

Molecular Confirmation of Forensic Important Flies Collected in Palembang, South Sumatra

Ahmad Ghiffari¹, Chairil Anwar^{2*}, Dalilah², Mansuri³, Elfinchia Tiara Switha⁴

¹Department of Parasitology, Faculty of Medicine, Muhammadiyah University of Palembang, South Sumatra, 30116 Indonesia

²Department of Parasitology, Faculty of Medicine, Sriwijaya University, Palembang, South Sumatra 30126 Indonesia

³Department of Forensic, Bhayangkara Hospital Palembang, South Sumatra, 30128 Indonesia

⁴Postgraduate School of Medicine Faculty, Sriwijaya University, Palembang, South Sumatra 30126 Indonesia

*Corresponding author. Email: chairil53@unsri.ac.id

ABSTRACT

The identification of insect species is an important key in forensic entomology. Molecular examination of the nucleotide sequences of cytochrome c oxidase subunit II (COII) gene has been widely performed before the morphology identification. We analyzed the partial conserved COII nucleotide sequence of the forensic related flies. After the DNA extraction from collected larvae, thus PCR amplification of conserved sequencing of the partial COII sequence was implemented. Obtained sequences were analyzed for a phylogenetic tree and a distance matrix. Our data showed a very low intraspecific sequence distance among Calliphoridae and Sarcophagidae to the flies' species of *Chrysomya megacephala*. Further research should attempt to determine the larval growth of newly discovered flies, to apply the time of death in forensic crime setting.

Keywords: Calliphoridae, Sarcophagidae, cytochrome C oxidase subunit II, morphology

1. INTRODUCTION

Judicial and forensic medicine has several strategies on how to determine the time of death in summing up the cause of finding bodies. Changes in the size of the cadaver skin bruising color can determine the hours after death [1], and the composition of the eyeball liquid potassium has been used in court for evidence [2]. The use of artificial intelligence by using metabolites and blood pH is promising as a biomarker measurement [3]. Molecular biology methods can determine the time of accurate and reliable than the age of the injury [4], and the ecology of microbes after the decomposition of corpses [5]. The order of arrival of the insect on the carcasses can also predict the estimated time of death [6]. Determining the right time can help the alibis of the suspects are precise and exact.

Some species of flies known to play a role in determining the time of death of a corpse. Flies of the family Calliphoridae came the first time of death whom larvae are cannibals against larvae of other species that infests the body [7]. Flies of the family Sarcophagidae came after Calliphoridae, larger and carcasses have been found in the larval stage, making them easier observed at the site of death [8]. Flies of the family Calliphoridae more often used in forensic examination, but the vast morphological variation makes it a bit challenging as the variant species of African countries [9]. The larval stage can determine the diagnosis of species, due to the specificity of the shape and numbers of spiracles posterior

and anterior lobe [10]. Fly species both adult stage, pupa, and larvae can act as a specimen diagnosis [11,12].

Chrysomya megacephala is closely associated with forensic entomology [13, 14]. The suspect of one murder from a burned corpses infested with flies was managed to be investigated and arrested due to the use of flies' predictions 50 hours after death [15]. The same species of flies may have different morphologies on the strain state [16]. Morphometric geometry use pupae as a benchmark in identifying the species *C. megacephala* [17]. Limited expertise in taxonomy [18], can be overcome with the use of a reference database of *C. megacephala* DNA [19, 20]. Proper identification of the fly species carried out by various methods exist, will further determine the time of death of a corpse.

Some related research regarding flies in Palembang. *Chrysomya megacephala* species documented in the market [21] and landfills [22]. An experimental studies using animal carcasses showed the presence of the species using morphological examination [23]. The species of *C. megacephala* seemed to be the only one of forensic importance. This is the first molecular attempt to utilize the mitochondrial COII nucleotide sequence analysis to confirm the forensically important flies of Palembang city.

1.1. Materials and Methods

This experimental analytic with a cross-sectional study design was conducted in September 2018 at the lab of Medical faculty Sriwijaya University, Palembang. The samples were larva of flies that infested the dead rats (*Rattus novergicus*) of 2-3 months old with weight around 150-200 grams. Before the morphological key identification by observing the posterior and anterior spiracles with a dissecting microscope, the DNA genomic was extracted using Qiagen Genomic DNA Kit 50 according to the manufacturer's instructions. Ethical approval obtained from the Ethics Committee of the Faculty of Medicine, Sriwijaya University, Palembang.

