Agrium ISSN 0852-1077 (Print) ISSN 2442-7306 (Online)
Oktober 2025 Volume 28 No.2

Morphology and Moleculer Sequencing *Bactrocera* sp. Fruit Fly from Deli Serdang District

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Abstrack

Due to some doubts regarding the morphological identification of fruit flies, molecular identification is necessary to more clearly determine the origin of the insect. With the PCR RAPD method, which can identify genetic markers, it can effectively and quickly distinguish closely related species. RAPD is used as a tool to create genetic maps, identify strains, species, populations, and systematics of various organisms. RAPD can differentiate laboratory populations that are morphologically indistinguishable. So the purpose of this study is to more clearly determine the identity of the fruit flies found, both morphologically and molecularly. This research method is a purpose sampling method, where fruit fly samples are obtained from traps using attractants in red guava plantations. The fruit fly species found in the red guava plantation location are Bactrocera sp., and Bactrocera kinabalu. The results of the study found that the fruit fly species Bactrocera sp. found in the village of Sawit Rejo was identified morphologically as having many similarities with Bactrocera papayae except for its abdominal pattern. To confirm the taxonomic status of Bactrocera sp. For further clarification, it is necessary to use molecular identification of cytochrome oxidase 1 and phylogenetic genes, with DNA sequencing results (500 bp) showing that the Bactrocera sp. species has a homology level of 88% with Bactrocera carambolae from the Genbank collection. The phylogenetic tree shows that Bactrocera sp. has ancestors from China.

Keywords: *Bactrocera, fruit fly, moleculer, morphology, sequencing.*

Received: 24 April 2025; **Revised:** 06 September 2025; **Accepted:** 10 October 2025

INTRODUCTION

The attack on fruit that can cause great damage is of concern to the world, in Indonesia it has increased the problem of fruit commodities that cause changes in fruit attacks (Suputa et al., 2010). Identification based on morphological characteristics is less accurate due to environmental changes (Pramudi et al., 2013). DNA differences are more widely used more stable in species development compared to morphological differences (William et al., 1990). According to Putra (2005) ordinary conversations use body characters such as morphology, anatomy, behavior and physiology, utilizing the DNA base camel provided to the insect as its marker. Required from species through conventional methods needed before using DNA analysis (Anggereini, 2008).

Bactrocera (Diptera: Tephritidae) are polyphag with a broad host range, although some are known to be oligophagic and monophagic (Suputa et al., 2010). added to the statement by Drew & Romig (2012), that it is very important to identify fruit fly species because several types of fruit flies have similar and almost identical morphological forms. Examples of mophological differences that are difficult to distinguish from each other The difference between B. carambolae and B. papayae is due to the close kinship which can be seen from the shape of the size body and the wings which look the same, in contrast to other species where the wings and abdomen can be easily distinguished (Pramudi et al., 2013).

The fruit fly puparium is oval in shape and brownish-yellow in color with a length of ± 5 mm (Ditlin Hortikultura, 2006). Fruit fly imago generally has exhibits important characteristics in the head, thorax, wings, and abdomen. The head consists of antennae, spots, and eyes; the thorax consists of medial postsutural vittae and lateral postsutural vittae (Siwi et al., 2006). The wings consist of basal ribs,

microtrichiae, ribs, anal line, costal band, and wing pattern. The abdomen consists of a T pattern on the terga, whether the terga are united or not, and a color pattern on the terga. (Pramudi et al., 2013).

Fruit flies' activity in determining host plants is based on the color and aroma of fruit flies (Suputa et al. 2007). Fruit fly behavior is stimulated by chemicals known as semiochemicals, one of which is kairomones (methyl eugenol in the form of a phenylpropanoid compound) (Tan et al., 2011). Ginting (2009) stated that fruit flies can develop at temperatures of (10 – 30) ^{o}C and can develop well at humidity of (62 – 90) %. Fruit flies start to be active in the morning until noon and their activity decreases in the afternoon (Suputa et al., 2007).

