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Morphometric and DNA barcoding of fruit fly *Bactrocera dorsalis* complex from Simalungun district, North Sumatra, Indonesia

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Abstract
This investigation aims to understand the morphometric characters and DNA barcoding of *Bactrocera* fruit flies, which originated from citrus plantations in Simalungun, North Sumatra, Indonesia. The research goals also to comprehend the relation between fruit flies' body length and other morphometric features. Fly trapping was done in Baringin Raya village using the Steiner trap. Classic taxonomy and DNA barcoding methods have been used to identify fruit flies. The character of morphometric: The length of the body (Y), the length of the wing (X_1), the width of the wing (X_2), and the length of tibia (X_3) were observed and measured under a microscope. DNA barcoding has been done by isolation, amplification, electrophoresis, sequencing, and blasting. MEGA XI software was used to blast the PCR product into the NCBI gene bank. The calculation of morphometric traits and their relations were determined using SPSS v. 23. The research finding revealed that the fruit flies collected from oranges plantation species were *Bactrocera dorsalis* complex. The body, wing, tibia length, and wing width of obtained fruit flies (n=30) were 6.91, 6.36, 1.98, and 2.43 mm, respectively. The equation of multiple regression that stated the relation among body length (n=30) into other morphometric features is Y = 0.24 X1+ 0.58 X2 + 3.91 (R2=73.20). Wing length and wing width are traits that have the highest value in determining a fruit fly's length body. The size of the nucleotide base chain of obtained *B.dorsalis* fruit fly was about 690 bp.

Keywords: morphometric, mtCOI gene, bactrocera dorsalis complex, simalungun

Introduction

Simalungun, Karo, and Dairi are central districts producing fruit and vegetables in North Sumatra, Indonesia. At the last time, fruit production, especially oranges or citrus fruit from that region, decreased. This is because fruit flies have been reported to attack the citrus fruit on the crops. The decreasing citrus production correlated with the increasing fruit fly infestation. Manurung *et al*. (2022 & 2020) [1, 2] have stated that the infestation of fruit flies just in the Karo district has damaged 17.000 ha of oranges plantation and caused citrus production became only 20 tons ha^{-1} [1] [2]. In this case, the female fruit flies punctured the soft fruits with their ovipositor organ and put the eggs under the fruit's skin. After some days, maggots will hatch from the eggs and enter deeper into the fruit. This situation causes destroying and rotting of the citrus fruit and, in the end, causes the fruit to fall to the ground.

The results of fruit fly investigations have shown that the fruit fly that attacks oranges crop and causes yield losses in North Sumatra, Indonesia, belongs to the *Bactrocera* genus. The fruit fly species that have been successfully identified were *B. dorsalis*, *B. umbrosa*, and *B. caudatus* [3] [4] [5]. This *Bactrocera* fruit fly belongs to the Tephritidae family of the Diptera order [6].

Regarding fruit fly identification that is infested on fruit and vegetable crops, the genetic (molecular) approach has been used by some experts in order to confirm or support the result of the classic or conventional approach, namely identification based on morphology and anatomy. The use of mt COI gene (mitochondrial cytochrome oxidase subunit I) as a molecular marker in animal taxonomy have been implemented by some researcher in order to determine *Bactrocera* species [2, 4, 5, 7].

As an insect that can act as a potential pest on horticulture crops, especially on citrus plantations, the comprehensive comprehension of fruit flies' morphology, morphometric and genetic traits are essential. In addition, this information is needed to control and monitor its population in supporting integrated pest management activities.

This research goal is to obtain the morphometric and genetic features of fruit flies that attack citrus plants in the Simalungun district according to classic/conventional taxonomy and molecular or DNA barcoding ways. This investigation's aim is also to know the relation between fruit fly morphometric traits into its body length that until this study has never been reported.