1.1.1. Larva preparation and morphology staining

Larvae were prepared in the solution before the staining for the microscopic identification. Specimens were first dipped in the hot water for immobilization before dissection of the abdomen for internal organ removal. Later, the solution of KOH 10%, solution of acetic acid, and the solution series of alcohol 70-80-90-96 % were performed 30 min respectively. The staining of specimens was using the clove oil solution for at least 30 min. Thus, the samples were ready to be observed using a microscope examination.

1.1.2. Polymerase chain reaction and automatic sequencing

Primer sequences for the COII gene were taken from the literature [19]. Amplifications of COII locus were performed using primers C1-J-2495 (5'-CAG CTA CTT TAT GAG CTT TAG G-3') and TK-N-3775 (5'-GAG ACC ATT ACT TGC TTT CAG TCA TCT-3') and PCRs were performed using a 2720 Thermal Cycler (Applied Biosystems, Foster City, CA, USA). The PCR reaction conditions consisted of an initial denaturation step at 95 °C for 5 min, annealing step at 93 °C for 1 min, extension step at 48 °C for 1 min, and final extension step at 72 °C for 2 min. Each reaction mixture was prepared using 50 ng of template DNA, 2.5 µL 10× AmpliTaq Gold Buffer, 0.5U AmpliTaq Gold DNA Polymerase (Applied Biosystems, Foster City, CA, USA), 10 pmol (each) upstream and downstream primers, 62.5 nmol MgCl₂, 5 nmol (each) dNTPs, and sterile distilled water to a final volume of 25 µL. After purification of the PCR products, sequencing reactions were performed according to the manufacturer's instructions using a BigDye v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA). The sequencing products were analyzed using an ABI 3730xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). Assembled sequences were deposited into the NCBI GenBank database.

1.1.3. Phylogenetic analysis and sequence comparison.

Phylogenetic trees were generated for 2 fly families by using the maximum likelihood method with 1,000 replicates of bootstrapping based on the Tamura-Nei model using MEGA6 software [24]. Initial trees for the heuristic search were obtained by applying the neighbor-joining method to a matrix of pairwise distances estimated using the maximum composite likelihood (MCL) approach. To make root for each tree, COII sequences for *Hemipyrellia liguriens* (KF037968), *Sarcophaga ruficornis* (NC_041069), *Sarcophaga dux* (MH765524), and *Chrysomya rufifacies* (GQ912623) were introduced as outgroup taxa. Average intraspecific and interspecific sequence distances were calculated for sequence comparison. Sequences obtained in this study were later aligned with to previously published in GenBank data.

1.2. Our Contribution

This paper presents the first reports of DNA-based COII nucleotide sequences from flies' species. *Hemipyrellia liguriens*, *Sarcophaga ruficornis*, *S. dux*, and *Chrysomya rufifacies*, were four flies species that share the large similarity the Palembang flies. However, the morphology identification of the larva assumed the sample as *C. megacephala*. Microscopy showed of the spine between the first and second thoracic segments and the morphology of the posterior spiracle make it difficult to carry out differential identification sequences obtained from the mitochondrial COII gene fragment of Calliphoridae, and Sarcophagidae are now available and can be used as an additional tool for the identification of populations from the City of Palembang, South Sumatra, Ind, especially in forensic applications.

1.3. Paper Structure

The rest of the paper is organized as follows. The first section introduces the morphological identification, which used the anterior and posterior spiracles as microscopical keys. The second section described of the primers applies the COII conserved gene of flies. The third section presents the phylogenetic analysis from the sequence samples compared to the GenBank data. The last section concludes the findings.

