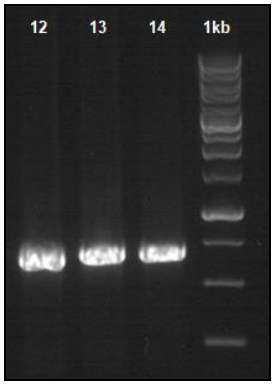
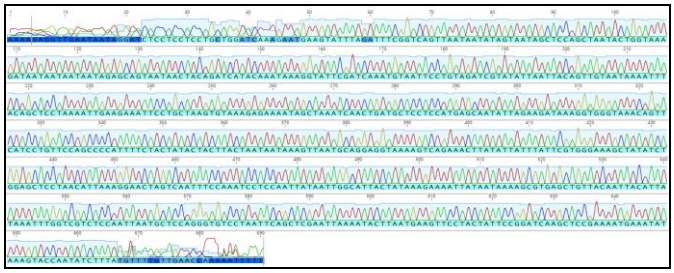


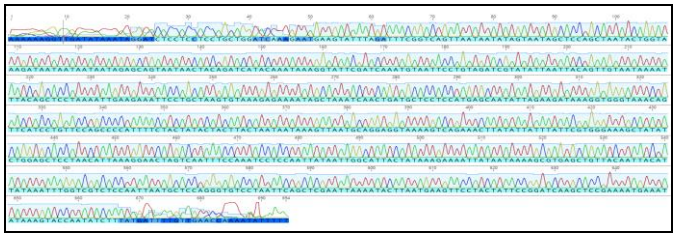
Fig 1: Amplicon visualized of CO1 gene of Flies from Luwuk (12=PSR, 13=RS1 dan 14 = RS2). Condition 0,8 % agarose gel, amount of the DNA ladder loaded per lane : 0,2 µg each; volume pf the sample loaded per lane : 1 µl each

Sequencing

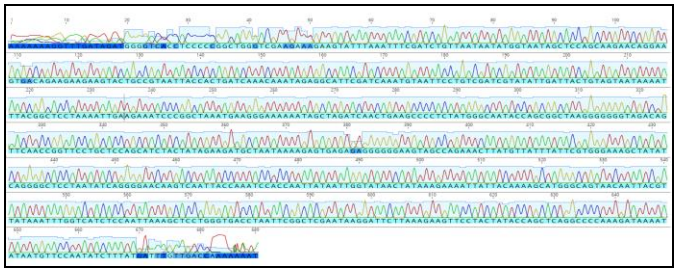
The result of the sequence of the fly CO1 gene from Luwuk (PSR, RS1 and RS2), read the sequence of nucleotides with the Geneous 11.0.1 Program. Based on the chromatogram of the sequencing results, the sequencing process proceeds well. This is proven, with a chromatogram type, representing a nucleotide type, separated completely or not coincidentally (Fig. 2).



a. PSR



b. RS1



c. RS2

Fig 2: Chromatogram of Sequence Results of the fly CO2 gene (a PSR, b. RS1 and c. RS2)

The length of the PS1 CO1 gene sequence was 690 bp with HQ (92.6%). Furthermore, the length of CO1 RS1 gene sequences and the CO2 RS2 gene were 694 bp (HQ: 91.9%) and 689 bp (HQ: L 93.3%), respectively. Comparison of Guanin and Cytosine, the gene sequence of CO1 PSR is 215 (31.2%), the sequence of the RS1 CO1 gene (214 (30.8%) and the CO2 RS2 gene sequence is 263 (38.2%) (Table 1). According to Herbert *et al*. (2003), the sequence of CO1 gene lies in the length of 600 - 700 bp. The sequence of the fly CO1 gene in this study lies in the sequence length of 689 bp - 694 bp, thus conforming to the characteristics of the CO1 gene as barcode molecular for animal identification.