Siwi et al. (2006) mention that the host plants of the Bactrocera albistrigata species are mango, guava, water apple, jackfruit, and plants of the curcubitaceae family. The host plants of the *Bactrocera tau* species are melon, guava, cucumber, and plants of the curcubitaceae family. The host plants of the fruit fly *Bactrocera carambolae* species are starfruit, water apple, guava, tomato, chili, breadfruit, jackfruit, lemon, mangosteen, sapodilla, mango, and others. The host plants of the Bactrocera papayae species are banana, papaya, guava, guava, sweet orange, sapodilla, starfruit, soursop, mangosteen, rambutan, jackfruit, mango, langsat, rambai, palm fruit, chili, eggplant, passion fruit and others (Sukarmin, 2011).

Genetic diversity refers to the variation in genes within and between species wich necessary to cope with environmental changes. The magnitude of diversity within a species depends on the number of individuals, the range of its geographic distribution, and the degree of isolation of the population (Julisaniah, 2008). Determining the value of gene flow between populations is also necessary to see the level of dispersal between these populations and to maintain genetic relationships within the population (Pratiwi, 2012). Natural selection and gene dispersal control the spatial pattern of genetic variation within populations. Over short geographic distances, gene flow affects the genetic structure within populations (Jenkins et al., 2012).

RAPDs reveal patterns of genetic diversity and provide markers specific to a species. These genetic markers can be used for population studies and inform population conservation (Jenkins et al., 2012). Elrod & Stansfield (2007) explain that genetic diversity arises due to mutation mechanisms, the free assemblage of alleles, and the migration of genes from one location to another. The use of polymerase chain reaction (PCR) is the basis of RAPD analysis, an in vitro method for adding DNA sequences and aims to analyze kinship, genetic markers, phylogenetics, and genetic mapping (Muladno, 2010).

Species DNA identification is very useful in entomology, where as it is not approved, morphological species (Pramudi et al., 2013). Information is needed about the genetic relationship between existing fruit participants and the new one, so that the source of the pest can be obtained, and controlling fruit pests can be further developed in the source of origin of fruit fly attacks (Anggereini, 2008).

Zhang et al. (2010) stated that *Bactrocera dorsalis*, a member of the subgenus Bactrocera, is monophyletic. Bactrocera dorsalis has different characteristics and forms but shares a common ancestor. This is in line with Dharmayanti (2011) statement that phylogenetic analysis aims to determine the families inherited during the evolutionary process. Individual species with higher genetic distance values indicate distant kinship, and lower genetic distance values indicate close kinship.

Large differences in genetic distance within populations may be due to ecological, distance, geographic, and reproductive isolation. If this occurs, it is possible to discover new species capable of adapting to their environment naturally over long periods (Schmitt & Karola, 2008). Lucic et al. (2011) state that the kinship between individuals, as shown in a dendogram, correlates with their genetic distance. Close relatives are those with low genetic distances, and distant relatives are those with high genetic distances.

Information about fruit fly species in Deli Serdang District is still small and there are still errors made based on morphology. This study discusses morphological and molecular fruit fly species (Gen Cox 1).

MATERIALS AND METHODS

Place and Time

This research was conducted from August 2023 until completion. Fruit flies were collected from red guava plantations in Deli Serdang Regency. Samples were taken to the Genetics and Molecular Biology Laboratory, Faculty of Mathematics and Natural Sciences, University of North Sumatra, for molecular characterization analysis.

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Materials and Tools

The materials used are attractants, 70% alcohol, Wizard Genomic DNA, Go Taq Green Master Mix, Agarose, Bench Top 100bp DNA Ladder, TAE Buffer, ethidium bromide. The tools used are: microscope, tweezers, brush, scissors, camera, thin wire, plastic container, specimen bottle, gauze, stationery, measuring cup, stirrer, dropper, label paper, Ependorf tube, micropestle, incubator, centrifuge, PCR machine, electrophoresis machine, UV illuminator.