2. RESULTS AND DISCUSSION

2.1. Morphological Results

Larval identification can be determined from the anterior and posterior spiracles. Larvae with papillae anterior spiracles showed 1 to 9 finger-shaped protrusions; Posterior spiracles figured the peritreme incomplete and do not cover the button; there are three slits which are straight and wide (Figure 1). These 3 slits show larvae of *C. megacephala* at the third instar stage (Figure 2). On the contrary, the *C. ruffifacies* has as many as 9-10 papillae anterior spiracles bulge fingers, which were arranged in a straight line. Posterior spiracles shaped slits width, also corresponding with larval instar stage, open peritreme, thin and slightly dark [24, 25]. It was concluded based on the anterior-posterior spiracles characteristics that samples found were identified as *C. megacephala*.

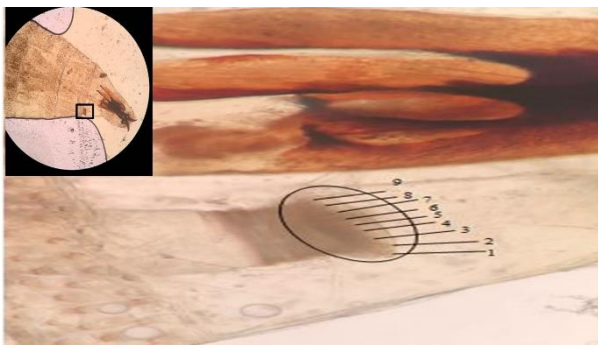


Figure 1 Microscopic examination of the larvae *Chrysomya megacephala*: the anterior spiracles with 9 protrusions (magnification 10x10x4.5). Caption: 1-9 end spine bands

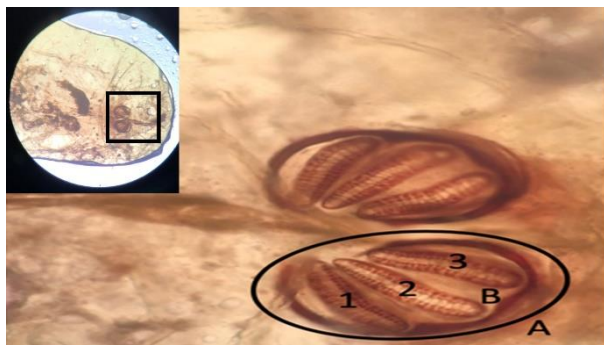


Figure 2 Microscopic examination of the larvae *Chrysomya megacephala*: The posterior part with posterior spiracles of third instar larvae (magnification 10x10x4.5). Caption: (A) incomplete peritreme; (B) three slits, straight shape, and width; 1-3: slit numbers

2.2. PCR Results

Positive samples resulted in the appearance of the white band in 1324 basepair. Negative samples showed no white band, indicated that not all microscopically positive samples showed positive PCR tests (Figure 3). Positive samples showed faint and thick white bands, which were the number 16, 21, 24, 34, 43, 48, 49, 50, 51, 52 and 53. Out of 11 that have been observed whit thick white band, thus 5 samples were taken randomly for the sequencing process. Positive samples were furthered test for sequence analysis.

2.3. Phylogenetic Analysis Maximum Likelihood

The five samples randomly chosen were aligned with GenBank COII flies' genes. The five samples were 99-100% similarity to *Hemipyrellia ligurriens* (KF037968), *Sarcophaga ruficornis* (NC_041069), *Sarcophaga dux* (MH765524), and *Chrysomya rufifacies* (GQ912623). The GenBank data showed that four Palembang Indonesia species were highly similarity same as in Brazil, Portugal, Thailand, and Australia. *Chrysomya megacephala* is difficult to differentiate from the microscopic identification due to a high similarity, therefore, require experienced taxonomist. The results of sequencing are more useful for species identification as *C. megacephala* flies [26].

In summary, sequences obtained from the mitochondrial COII gene fragment of *Calliphoridae*, and *Sarcophagidae* which now available online can be used as an additional tool for the confirmation of flies' identification especially in forensic applications to the estimated time of the death investigation.