Table 2: Characteristics of the CO1 Genes Flies from Luwuk

No	Sample	Characteristics					Length (bp)	MW of dsDNA (kDa)
		A	C	G	T	% GC		
1	PSR	261 (37,8 %)	109 (15,8%)	106 (15,4%)	214 (31,0%)	215 (31,2%)	690	426.224
2	RS1	264 (38,0%)	109 (15,7%)	105 (15,1%)	216 (31,1%)	214 (30,8%)	694	428.692
3	RS2	250 (36,3%)	121 (17,6%)	142 (20,6%)	176 (25,5%)	263 (38,2%)	689	425.653

Alignment Analysis With the NCBI BLAST Method (https://blast.ncbi.nlm.nih.gov/Blast.cgi)

The consensus areas of the CO1 gene of PSR, RS1 and RS2 were used for alignment analysis with the BLAST method on the NCBI website. The BLAST results showed that the sequence of the PS1 CO1 gene has the highest percentage of similarity with *Chrysomya megacephala* SCAU-DE-LD-C26 [KY020769] (99%). While the gene sequences of CO1 RS1, and RS2 each showed the highest level of similarity with *Chrysomya megacephala* CSU160701CB14 [KY001899.1] (99%) and *Hermetia illucens* [HQ541186.1].

From the homology analysis of PSR fly gene sequences, the RS1 CO1 gene with the identical sequence of *Chrysomya megacephala* SCAU-DE-LD-C26 [KY020769] obtained a 99% (694bp / 692 bp) equivalent percentage. Sites where the nucleotide difference occurs are nucleotides to 662 (T > C),

668 (T> C), 683 (A>T) and 689 (T>A). The homology analysis of the sequence of CO2 RS2 (query) genes with identified sequences of *Hermetia illucens* [HQ541186.1], obtained 99% (648bp / 648 bp) similarity, no site found where the nucleotide difference occurred (Table 3, Table 4 and Table 5).

Table 3: Nucleotide Sequence Comparison Between PSR CO1 Gene and Identical Sequence from BLAST Results

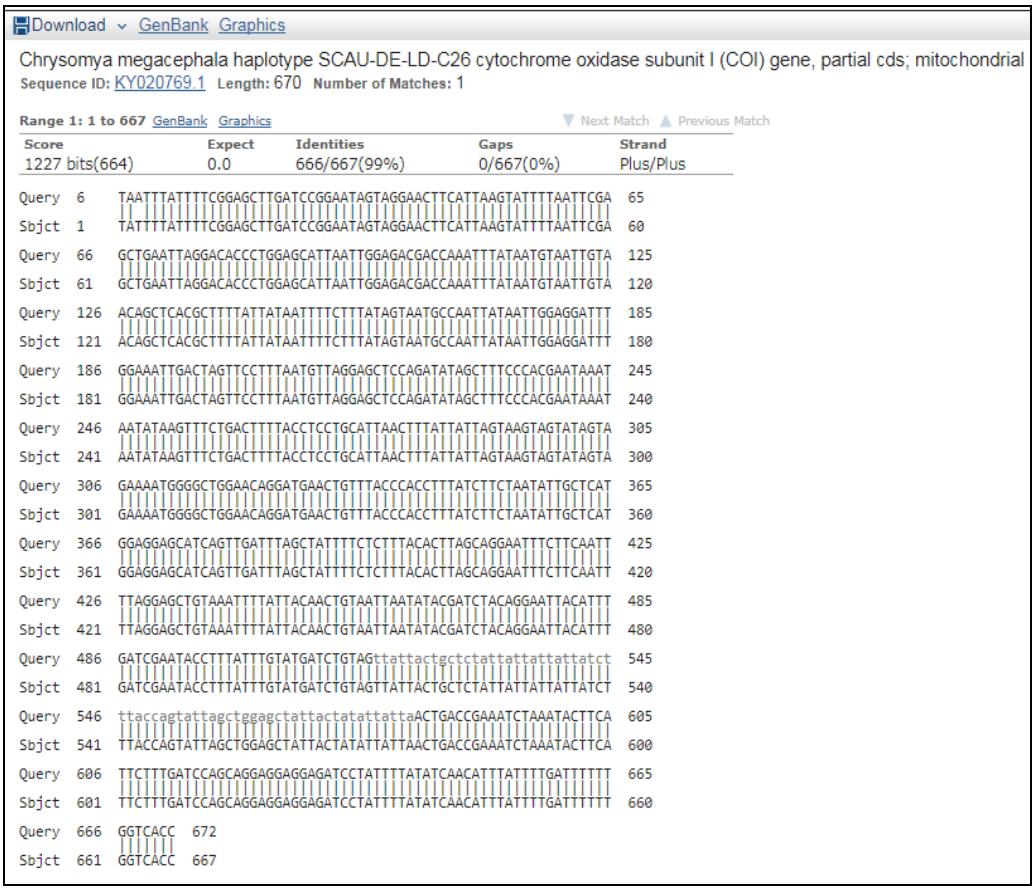


Table 4: Nucleotide Sequence Comparison Between RS1 CO1 Gene and Identical Sequence from BLAST Results