Research Method

Insect Inventory

Insects were collected from five *Psidium guava* plantations in Deli Serdang District. *Bactrocera* sp. insects were collected by collecting fruit flies captured using a yellow device containing an attractant mixture.

Morphological Identification

Morphological identification of samples was carried out by observing the shape of the caput, thorax, wings, and abdomen of the insect through a microscope, and using reference books on fruit flies, namely: Taxonomy and Bioecology of Important Fruit Flies *Bactrocera* spp. In Indonesia (Siwi & Purnama, 2004); The Australian Handbook for Identification of Fruit Flies (Drew, 2018); Tephritid Flies (White, 1988), where sample identification was carried out at the Laboratory of the Biology Research Center of the Indonesian Institute of Sciences.

DNA Isolation

The fruit fly DNA extraction method is based on the procedure written on the Genomic DNA Wizard product, namely: the insects are ground using a mortar until smooth in cold Nuclei Lysis solution. Then the sample is placed in a tube, after which Nuclei Lysis Solution is added to the tube, and vortexed for 10 seconds. Then the sample is incubated at 65 ^{0}C for 15-30 minutes. After that, 3 µl of RNase Solution and 17.5 µl of proteinase K are added to the sample. Then the extract is mixed by inverting the tube 2-5 times, after which it is incubated at 37 ^{0}C for 15-30 minutes. The sample is cooled to room temperature. Then 200 µl of Protein Precipitation Solution is added, vortexed again for 20 s. Centrifuged for 4 minutes with a speed of 14,000 rpm. Unnecessary supernatant was discarded, the pellet solution was transferred into another tube containing 600 µl of isopropanol at room temperature. Then the solution was stirred gently. After that, it was centrifuged again for 3 minutes at a speed of 14,000 µg. Then 600 µl of 70 % ethanol at room temperature was added, while stirring gently. Then it was centrifuged again for 1 minute at a speed of 14,000 µg. The ethanol was discarded and the pellet was dried for 15 minutes. Then the 100 µl DNA solution was incubated at 65 °C for 1 hour, and the DNA sample can be stored at 4 °C.

PCR amplification

Primary used based on previous research Zhang et al. (2010) who used the Cox I gene with forward mtD7 primer (5' ATT AGG AGC 3 'HCC HAT AGC ATT) and reverse mtD9 primer (5' GAG GCA AGA TTA AAA TAT 3 CTG CTG AAA CTT '). The solution mixture, namely: put into the PCR tube as much as 12.5 µl of Go Taq Green PCR master mix, 2.5 µl of fruit fly species DNA, 8 µl of Nuclei-Free Water, 1 µl of mtD9 reverse primer, and 1 µl of mtD7 forward primer. By using 35 cycles in the PCR program settings, in Table 1.

Table 1. PCR Settings for Amplification

Process	Temp (^{0}C)	Time
Pre Denaturation	94	3 min
Denaturation	94	15 sec
Annealing	53	15 sec
Extension	70	1 min
Post Extention	72	1 min

Electrophoresis

4 µl of fruit fly DNA derived from PCR was taken, then inserted into a 1.5% agarose gel stained with ethidium bromide in 1X TAE buffer. Then, 2 µl of 100 bp DNA ladder was added as a marker. Electrophoresis was performed for 60 minutes at 80 Volts. After that, visualization was carried out in a UV illuminator to see the DNA bands.

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Sequencing

Fruit fly species identified morphologically, followed by identifying based on cox gene DNA sequences 1. DNA sequencing was carried out to determine nucleotide sequences in the cox region 1. Sequencing was carried out by Macrogen in Korea.

Sequencing results analysis

Sequences of fruit fly species DNA then alignment with the Bioedit program. Then the results of DNA sequences were matched by utilizing the DNA sequence information available in the genebank (http://www/ncbi.nlm.nih.gov). Then analyzed with Mega 5 data processing program.