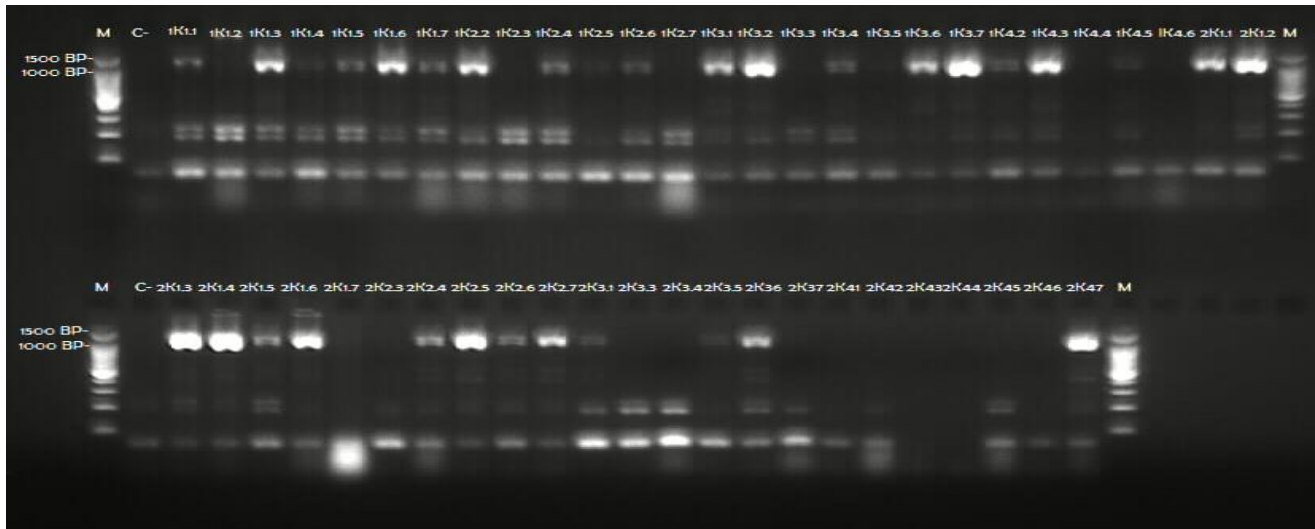


Figure 3 Agarose gel with ethidium bromide electrophoresis UV visualization of 50 samples. Positive PCR amplicons of COII were at 1324 bp. Caption: (M) marker; (C) negative control; (BP) basepair; (1K1.1-2K47) samples' numbers