Materials and Methods

1. Research location and catching time

The sample of fruit flies which are the object of this investigation, was collected on the citrus plantation that be found in Baringin Raya village at Simalungun district, North Sumatera, Indonesia (E: 948019° 98.846297° 104°; W: 948001°098.846326° 278°), at 997.8 m above sea level. The catching of fruit flies took place in June 2022.

2. Catching, identification and morphometric traits assesment

The fruit flies as objects of the study were caught using Steiner traps (modification) in the citrus plantation. Cotton in the traps was given methyl eugenol lure [1] [2] [8]. The obtained fruit flies were conserved in alcohol, tagged, and brought to the laboratory for curation and identification. Determination of fly species based on morphology traits was done under the stereo microscope in the invertebrate taxonomy laboratory of the Biology Department,

Universitas Negeri Medan. The species identification refers to Siwi *et al*. (2006) [9] and Hasinu *et al*. (2020) [10].

Furthermore, the assessment for morphometric traits was done according to Manurung *et al*. (2022, 2021) [1] [2]. The body length, wing length/wing expanse, wing width, and tibia length of 30 fruit flies were observed and measured under Stereo Zeiss-Stemi 2000-C microscope with helping of software Carl Zeiss Imaging System Axio Vision LE Release 4.8.2. The relation among the length of the body into other morphometric features was analyzed using the stepwise multiple regression method using the software SPSS Statistics v. 23.

3. Molecular Identification

Identification based on molecular, namely DNA barcoding method (mtCOI DNA gene marker) on obtained fruit flies was done to validate or confirm the result of the morphological approach (classic/conventional taxonomy). The steps in the DNA barcoding method were referred on Manurung *et al*. (2020) [2]. In this case, the step of working consisted of extraction, amplification, electrophoresis, sequencing, and blasting. The DNA genomic of the fruit fly was extracted from the head and leg tissues using ZR Tissue and Insect DNA Mini-Prep Kit (Zymo Research, D6016). This extraction step consisted of preparing lysis cells, DNA binding, washing, and DNA elution. The result of DNA isolation was confirmed by using 1% TBE agarose. Meanwhile, for DNA/mt COI gene amplification in a thermal cycler, My Taq Red Mix (Bioline, Catalog No Bio-25043) has been used as a PCR master mix (Table 1). The primer LCO-1490 and HCO-2198 have been utilized as forward and reverse in mtCOI amplification [11]. The sequence of both primers is listed in Table 2. Furthermore, the PCR condition for amplifying the cytochrome oxidase I region are given in Table 3.

Table 1: PCR master mix for DNA amplification

Component	1 x 25µl
dd H_2O	9.5
MyTaq Red Mix, 2x	12.5
20 µmol / µl LCO 1490 Primer*	1
20 µmol / µl 2198 Primer**	1
DNA Template	1

Table 2: Oligonucleotide primer for DNA amplification

Primer name	Sequence
LCO-1490 as forward	5′-GGTCAACAAATCA TAAAGATATTGG-3′
HCO-2198 as reverse	5′-TAAACTTCAGGGTGA CCAAAAAATCA-3′

Table 3: PCR condition in amplification of mt COI

Step	Temperature (º C)	Duration	Cycles
Initial Denaturation	95	1 min	1
Denaturation	95	15 sec	35
Annealing	52	30 sec	
Extension	72	45 sec	
Hold	4	∞	1

PCR amplification result was purified using Zymoclean Gel DNA Recovery Kit (Zymo Research, D4002) and assessed by electrophoresis with 1% TBE agarose. The running agarose took place at 100 volts for 60 min (Wealtec). The purified PCR amplification product was sequenced with Bi-directional Sequencing using an ABI PRISM 3730 XL Genetic Analyzer and took place at the genetic lab of PT Genetika Science Indonesia, Jakarta. The existing mtCOI gene fragments were aligned by employing ClustalW-MEGA XI. The sequencing homology was evaluated using the BLAST program at the National Center for Biotechnology Information (NCBI) website. The sequence homology was interpreted by comparing the Simalungun fruit fly COI sequence with the NCBI Gen Bank database.

