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BIODIVERSITY OF *DROSOPHILA* SP. FROM THE NATURAL ENVIRONMENT BASED ON THE CYTOCHROME OXIDASE SUBUNIT 1 GENE

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ABSTRACT

Research on insect phylogenetics is intricated by their similar morphology and significant genetic diversity. The cytochrome oxidase subunit 1 (CO1) gene is the most widely utilized mitochondrial DNA gene in the identification and study of animal molecular biodiversity. This study aims to identify and reconstruct the phylogeny of fruit flies from North Sulawesi using the cytochrome oxidase subunit 1 (CO1) gene. Fruit flies were obtained from 5 (five) areas in North Sulawesi, namely Siau (L1), North Minahasa (L2), Minahasa (L3), Southeast Minahasa (L4), and Bolaang Mongondow (L5). Fruit fly imago limbs were used as a tissue source for genomic DNA extraction. Genomic DNA extraction was carried out using the Quick-DNA™ Miniprep Kit manufacture protocol. The CO1 gene amplification was carried out by the PCR method, and the visualization of the amplicons was carried out by the 1.5% gel electrophoresis method. Nucleotide sequencing used a sequencing service at First BASE Singapore with a bidirectional sequencing method. CO1 gene amplification of each sample was visualized at 690 bp to 702 bp length. After analyzing the CO1 gene concession area using the MEGA XI program, it is found that Drosophila at L1 has 702 bp, L2 has 703 bp, L3 has 698 bp, L4 has 700 bp, and L5 has 697 bp. Based on alignment analysis using the BLAST method, it is found that the L1 fruit fly has a similarity rate of 99.29% (E=0.0) to *Drosophila parapallidosa* [MK659836.1]. The L2 fruit fly also has a similarity rate of 96.86% with *Drosophila parapallidosa* [MK659836.1]. The L3 fruit fly has a similarity level of 94.94% with *Drosophila parapallidosa* [MK659836.1]. The L4 fruit fly has a similarity rate of 94.43% with *Drosophila parapallidosa* [MK659836.1]. However, the L5 fruit fly shows a similarity rate of 96.86% with *Drosophila rubida* [EU493593.1]. The reconstruction results with the MEGA XI program using the Minimum Evolution model obtain two monophyletic groups where the fruit fly in Bolaang Mongondow is in a monophyletic group different from other fruit flies. The results of this study prove the variation in fruit fly species in North Sulawesi based on the identification of the CO1 gene.

Keywords: CO1 gene; fruit flies; phylogeny reconstruction; North Sulawesi

INTRODUCTION

The family Drosophilidae consists of more than 3,750 species worldwide. About 2000 species are members of the genus Drosophila (O'Grady & DeSalle 2018; Khali et al., 2022). Drosophila species are famous for their extensive use in genetic, biomedical, biophysical, and other studies (Nourmohamad et al., 2017; Yamaguchi & Yoshida, 2018; Mirzoyan et al., 2019). Fruit flies are also known as pests on fruit crops. Fruit flies can be found easily in the tropics. The habitat of fruit

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flies on fresh fruit or rotten fruit residues in fruit markets, grocery stores, restaurants, and even on fruit crops. Drosophila is used in many genetic studies due to its small size, short life cycle (10 - 14 days at 25^0C), high reproduction rate (an adult female can lay 400-500 eggs in 10 days), and inexpensive ease of cultivation.

Further, the diversity of Drosophila is very high, and the population can be bred in large quantities quickly. Genetic variations in mitochondrial and nucleus DNA genes are also widely objected to in genetic studies. The fruit fly *Drosophila melanogaster* is a versatile model organism used in biomedical research for more than a century to study various phenomena. There are many technical advantages of using Drosophila over vertebrate models; Drosophila is easy and inexpensive to culture in laboratory conditions, has a much shorter life cycle, produces many externally laid embryos, and can be genetically modified in various ways. Genome mapping that has been successfully carried out shows that Drosophila has a genetic similarity of about 75% to humans (Irion & Nüsslein-Volhard, 2022).