RESULTS AND DISCUSSION

From the results of identification at at Laboratory there were 2 species of *Bactrocera*, namely *Bactrocera* sp. found in the red guava plantations of Sawit Rejo village and *Bactrocera kinabalu* in the red guava village of Kolam village which have morphological characters similar to *Bactrocera papayae* and *Bactrocera carambolae*. The morphology of sample can be seen from the wing and abdomen patterns, namely:

1. *B. carambolae*, the wings have black bands on the anal and costal lines, and the wingtips are shaped like fishing line. The abdomen has a clearly visible T-pattern and is rectangular in shape, and is black on terga IV.
2. *B. papayae*, the wings have clearly visible black bands on the anal and costal lines. The abdomen has a clearly visible T-pattern with a yellow-brown color. In males, there are pecten (bristles) on each side of terga III, and a pair of ceromata (spots) on terga V.
3. *B. kinabalu* has wings with a costal band that passes very thinly at the R2 + 3 section, and extends to the apex; The abdomen has a brownish-yellow T-pattern with clearly visible vertebrae, and has a black pattern that extends to terga III-IV.

P. guajava (Red guava) is known as the main host of various fruit fly species (Drew, 2016). Hosts that are only associated with one fruit fly species are *Musa sp., C. papaya, S. melongena, A. bunius, C. annum, N. lappaceum, A. comorus,* and *S. lycopersicum*. The different host ranges of each fruit fly species can be influenced by volatile chemical compounds, and the color spectrum released by the host plant, the fruit fly's perception and preference for nutrition, morphology, quantity of host plants, distribution, and interactions with other individuals (Binyameen, 2013). You can see the head, thorax, abdomen and wing pattern in Table 2.

B. papayae and *B. carambolae* are the species fruit fly with the highest populations found at the sampling location. It is known that *B. papayae* and *B. carambolae* are polyphagous pests, which means that these fruit flies can be found on many fruit plants. According to Siwi et al. (2006) the host plants of the fruit fly species Bactrocera carambolae are sapodilla, star fruit, guava, breadfruit, tomato, chili, jackfruit, cempedak, lemon, apple, mango, and others. The host plants of the species *Bactrocera papayae* are banana, papaya, guava, guava, sweet orange, sapodilla, star fruit, soursop, mangosteen, rambutan, jackfruit, mango, langsat, rambai, palm fruit, chili, eggplant, passion fruit and others (Sukarmin, 2011). Both species are important pests because they are widespread in very high populations.

Electrophoresis results from *Bactrocera* sp. shows a ribbon pattern that looks vague while *Bactrocera kinabalu* looks clearer, can be continued for the sequencing process. The DNA amplification band pattern can be seen in Figure 1.

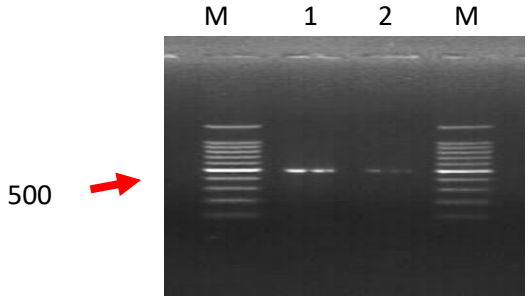

Figure 1. PCR amplification DNA results (1) *Bactrocera kinabalu*; (2) *Bactrocera* sp. with (M) DNA ladder 100 bp marker.