Results and Discussion

1. Fruit flies species based on morphology and molecular (mtCOI) gene traits

Based on the result of the observation done on the morphology of received fruit flies, the species of fruit flies that are trapped on oranges/citrus crops in the Simalungun district belongs to the *Bactrocera dorsalis* complex. The confirmation of this fruit fly species was based on the traits found on its head, thorax, leg, wing, and abdomen. On the face of the fruit fly could be found a dark spot, especially in its antennal furrow.

There is reduced chaetotaxy on the head and thorax. At the scutum, there are anterior supra-alar setae and prescutellar acrostichal setae. The colour of its scutum is predominant black; meanwhile, its lateral is yellow. At its wings, the costal band is confluent and overlaps with R2+3. The subcostal vein is abruptly bent forward at nearly 90o. The abdominal tergites have a distinct black T-shaped mark. On terga III and IV could be found a narrow medial dark section. Triangular ends of the lateral bands could be found on its abdomen. On the tergit of males, there is the row of setae or pecten [8] [9] [10]. Furthermore, the result of the PCR product of fruit fly *Bactrocera* sample 1 and 2 in photo gel was displayed in Figure 1. A single fragment of approximately 700 bp was amplified for *Bactrocera*. This fragment has a size of 690-691 bp, and their sequence is as in Figure 2.

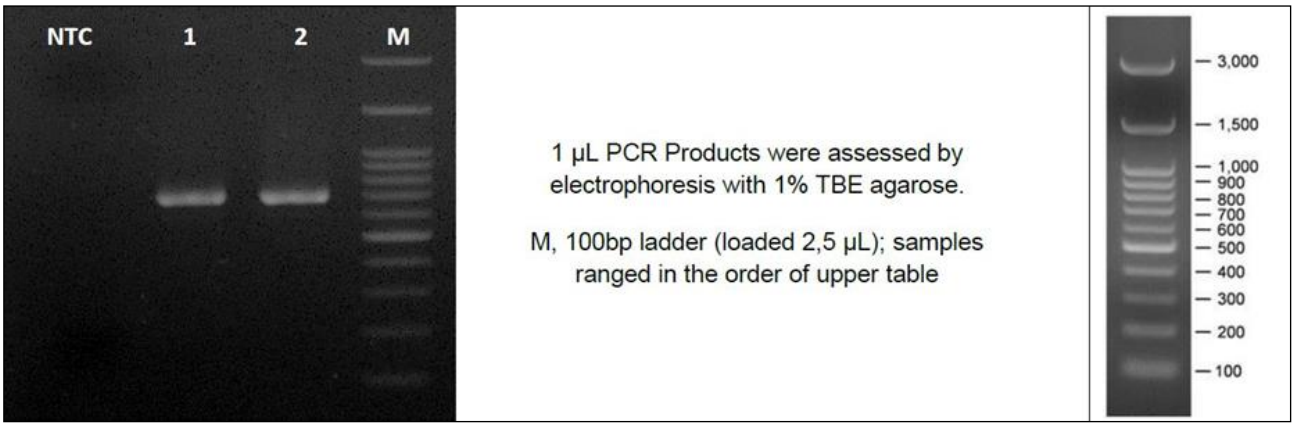

Fig 1: Gel photo of PCR product of fruit fly originated Simalungun district (sample 1 and 2), North Sumatra