Table 5: Nucleotide sequence comparison between RS2 CO1 gene and identical sequence from BLAST results

Download ▾ GenBank Graphics

Hermetia illucens voucher Suwon-4 cytochrome oxidase subunit I gene, partial cds; mitochondrial

Sequence ID: HQ541186.1 Length: 657 Number of Matches: 1

▸ See 24 more title(s)

Range 1: 10 to 657 GenBank Graphics

Score	Expect	Identities	Gaps	Strand
1197 bits(648)	0.0	648/648(100%)	0/648(0%)	Plus/Plus

```
Query  7    TTTATCTTTGGGGCCTGAGCTGGTATAGTAGGAACTTCTTTAAGAATCCTTATTCGAGCC  66
Sbjct  10   TTTATCTTTGGGGCCTGAGCTGGTATAGTAGGAACTTCTTTAAGAATCCTTATTCGAGCC  69
Query  67   GAATTAGGTCACCCAGGAGCTTTAATTGGAGATGACCAAATTTATAACGTAATTGTTACT  126
Sbjct  70   GAATTAGGTCACCCAGGAGCTTTAATTGGAGATGACCAAATTTATAACGTAATTGTTACT  129
Query  127  GCCCATGCTTTTGTAATAATTTTCTTTATAGTTATACCAATTATAATTGGTGGATTTGGT  186
Sbjct  130  GCCCATGCTTTTGTAATAATTTTCTTTATAGTTATACCAATTATAATTGGTGGATTTGGT  189
Query  187  AATTGACTTGTTCCCCTGATATTAGGAGCCCCTGATATAGCTTTCCCACGAATAAATAAC  246
Sbjct  190  AATTGACTTGTTCCCCTGATATTAGGAGCCCCTGATATAGCTTTCCCACGAATAAATAAC  249
Query  247  ATAAGTTTCTGGCTACTTCCCCCCTCTCTCACTCTTTTATTAGCATCTTCTATAGTAGAT  306
Sbjct  250  ATAAGTTTCTGGCTACTTCCCCCCTCTCTCACTCTTTTATTAGCATCTTCTATAGTAGAT  309
Query  307  GCTGGAGCAGGAACCGGTTGAACTGTCTACccccccTTAGCCGCTGGTATTGCCCATAGA  366
Sbjct  310  GCTGGAGCAGGAACCGGTTGAACTGTCTACCCCCCCCTTAGCCGCTGGTATTGCCCATAGA  369
Query  367  GGGGCTTCAGTTGATCTAGCTATTTTTTCCCTTCATTTAGCCGGGATTTCTTCAATTTTA  426
Sbjct  370  GGGGCTTCAGTTGATCTAGCTATTTTTTCCCTTCATTTAGCCGGGATTTCTTCAATTTTA  429
Query  427  GGAGCCGTAAATTTTATTACTACAGTAATCAATATACGATCGACAGGAATTACATTTGAT  486
Sbjct  430  GGAGCCGTAAATTTTATTACTACAGTAATCAATATACGATCGACAGGAATTACATTTGAT  489
Query  487  CGAATGCCTCTATTTGTTTGATCAGTGGTAATTACGGCAGTACTTCTTCTTCTGTCACTT  546
Sbjct  490  CGAATGCCTCTATTTGTTTGATCAGTGGTAATTACGGCAGTACTTCTTCTTCTGTCACTT  549
Query  547  CCTGTTCTTGCTGGAGCTATTACCATATTATTAACAGATCGAAATTTAAATACTTCTTTC  606
Sbjct  550  CCTGTTCTTGCTGGAGCTATTACCATATTATTAACAGATCGAAATTTAAATACTTCTTTC  609
Query  607  TTCGACCCAGCCGGGGGAGGTGACCCCATTCTTTATCAACATTTATTT  654
Sbjct  610  TTCGACCCAGCCGGGGGAGGTGACCCCATTCTTTATCAACATTTATTT  657