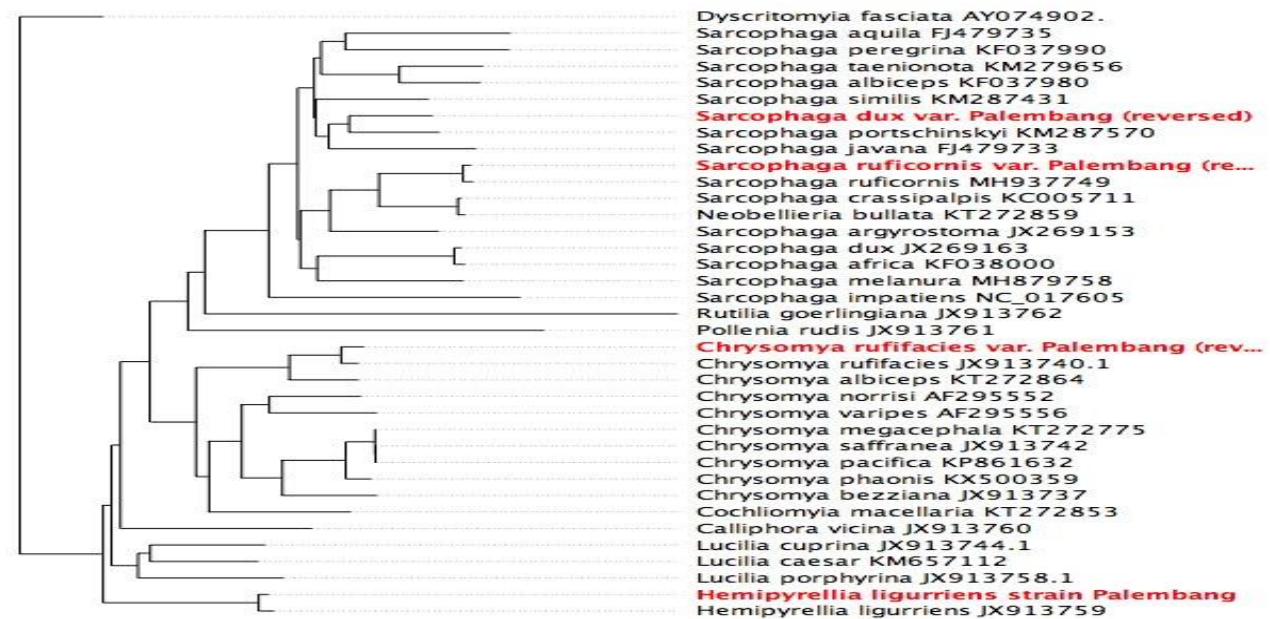


Figure 4 The phylogenetic tree using the conserved partial gene of COII showed confirmation of Calliphoridae and Sarcophagidae species. The five randomized samples of Palembang fly strain were the Calliphoridae family (*Chrysomya rufifacies* and 2 *Hemipyrellia ligurriensis*); and the Sarcophagidae family (*Sarcophaga dux* and *S. ruficornis*)

3. CONCLUSION

We concluded that molecular reconfirmation is necessary for flies related to forensic identification, while morphology identification is quite not specific to public practice. Research should further investigate the larval growth of newly discovered flies related forensic flies in a

laboratory setting, to understand the correlation with its finding in crime scene-setting.

ACKNOWLEDGEMENT

This work was supported by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia, and Universitas Sriwijaya.

REFERENCES

- [1] A.P. Rayamane, M.P. Kumar, S.D. Nanandkar, G.S. Chavan, S.S. Bhise, R. Dayananda, Medicolegal significance of postmortem lividity in determination of time since death, *JH J. Forensic Med. Toxicol.* **31** (2014) 12–17
- [2] C. Cordeiro, L. Ordóñez-Mayán, E. Lendoiro, M. Febrero-Bande, D.N. Vieira, J.I. Muñoz-Barús, A reliable method for estimating the postmortem interval from the biochemistry of the vitreous humor, temperature and body weight, *Forensic Sci. Int.* **295** (2019) 157–68
- [3] M. Hachem, B.K. Sharma, Artificial Intelligence in Prediction of PostMortem Interval (PMI) Through Blood Biomarkers in Forensic Examination-A Concept, In: *Proceeding of 2019 Amity International Conference of Artificial Intelligent (AICAI)*, 2019, pp. 255–258
- [4] N. Li, Q. Du, R. Bai, J. Sun, Vitality and wound-age estimation in forensic pathology: review and future prospects *Forensic Sci. Res.* 1790 (2008) 1–10
- [5] J.L. Metcalf, Estimating the postmortem interval using microbes: Knowledge gaps and a path to technology adoption *Forensic Sci. Int. Genet.* **38** (2019) 211–218
- [6] C. Li, Q. Wang, Y. Zhang, H. Lin, J. Zhang, P. Huang, Z. Wang, Research progress in the estimation of the postmortem interval by Chinese forensic scholars, *Forensic Sci. Res.* **1** (2016) 3–13
- [7] D. Klekovska, V. Slavevska-stamenkovi, S. Smiljkov, J. Hinić, K. Rebok, B. Janeska, Forensic use of *Chrysomya albiceps* (Wiedemann, 1819): the first cases indicating postmortem interval for human corpses in Republic of Macedonia, *J. Entomol. Zool. Stud.* **5** (2017) 320–323
- [8] L. Ren, Y. Shang, W. Chen, F. Meng, J. Cai, G. Zhu, L. Chen, Y. Wang, J. Deng, Y. Guo 2018 A brief review of forensically important flesh flies (Diptera: Sarcophagidae), *Forensic Sci. Res.* **3** (2018) 16–26
- [9] L. Lutz, K.A. Williams, M.H. Villet, M. Ekanem, K. Szpila, Species identification of adult African blowflies (Diptera: Calliphoridae) of forensic importance, *Int. J. Legal Med.* **132** (2018) 831–842
- [10] K.L. Sukontason, T. Bhoopat, A. Wannasan, N. Sontigun, S. Sanit, J. Amendt, C. Samerjai, K. Sukontason, *Chrysomya chani* Kurahashi (Diptera: Calliphoridae), a blow fly species of forensic importance: morphological characters of the third larval instar and a case report from Thailand, *Forensic Sci. Res.* **3** (2018) 83–93
- [11] M.R. Sanford, Comparing species composition of passive trapping of adult flies with larval collections from the body during scene-based medicolegal death investigations, *Insects* **8** (2) (2017) 36
- [12] Y. Ramos-Pastrana, C.A. Londono, M. Wolff, Intra-puparial development of *Lucilia eximia* (Diptera, Calliphoridae). *Acta Amaz.* **47** (2017) 63–70
- [13] R. Badenhorst, M.H. Villet, The uses of *Chrysomya megacephala* (Fabricius, 1794) (Diptera: Calliphoridae) in forensic entomology, *Forensic Sci. Res.* **3** (2018) 2–15
- [14] P.B. Waghmare, Establishing Identity and Cause of Death in Mutilated and Un Identifiable Corpses: A Challenging Task for Medico Legal Expert, *J. Forensic Biomech.* **06** (2015) 1–5
- [15] C.Y. Pai, M.C. Jien, L.H. Li, Y.Y. Cheng, C.H. Yang, Application of Forensic Entomology to Postmortem Interval Determination of a Burned Human Corpse: A Homicide Case Report from Taiwan, *J. Formos. Med. Assoc.* **106** (2007) 792–798
- [16] K. Akbarzadeh, J.F. Wallman, H. Sulakova, K. Szpila, Species identification of Middle Eastern blowflies (Diptera: Calliphoridae) of forensic importance, *Parasitol. Res.* **114** (2015) 1463–1472
- [17] T. Chaiphongpachara, T. Tubsamut, Geometric morphometry of pupae to identify four medically important flies (Order: Diptera) in Thailand, *Biodiversitas* **20** (2019) 1504–1509
- [18] K. Szpila, J.F. Wallman, Morphology and identification of first instar larvae of Australian blowflies of the genus *Chrysomya* of forensic importance, *Acta Trop.* **162** (2016) 146–154
- [19] N. Sontigun, K.L. Sukontason, J. Amendt, B.K. Zajac, R. Zehner, K. Sukontason, T. Chareonviriyaphap, A. Wannasa, Molecular analysis of forensically important blow flies in Thailand, *Insects* **9** (4) (2018) 159
- [20] S.Z. Yusseff-Vanegas, I. Agnarsson, DNA-barcoding of forensically important blow flies (Diptera: Calliphoridae) in the Caribbean Region *Peer J.* **5** (2) (2017) e3516
- [21] Y.P. Putri, Taksonomi Lalat di Pasar Induk Jakabaring Kota Palembang, *Sainmatika J. Ilm.*