Molecular identification of fruit flies based on mitochondrial DNA has been successfully carried out. 164 species of the family Drosophilidae were discussed using the Gene Amyrel, a member of the multigene-amylase family. Analysis of fruit fly phylogeny based on the CO1 gene in *D. ananassae* is recommended to be supplemented with core DNA genes, including Gpdh (glycerol-3-phosphate dehydrogenase) (Dzaki & Azzam, 2019). Likewise, the molecular identification of Drosophila (subgenus Sophophora) obtained a good phylogeny construction when combining mitochondrial DNA genes and genes from core DNA (Rand et al., 2022; Suvorov et al., 2022). Drosophila sp, which was almost morphologically indistinguishable, was successfully identified using mtDNA (Parakatselaki & Ladoukakis, 2022). However, the molecular identification of fruit flies in Indonesia is still little reported. As a tropical country with a very high diversity of fruit plants, it is believed that many species of fruit flies native to Indonesia have not been identified.

Moreover, local fruit flies live naturally in Sulawesi, an area with high species endemic. North Sulawesi has many endemic fruit plants as a food source for fruit flies, including *Ficus minahassae*, Pala Sanger, Pakoba, Langsat, and other fruit plants. It is believed that fruit flies have an excellent coevolution ability with food source plants. North Sulawesi has many types of plant fruits and endemic fruits. The molecular identification of fruit flies is necessary for biodiversity studies and can be used as an object of genetic studies in college and high school. It has been carried out to reconstruct the phylogeny of the North Sulawesi fruit fly based on the cytochrome oxidase subunit one gene (CO1).

Geographical isolation and natural selection greatly influence the species diversity of Drosophila sp (Asada et al., 2015; Andreazza et al., 2017). Sister species studies of *D. mojavensis* and *D. arizonae* in South America and Mexico strengthen the hypothesis that the geographic isolation of Drosophila causes diversity and speciation (McGiir et al., 2017). Heterogeneity in Drosophila populations in an area amplifies intraspecies variation and diversity (Curtsinger, 2020). A study on 1500 generations of Drosophila selection from nature and the laboratory concludes that geographic isolation can lead to reproductive isolation (Kezos et al., 2022; Robinson et al., 2023). Furthermore, food preferences, nutritional content, and climate affect Drosophila's adaptive evolution, which lives in a specific area (Stockton et al., 2019; Xiao et al., 2019; Bitner et al., 2021). Furthermore, global climate change also significantly affects terrestrial insect adaptation patterns, especially regarding food preferences, behavior, gardening, and phenology (Kellermann & Heerwaarden, 2019; Wilson & Fox, 2021; Rudman et al., 2022). Drosophila sp. in North Sulawesi, both from the mainland and islands, is not a local species. The above research results strengthen the hypothesis that there is a local Drosophila genetic variation in North Sulawesi. Therefore, this study aims to determine the species diversity of Drosophila sp. in North Sulawesi based on the CO1 gene.

METHODS

This research used a descriptive method. This descriptive study described the results of *Droshopila* sp DNA analysis, where the research data were obtained through laboratory experiments. Data from DNA analysis based on the tool's output was interpreted descriptively using bioinformatics applications. DNA extraction used the kit method with a protocol based on the manufacturing kit. The researchers modified several stages of DNA analysis. The CO1 gene was a marker in identifying and analyzing Drosophila diversity. The CO1 gene was selective in differentiating intra and interspecies in animals (Mege et al., 2019; Aminisarteshnizi, 2022; Sittenthaler et al., 2023).

Fruit fly isolate samples were obtained from East Siau District, Sitaro Regency, Dimembe, North Minahasa Regency, Langowan, Mina-

hasa Regency, Ratahan, Southeast Minahasa Regency, and Lolak Bolaang Mongondow Regency. In each location, ten individual imago fruit flies were preserved in 95% alcohol for 24 hours.

Figure 1. Location of Origin of Fruit Fly Isolated Samples: L1 (East Siau, Sitaro Regency), L2 (Dimembe, North Minahasa Regency), L3 (Langowan, Minahasa Regency), L4 (Ratahan, Southeast Minahasa Regency), L5 (Lolak, Bolaang Mongondow Regency)

Extraction and Purification of DNA

Genomic DNA was extracted using two to three legs from one side of the fruit fly to preserve the remainder of the dried specimen for future reference. The entire thorax was extracted when the specimen was damaged or incomplete. The tissue was homogenized (SPEX Sample Prep 1600 Mini G) and then ingested overnight at 56◦ C. According to the manufacturer's specifications, DNA extraction was performed using a Quick-DNA™ Miniprep Kit. Total DNA was transmitted on a Genomic Lysis Buffer of 500 µL and stored at −20◦C. Protocol modifications were made during the time of tissue immersion with K-proteinase. DNA extraction was done by analyzing purity and concentration at A260/A280 nm, using NanoPhotometer, Implant.