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Discussion

DNA extraction and purification of flies using thoracic and legs of flies preserved with ethanol 70^0C for 24 hours resulted in a good purity and concentration of DNA. Extraction and purification of insect DNA using thoracic and legs, success extracted mitochondrial DNA optimally in *Aedes* sp. (Timah and Mokosuli, 2017) [15, 17]; *Anopheles* sp. (Manuahe *et al*. 2016) [10], *Droshophila* sp. (Sumampouw and Mokosuli, 2017) [15, 17] and bed bugs (Kalangi et.al. 2017), *Apis dorsata* Binghami (Mokosuli *et al*. 2013) [11]. However, thoracic and legs are best used for the isolation of mitochondrial DNA in insects.

The gene sequence of CO1 gene of flies from Luwuk City, Central Sulawesi, i.e. PSR and RS1 shows the highest similarity level with *Chrysomya megacephala*, on the other hand the CO1 gene of RS2 showed the highest similarity level with *Hermetia illucens.* This showed there has been a mixed population of flies in the city of Luwuk. Some species of flies feed for the same niche in the same time. From alignment analysis, the discovery of sites where there has been a nucleotide difference in the CO1 fly sequence of PSR and RS1 with the closest sequence. However, showing the process of CO1 gene mutation, occurs in flies in Luwuk City. However, the CO2 gene RS2 is not found on the site where there is a difference of nucleotides with BLAST sequenced sequences. In morphology flies PSR, RS1 and RS2 have differences (Figure 3).

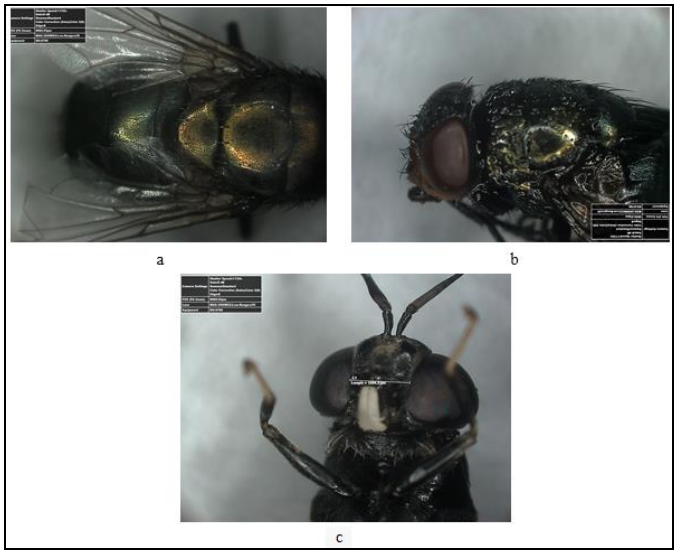

Fig 3: The fly from Luwuk is observed with Stereomicroscope Digital Hirox KH8700, with a magnification of Low Range 35x. a. PSR (derived from traditional Market). b. RS1 (originally from Hospital Location 1). C. RS2 (originating from hospital and residential area)

The reconstruction of phylogenetic trees is done on line at the NCBI website and uses the MEGA 7.0 program. The phylogenetic tree formed using Neigbor Joining model with bootstrap 1000 x. The phylogenetic tree constructed with 31