Sequence Assembly 690 bp

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1     GGTGACCAAA AAATCAAAAT AAATGTTGGT AAAGAATAGG ATCTCCTCCT CCGGCAGGGT
61    CAAAAAAGGA AGTATTTAAG TTTCGGTCTG TTAGTAGTAT AGTAATAGCC CCTGCTAAAA
121   CTGGTAATGA TAATAAAAGT AATAAAGCTG TTAATACAAC TGCTCAAACG AATAGAGGTA
181   TTCGATCAAA AGTGATTCCT GTTGATCGTA TATTAATTAC TGTTGTAATG AAATTTACTG
241   CTCCTAAAAT TGAGGAAATA CCCGCTAAGT GAAGTGAAAA AATAGCTAGG TCAACTGAAG
301   CTCCTCCGTG TGCAATAACA GATGATAGGG GTGGGTAAAC TGTTCAACCT GTACCAGCTC
361   CGTTTTCTAC TATACTTCTT ACTAATAGTA ATGTAAGGGA AGGAGGTAAT AATCAAAATC
421   TTATATTATT CATTCGTGGA AATGCTATAT CGGGAGCTCC TAATATTAAA GGAACAAGTC
481   AATTTCCAAA TCCACCAATT ATAATTGGTA TAACTATAAA GAAAATTATT ACGAAAGCAT
541   GAGCTGTTAC AATTACATTA TAAATTTGAT CGTCACCGAT TAAAGCTCCT GGGTGACCGA
601   GTTCAGCTCG GACTAAAATT CTAAGGGATG TTCCTACTAT TCCTGCTCAG GCTCCGAAGA
661   TAAAATATAA AGTTCCAATT ATCTTTATGA
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Sequence Assembly 691 bp

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1     CAGGTGACCA AAAAATCAAA ATAAATGTTG GTAAAGAATA GGATCTCCTC CTCCGGCAGG
61    GTCAAAAAAG GAAGTATTTA AGTTTCGGTC TGTTAGCAGT ATAGTAATAG CCCCTGCTAA
121   AACTGGTAAT GATAATAAAA GTAATAAAGC TGTTAATACA ACTGCTCAAA CGAATAGAGG
181   TATTCGATCA AAGGTGATTC CTGTTGATCG TATATTAATT ACTGTTGTAA TGAAATTTAC
241   TGCTCCTAAA ATTGAGGAAA TACCCGCTAA GTGAAGTGAA AAAATAGCTA GGTCAACTGA
301   AGCTCCTCCG TGTGCAATAA CAGATGATAG GGGTGGGTAA ACTGTTCAAC CTGTACCAGC
361   TCCGTTTTCT ACTATACTTC TTACTAATAG TAATGTAAGG GAAGGAGGTA ATAATCAAAA
421   TCTTATATTA TTCATTCGTG GAAATGCTAT ATCGGGAGCT CCTAATATTA AAGGAACAAG
481   TCAATTTCCA AATCCACCAA TTATAATTGG TATAACTATA AAGAAAATTA TTACGAAAGC
541   ATGAGCTGTT ACAATTACAT TATAAATTTG ATCGTCACCG ATTAAAGCTC CTGGGTGACC
601   GAGTTCAGCT CGGACTAAAA TTCTAAGGGA TGTTCCTACT ATTCCTGCTC AGGCTCCGAA
661   GATAAAATAT AAAGTTCCAA TTATCTTTAT G
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Fig 2: Consensus sequences of 690-691 bp fragment from the mtCOI gene for fruit fly samples from Simalungun district, North Sumatera

The blasting result of the above mtCOI gene fragment on NCBI gen bank revealed that the Simalungun sequence has high similarity until 99.85% with the mtCOI sequence of fruit flies *Bactrocera dorsalis* that originated from Papua New Guinea (MG689794.1, MG689795.1, MG689801.1, MG689802.1, MG689803.1) (Table 4). The result of that blasting showed that the Simalungun fruit fly was *Bactrocera dorsalis* species. The result of this study displayed that the use of the DNA barcoding method, primarily through the use of the mtCOI DNA gene as a marker, has corroborated the classic or conventional taxonomy that is based on morphology traits [2] [4] [5] [7]. The result of homologous about 99.85% between Simalungun, Indonesia, and Papua New Guinea gives meaning that geographical distance and ecological differences between both countries may not significantly affect the mtCOI DNA gene variation created in the two populations. Therefore, both fruit fly populations (Indonesia and Papua New Guinea) probably have the same ancestor.