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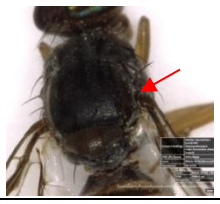
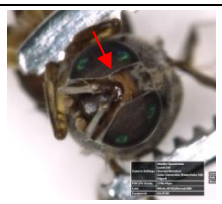
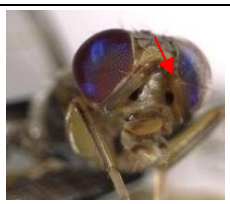
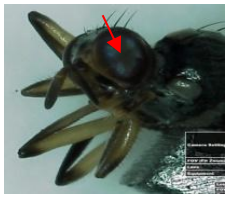
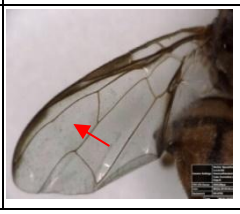
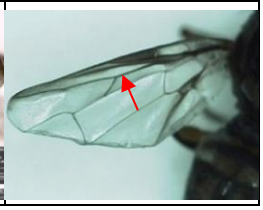
Table 2. Morphology of Bactrocera sp., B. papayae, B. carambolae, and B. Kinabalu

Character	*Bactrocera* sp.*	*Bactrocera papayae*	*Bactrocera carambolae*	*Bactrocera kinabalu**
Thorax				
Abdomen				
Head				
Wing				

Note: (Sign (*) is a sample of fruit flies to be sequenced)

The results of checking the quantity with the nanofotometer *Bactrocera* sp. and *Bactrocera kinabalu*, each of which has a purity and concentration level of 1.26 and 190; 1.98 and 1070. Good purity levels are in accordance with the comparison ratio of Sambrook & Michael (2012), namely 1.80 - 2.00. Shows that the purity level of *Bactrocera* sp. outside the ratio means that there are still contaminants in the form of phenolics, carbohydrates, proteins and RNA. According to Zein & Dewi (2013), if the concentration is too low, the primer cannot find the target. In fact, if the print DNA concentration is too high, it increases the chance of mispriming. Besides that, the purity of the DNA template is also important, because it can affect the reaction results.

Figure 1 shows the smear condition of the extraction results, indicated by several bands. This is caused by the presence of contaminants, an extraction process that is not optimal, or is the remaining solutions carried over during isolation. Mulyani et al. (2011) stated that smears can be the remaining solutions carried over during the isolation process or can also be DNA that is degraded during the isolation process. The presence of these compounds can be a factor that hinders the success of the DNA extraction process (Maftuchah & Zainuddin, 2013).

Faint bands contained in the amplified fruit fly DNA sample, as seen in Figure 1. This was due to the lack of amplification, as the primers used were suspected to be incompatible with the template DNA. According to Azizah (2009), specific primer mismatches can result in no amplified genome or amplification of other untargeted genome regions. A single base pair difference in an experiment is sufficient to demonstrate a mismatch between primer templates, preventing amplification (Asokan et al., 2007). This is reinforced by the statement by Pramudi et al. (2013) that if a change in the reaction, no matter how small, can alter the intensity and quantity of amplification products, it will be difficult to maintain replication. Muladno (2010) also added that the RAPD method cannot only differentiate between heterozygous and homozygous samples because they become difficult to detect and the dominant marker if there is a small change in DNA structure.

Bactrocera have distinct RAPD banding profiles, demonstrating the potential of the method RAPD for detecting genetic differences between Bactrocera. This means that each Bactrocera species has a

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distinct RAPD fingerprint or banding profile based on the primers used. These distinct individuals can be identified by varying levels of polymorphism, the number of amplified loci, and the diversity of RAPD loci (Jiang et al., 2014).