sequences with the highest similarity level, from the BLAST result, forms two monophyletic clades. The number of nodes that are formed, indicating a high genetic variation, based on the CO1 gene. The three sequences of the fly fly CO2 gene from Luwuk, are on the same monophyletic clade, but differ similarities with the closest species. Flies RS1 and RS2 have the closest evolutionary or similiarity relationship with *Chrysomya megacephala* Voucher CM1, whereas PSR has evolutionary relationship or closest resemblance to *Chrysomya megacephala* Haplotype SCAU-DE-LD-C26. Although waking from the same monophyletic clade but flies RS1 and RS2 have shown a very striking difference as it is formed on two different clades. Thus, it can be concluded the flies RS1 and RS2 have the closest evolutionary relationship with the flies coming from Thailand while the PSR flies have the closest evolutionary relationship with the fly Cina.

Chrysoma megacephala is one species of fly whose reproductive ability is very high (Sukontason *et al*., 2005) [14]. Boonchu *et al*. (2003) [2] found that *C. megacephala* (96.3%) represented the majority of all species whereas *C. rufifacies* (0.2%) was rarely captured by this trapping method. A possible explanation for their abundance is to utilize a wide range of ecological niches.

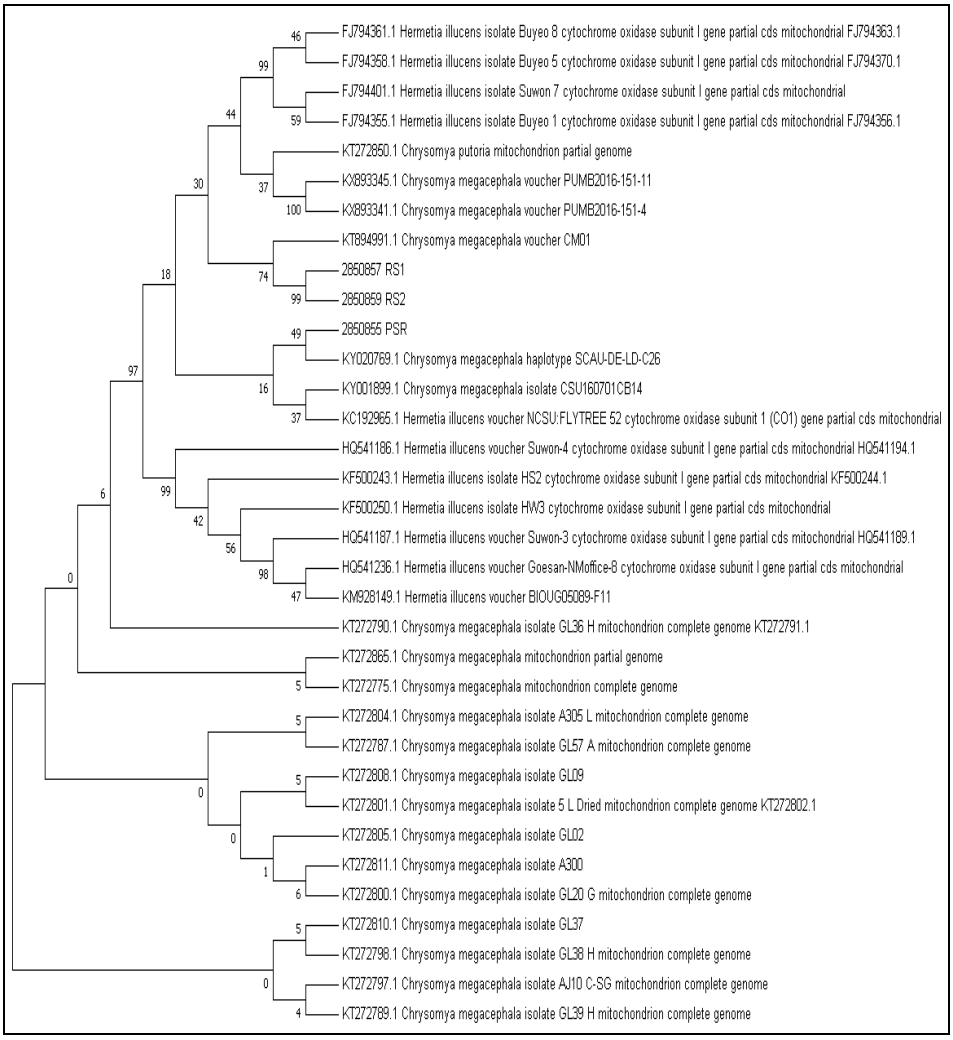

Fig 4: Reconstruction of the phylogeny tree Flies from Luwuk, with 31 sequences of BLAST result

Conclusion

The sequence of CO1 gene flies from Luwuk is the Sequence of the PS1 CO1 gene has the highest percentage of similarity with *Chrysomya megacephala* SCAU-DE-LD-C26 [KY020769] (99%). While the gene sequences of RS1 and RS2 CO1 each, show the highest similarity with *Chrysomya megacephala* CSU160701CB14 [KY001899.1] (99%) and *Hermetia illucens* [HQ541186.1]. Based on the phylogenetic tree formed The RS1 and RS2 flies have the closest evolutionary or resemblance relationship with *Chrysomya megacephala* Voucher CM1, whereas the PSR has an evolutionary relationship or closest resemblance to *Chrysomya megacephala* Haplotype SCAU-DE-LD- C26

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