CO1 Gene Amplification and Amplicon Visualization

A 700 bp fragment of the mitochondrial cytochrome c oxidase subunit 1 (COI) gene was targeted for amplification using the following primary pairs: **HCO**, 5' -TAAACTTCAGGGTGACCAAAAAATCA-3' (Rach et al., 2017; Russell et al., 2022); **LCO**, 5' GGTCAACAAATCATAAAGATATTGG-3' (Russell et al., 2022). The polymerase chain reaction was prepared in a 20 µL reaction consisting of PCR amplification with (2x) My Taq HS Red Mix (Bioline, BIO-25048), one µL of each primer (resulting in a final concentration of 0.5 µM), template DNA of 4 µL and four µL of H2O. The PCR conditions applied are as follows: initial denaturation for 10 s at 98◦C, five cycles of 98◦C for 8 s, 50◦C for 15 s, and 72◦C for 30 s, followed by 35 cycles of 98◦C for 8 s, 55◦C for 15 s, 72◦C for 30 s, and a final extension of 72◦C for 1 minute. The reinforced PCR amplicons were then examined on a 1.5% agarose gel stained with GelRed (Biotium Inc., 46117 Landing Parkway Fremont, CA, USA).

Sequencing

The result of amplifying the CO1 gene using the PCR method, as much as 100 ml, was used as a sequencing template. Sequencing was carried out through Singapore's First BASE sequencing service. Sequencing was carried out using the Bi-directional method. Product sequencing was in the form of a seq file to be analyzed using Bioinformatics software.

Bioinformatic Analysis

Sequence analysis using Geneous Program 9.0. Alignment was done using the Basic Local Alignment Searching Test (BLAST) on the NCBI website (www.ncbi.com). Reconstruction of phylogeny used the MEGA XI program. The model of the phylogeny tree was determined by the analysis of the substitution model.

RESULTS AND DISCUSSION

Genomic DNA extraction of fruit fly limbs (Figure 2) was successfully performed using The Quick-DNA™ Miniprep Kit.

Figure 2. Imago samples of Drosophila as a source of DNA extraction tissue, observed with Stereo Microscope 3D Digital Hirox KH 8700 at a magnification of 150 x

The highest total DNA concentration was obtained in the L1 sample (102.7 ng/µl) while the lowest concentration was in the L2 sample (12.6 ng/µl). All samples showed good total DNA purity at a distribution of 2.05 to 2.22 (A260/280) (Table 1).

Table 1. DNA Concentration and Purity Measured with a Nanodrop Spectrophotometer

No	Sample	Conc. (ng/µl)	$A_{260/280}$	$A_{260/230}$	Volume (µl)
1	L1	102.7	2.22	2.10	35
2	L2	12.6	2.09	0.26	35
3	L3	23.4	2.05	1.36	35
4	L4	52.3	2.19	1.90	35
5	L5	20.5	2.22	0.63	35

As evidenced by electrogram visualization of electrophoresis results, amplifying the CO1 gene of fruit flies from all five samples was successfully carried out. The formed band is clearly at a length of about 700 bp (Figure 3).

Figure 3. CO1 Gene Electrograms of Fruit flies Isolated 1 (L1: Siau), 2 (L2: Dimembe North Minahasa, 3 (L3: Langowan, Minahasa), 4 (L4: Ratahan, Southeast Minahasa), 5 (L5: Poigar Bolaang Mongondow)

The nucleotide sequencing results after assembly obtained each sample's nucleotide length, namely L1 702 bp, L2 703 bp, L3 698 bp, L4 700 bp, and L5 697 bp. Based on the electrogram of sequencing results, the sequencing process was progressing well, as evidenced by the absence of a chromatogram crush of each nitrogenous base visualized with the MEGA XI and Geneous programs.

Figure 4. The Sequencing of the CO1 Gene of Fruit Flies in North Sulawesi

The CO1 gene sequence was then used as a template for alignment analysis with similar sequence data in the gene bank. Alignment using the BLAST method on the NCBI site obtained 100 sequences reported in the NCBI bank gene. The ten sequences with the highest degree of similarity are further shown in the BLAST result figure (Figure 5). Blast results show that the L1 fruit fly has a similarity rate of 99.29% (E= 0.0) with *Drosophila parapallidosa* [MK659836.1]. The L2 fruit fly also has a similarity rate of 98.86 % with *Drosophila parapallidosa* [MK659836.1]. The L3 fruit fly has a similarity rate of 94.94 % with *Drosophila parapallidosa* [MK659836.1]. The L4 fruit fly has a similarity rate of 94.43 % with *Drosophila parapallidosa* [MK659836.1]. However, the L5 fruit fly shows a similarity rate of 96.86 % with *Drosophila rubida* [EU493593.1]. Thus, the L5 fruit fly shows a different species similarity than the other four fruit flies (Table 2).