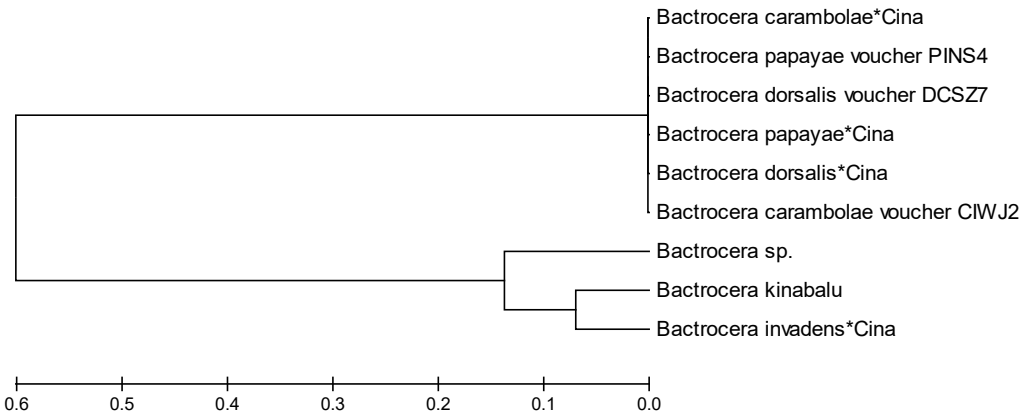

Figure 2. Dendogram of *Bactrocera* sp., and *Bactrocera kinabalu* Which are Compared with the GeneBank collection.

Species DNA sequences were processed with Mega 5 software, phylogeny construction was obtained (Tamura et al., 2007; Pramudi et al., 2013) from *Bactrocera* sp., and *Bactrocera kinabalu* samples compared to the GeneBank collection (Figure 2). Based on the phylogenetic tree analysis, kinship can be identified by occupying adjacent outgroups that form phylogenetic trees. Shows the origin of the ancestors of *Bactrocera* sp. and *B. kinabalu* originating from China are included in *Bactrocera dorsalis* complex (Oriental fruit fly) (Figure 2).

The nucleotide base sequence results from *Bactrocera* sp., and *Bactrocera kinabalu* sequencing, namely

>*Bactrocera* sp.

AA TA TA GTC TCC TCC TCC GGC AGG GTA AGA AAG GAA GTA TTT AA GT TT CG GT CT GT TA GT CA TA TA GT AA TA GC CC CT GA TA AA AC TG GG CA TG AC AA TA AA AG TA AT AA AG CT GT TA AT AC TT CT GC TC TT AC GA AT AG AG GT AT TC GA TC AA AA GT GA TT CC TG TT GA TC TC AT AT CA AT TA CT GT TG TA AT GA AA TT TA CT GC TC CT AC TT TT GA GG AA AT AC CT GC TA AC GT TT GA GC AG AA AT AG CT AG AT CA TC TG AA GC TC CA TC GT GT GC AA TA GC TT AA GA TA GG GC TG GG AC AA CT GT TC TA CC TG TA CG AG CT CC G TT TT CT AC TA TT CT TC TT AC TA AT AG TG AT GT AA GG CA AG GA GG TT AC CA TC AT AA TC TT AT GA TT CT TT GT CG TG GA AA AGT TTA TAT ATA ATT CCT TGA TAC

>*Bactrocera kinabalu*

AA TG TTG GTA AG AA TA GG GT CT CCT CC TCC GG CA GG GTC AA AAA AG GA AG TA TT TA AG TT TC GG TC TG TT AG TA AT AT AG TA AT AG CC CC TG CT AA AA CT GG TA AT GA CA AT AA AA GTA ATA AAG CTG TTA ATA CAA CTG CTC ATA CGA AT AGA GGT ATT CGA TCA AAG GTG ATT CCT GTC GAT CG TA TA TC AA TT AC TG TT GT AA TG AA AT TT AC TG CT CC TA AC AT TG AG CA AA TA CC TG CT AA GT GA AG TG AA AA AA TA GC TA GG TC AA CT GA AG CT CC AC CG TG TG CA AT AA CA GA TG ATA GGG CTG GGT CAA CT GT TCA ACC TGA ACC AAC TCC ATT TTC TA CC AT TC TT CA TA CT AA TA GT AA TG TA AG AG CA AG GA GG TT AT AA TC AT AAT CTT ATA TTA TTT ATT CA TG GA AA TG TAT ATC TTG GCT CC CT GA AT AC AG GTT TTG GGG TGT GGG TTT

Based on the results of the BLAST analysis found many nucleotide base differences in *Bactrocera* sp. with GeneBank Acc Number KF998788.1 (*Bactrocera carambolae*) with a level of similarity (homology) of 88 % (Table 2.). Morphologically *Bactrocera* sp., has similarities in the wing parts of *Bactrocera papayae* and the thorax in the *Bactrocera carambolae* collection at LIPI.