Table 4: The Result of BLASTN analysis on Simalungun fruit flies sample

Species	Accession number	Query Cover	Percent Identity
Bactrocera dorsalis COI	MG689794.1	98%	99.85%
Bactrocera dorsalis COI	MG689795.1	98%	99.85%
Bactrocera dorsalis COI	MG689801.1	98%	99.85%
Bactrocera dorsalis COI	MG689802.1	98%	99.85%
Bactrocera dorsalis COI	MG689803.1	98%	99.85%

2. Morphometric traits of *Bactrocera dorsalis* fruit fly

The result of the investigation about the morphometric features of fruit flies *Bactrocera dorsalis* originating from the Simalungun district were presented in Table 5. The mean size of body length, wing length, wing width, and tibia length were 6.91, 6.36, 2.43, and 1.98 mm, respectively. In comparison to the body length of *Bactrocera dorsalis* from India, as stated by Sharma and Gupta (2018) [6], the size of the Simalungun fly was smaller and shorter, whereas

compared with *Bactrocera dorsalis* originated from Karo district-North Sumatera was bigger and longer [1]. Meanwhile, compared to *Bactrocera umbrosa* size from Namoriam village, Deliserdang district, North Sumatra[2], the size of obtained *Bactrocera dorsalis* in this study was shorter or smaller.

Table 5: Morphometric characters of *Bactrocera dorsalis* from Simalungun district in North Sumatra, Indonesia

No	Morphological traits	Mean ± SE (n=30) (mm)	Range (mm)
1	Body length-BL (Y)	6.91 ± 0.05	6.5-7.5
2	Wing length-WL (X_1)	6.36 ± 0.06	5.9-7.0
3	Wing width-WW (X_2)	2.43 ± 0.05	2.0-3.0
5	Tibia length-TL(X_3)	1.98 ± 0.01	1.9-2.1

3. The relation among *B. dorsalis* body length into other morphometric features

The equation of regression that showed the relation among body length (Y) and other morphometric features (X_1 to X_3) of *the B.dorsalis* fruit fly is presented in Table 6. The relation among body length (Y) with wing length (X_1) and wing width (X_2) is stated in equation Y=3.91+0.24X1+0.58X2. According to this multiple regression, two morphometric attributes significantly contributed to the determination of fruit fly body length (Y). Both traits were wing length (X_1) and wing width (X_2). This study finding is in line with the research result reported by Manurung *et al*. (2022) regarding *B.dorsalis* morphometric originating from the Karo district [1]. The contribution of wing length as the dominant determinator was 57.50%, and the wing width was 73.20%. Meanwhile, the investigation finding in this research was very different compared to the fruit fly *Bactrocera umbrosa*. Manurung *et al*. (2020) wrote that tibia length was a morphometric trait that significantly contributed in determination of *B.umbrosa* body length with a value of 56.80% [2].

Table 6: Regression equation and determinant coefficient of morphometrical traits in the determination of Simalungun fruit flies *B. dorsalis* body length

No	Regression equation	Determinant coefficient (R^2)
1	$Y = 2.81 + 0.64X_1$	57.50%
2	$Y = 3.91 + 0.24X_1 + 0.58X_2$	73.20%

Conclusion

The flies collected from the citrus plantation at Simalungun district, North Sumatra, Indonesia, were identified as *Bactrocera dorsalis* complex both based on its morphology character and DNA barcoding or the sequence of cytochrome oxidase I gene. The size of its nucleotide base chain was 690-691 bp. The body length, wing length, wing width, and tibia length of obtained fruit flies were 6.91, 6.36, 2.43, and 1.98 mm, respectively. The wing length (57.50%) was the feature with the highest contribution in determining fruit fly body length.

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