Table 2. The Most Similar Species to the CO1 Gene of Fruit Flies from North Sulawesi based on BLAST: results at the NCBI gene bank (https://blast.ncbi.nlm.nih.gov/Blast.cgi#)

Sample	Max Score	Total Score	Query Cover	E value	Per. ident	Acc. Len	Accession	Species Name
L1	1262	1262	**99%**	0	**99.29**	15827	MK659836.1	*Drosophila parapallidosa*
L2	1245	1245	**99%**	0	**98.86**	15827	MK659836.1	*Drosophila parapallidosa*
L3	1079	1079	**99%**	0	**94.94**	15827	MK659836.1	*Drosophila parapallidosa*
L4	1267	1267	**100%**	0	**99.43**	15827	MK659836.1	*Drosophila parapallidosa*
L5	1240	1240	**100%**	0	**98.85**	2008	EU493593.1	*Drosophila rubida*

The phylogeny tree reconstruction was carried out with the MEGA XI program. Sequences were sequenced to reconstruct phylogenies. Based on the phylogenetic construction of the CO1 gene of the fruit fly, the fruit fly from Bolaang Mongondow does not belong to one monophyletic clade with four fruit flies from different regions. The Siau (Sitaro) fruit fly has the closest phylogenetic relationship with the fruit fly of the partner Ratahan. Subsequently, fruit flies from North Minahasa form a monophyletic clade with fruit flies from Sitaro and partners but are on separate nodes. Based on the CO1 gene, fruit flies from North Minahasa have had different phylogeny relationships. Minahasa fruit flies are also in a monophyletic group with Sitaro, North Minahasa, and Southeast Minahasa fruit flies but are in different nodes. This shows that Minahasa fruit flies have differences in CO1 gene DNA sequences with one other monophyletic group (Figure 5). From the results of this study, it can be concluded that there is a genetic diversity of Minahasa fruit flies based on the CO1 gene sequences. Further research must be carried out using other mitochondrial DNA barcode genes and genes from core DNA. In Figure 5, it is shown that the time tree of the North Sulawesi fruit fly is built with the MEGA XI Program. Timetree is a phylogenetic tree that is scaled over time. It shows the evolutionary relationship of a group of organisms within the temporal framework. The main result of molecular dating, the time tree, provides important information for understanding the historical evolution of bloodlines and is a requirement of some evolutionary analyses.

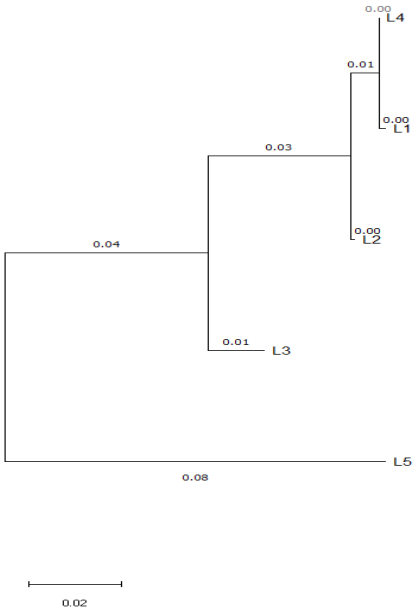

Figure 5. Reconstruction of the North Sulawesi Fruit Fly Phylogeny Tree with the Minimum Evolution Method Using the MEGA XI Program