Based on the morphology of *Bactrocera kinabalu* can be identified (Figure 1), but based on the NCBI Blast results from the sequence identified *Bactrocera kinabalu* with 95 % homology level in *Bactrocera dorsalis* GenBank collection Acc Number KF998620.1 (Table 2). *Bactrocera kinabalu* is still a fly that is rarely found in the location of fruit trees in Indonesia. *Bactrocera kinabalu* is included in the Asian complex *Bactrocera dorsalis* which is still only found in the Hala-Bala Nature Reserve (Danjuma et al., 2013).

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Table 3. Results of the Blast Analysis of Sequences of Cytochrome Oxidase I DNA

	Bactrocera kinabalu	***Bactrocera* sp.**
Character	*Bactrocera dorsalis* *China	*Bactrocera carambolae* *China
Homology	95 %	88 %
Gaps	0	0
Acc Number	KF998620.1	KF998788.1
Total Score	668	497
E-Value	0.0	1e-136

Table 4. Results of the Calculation of the Genetic Distance of *Bactrocera* sp., and *Bactrocera kinabalu*

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
1. Bactrocera sp.								
2. Bactrocera kinabalu	0.283							
3. Bactrocera _ dorsalis *China	1.208	1.166						
4. Bactrocera _ papayae *China	1.208	1.166	0.000					
5. Bactrocera _ carambolae *China	1.253	1.208	0.013	0.013				
6. Bactrocera _ invadens *China	0.266	0.139	1.208	1.208	1.253			
7.Bactrocera_carambolae_voucher_CIWJ2	1.208	1.166	0.000	0.000	0.013	1.208		
8. Bactrocera _ dorsalis _ voucher _ DCSZ7	1.208	1.166	0.000	0.000	0.013	1.208	0	
9. Bactrocera _ papayae _ voucher _ PINS4	1.208	1.166	0.000	0.000	0.013	1.208	0	0

The results of genetic distance calculations analyzed using Mega 5 software (Tamura et al., 2007; Pramudi et al., 2013), are shown in Table 4. The close kinship relationship between *Bactrocera* sp. and *Bactrocera kinabalu* is evident from the analysis used in Pairwise Distance Calculation that describes the genetic distance between sample species. From the calculated data, which ranges from 0.00-0.266 (Table 4), it is a close genetic distance value so that the two individuals have a close kinship relationship. This is reinforced by the statement (Dharmayanti et al., 2011) that individuals with a low genetic distance range have close kinship relationships, and vice versa. Therefore, large differences in genetic distance can give rise to new species that are able to adapt naturally to its environment over a long period of time (Schmitt & Karola, 2008).

CONCLUSION

The fruit fly species found in the red guava plantation location are Bactrocera sp., which originates from the red guava plantation in Sawit Rejo village and Bactrocera kinabalu which originates from the red guava plantation in Kolam village. The results of the study were found that the fruit fly species Bactrocera sp. found in the village of Sawit Rejo was identified morphologically as having many similarities with Bactrocera papayae except for its abdominal pattern. To confirm the taxonomic status of Bactrocera sp. For further clarification, it is necessary to use molecular identification of cytochrome oxidase 1 and phylogenetic genes, with DNA sequencing results (500 bp) showing that the Bactrocera sp. species has a homology level of 88% with Bactrocera carambolae from the Genbank collection. The phylogenetic tree shows that Bactrocera sp. has ancestors from China.

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