Mat. dan Ilmu Pengetah. Alam 15 (2018) 105–111

- [22] ,Y.P. Putri Keanekaragaman Spesies Lalat (Diptera) dan Bakteri pada Tubuh Lalat di Tempat Pembuangan Akhir Sampah (TPA) dan Pasar, J. Tek. Lingkung. Unand 12 (2015) 79–89
- [23] E.T. Switha, C. Anwar, D. Dalilah, A. Ghiffari, Pengaruh Beda Tempat Peletakan Bangkai Dengan Pertumbuhan Larva Lalat pada Tikus (*Rattus norvegicus*), Syifa Med. 10 (2019) 46–54
- [24] A. Brundage, M.E. Benbow, J.K. Tomberlin, Priority effects on the life-history traits of two carrion blow fly (Diptera, Calliphoridae) species, Ecol. Entomol. 39 (2014) 539–547
- [25] R.M.Z.H.K. Bashah, W.O. Abdullah, M.A. Marwi, J. Jeffery, A.F.M. Saleh, B. Omar, Descriptions of the larval instars of *Chrysomya rufifacies* (Macquart) (Diptera: Calliphoridae), a species of forensic importance in Malaysia, J. Sains Kesihat. Malaysia 6 (2) (2008) 35–41
- [26] T. Monum, K.L. Sukontason, P. Sribanditmongkol, K. Sukontason, Forensically Important Blow Flies *Chrysomya pinguis*, C . villeneuvei, and *Lucilia porphyryna* (Diptera: Calliphoridae) in a Case of Human Remains in Thailand, Korean J. Parasitol. 55 (2017) 71–76