The success of DNA extraction is highly dependent on sample preparation and the determination of primers for amplification using the PCR method (Mege et al., 2019; Suddin et al., 2019; Rombot & Mokosuli, 2021). Insect DNA extraction has difficulty because the exoskeleton has many molecules that can contaminate DNA (Marquina et al., 2021; Kirse et al., 2023). DNA extraction of Drosophila sp. from North Sulawesi was successfully done by modifying the extract kit protocol, especially in the protease enzyme treatment. Modifications are needed to minimize contamination that can reduce the concentration and purity of extracted DNA. The CO1 gene amplification was successfully carried out using universal primers, indicating that the level of selectivity of these primers can still be used in Drosophila (Jones et al., 2020; Piper et al., 2022). However, it is necessary to use several barcode genes to ensure the species' position further. Mitochondrial DNA shows recent gene flow across species boundaries (Revolson et al., 2019; Ishikawa et al., 2022). Using CO1 for reconstructing the Drosophila phylogeny resolves most of the basic relationships within the melanogaster species group and provides a framework that can be expanded to include more species (Jezovit et al., 2017). The advantages of the CO1 gene as a molecular barcode include that the universal primary primer has an extreme sensitivity that can cover the 5' end of almost all animal taxonomies (Folmer et al., 1994; Andújar et al., 2018); furthermore, the CO1 gene has a more pronounced phylogenetic signal than other mitochondrial DNA genes (Karthika et al., 2021; Manchola et al., 2021). The rapid and useful evolution of the CO1 gene shows differences at the level of closely related species and between phylogeographic groups within a single species (Song et al., 2021; Doorenweerd et al., 2022).

The genus Drosophila is paraphyletic concerning several other genera, but there is still much uncertainty about different aspects of the phylogeny (Koshikawa, 2020; Li et al., 2022). Genes in mitochondrial DNA are still markers sensitive enough to analyze intraspecies variation in Drosophila (Camus et al., 2017). The mitochondrial genome is represented by circular double-stranded DNA molecules with 16 to 19 kb length. The mitochondrial genome does not contain introns involved in recombination. The entire mitochondrial genome can be divided into three parts: (1) protein-coding genes; (2) genes encoding rRNA and tRNA; and (3) non-coding regulatory region (region A + T). The particular importance of mutations in different mtDNA regions is dissimilar. In Drosophila, the content of A + T pairs in mtDNA is very low. The nucleotide substitution pattern is characterized by a low transition/transversion ratio (and a low mutation saturation threshold). Deletions and duplications are common in the mitochondrial genome. The rate of evolution is highly heterogeneous across the mitochondrial proteome, with NADH dehydrogenase accumulating more amino acid substitutions than the Cytochrome Oxidase. The specific evolutionary rate of this oxidative phosphorylation complex varies across lineages and may reflect physiological and ecological changes across the Drosophila phylogeny (Kellerman et al., 2018). Therefore, tracing animal genetic variation using mtDNA is still quite selective (Palozzi et al., 2018; Dapporto, 2019; Kurbalija Novičić et al., 2020; Dong et al., 2021; Antil et al., 2022; Rand, 2022). The distribution pattern of Drosophila CO1 haplotypes is geographically partitioned, which may result from limited gene flow between species groups that exhibit a more extended history of differentiation than previously hypothesized (Wolff et al., 2013; Bevers et al., 2019).

The results of this study prove that genetic variations in the CO1 gene occur in fruit flies from several regions in North Sulawesi. The position of the species on the phylogeny tree places the L5 fruit fly not in the same monophyletic group as the other fruit flies. This shows that based on the CO1 gene, Drosophila from Bolaang Mongondow has the highest genetic variation. The L3 (Minahasa), although in a monophyletic group with L2, L1, and L4, are not in the same node. This also indicates that Droshophila minahasa has genetic variations compared to other Droshophila from North Sulawesi. Drosophila, which shows the closest level of gene kinship, is L4 (Minahasa Tenggara) and L1 (Sitaro) because they are in the same node. L2 (North Minahasa) even though it is in the same monophyletic group as L1 and L4 but not in the same node, indicating that there is genetic variation in the L2 CO1 gene compared to L1 and L4. The results of this study prove that the genetic variation of North Sulawesi Drosophila based on the CO1 gene is high. However, in the future genetic variation research is needed involving multi-gene barcodes from mitochondrial DNA. The use of multi genes to further strengthens the conclusion of high genetic variation in fruit flies based on genes in mitochondrial DNA.

CONCLUSION

Based on the CO1 gene, the construction of the phylogeny of *Drosophila* sp from North Sulawesi results in two groups. Drosophila from Sitaro, North Minahasa, Minahasa, and Southeast Minahasa are in one monophyletic group. Meanwhile, *Drosophila* from Bolaang Mongondow is in the outer group. The results of the phylogeny reconstruction show the genetic variation of the *Drosophila* sp CO1 gene in North Sulawesi.

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