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Identification of Morphology and Molecular PCR-RAPD *Bactrocera* spp. in the Location of Red Guava Crops, Deli Serdang District

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ABSTRACT

Various fruit flies are in Indonesia, but only a few species of fruit flies have been found at the red guava plant in Deli Serdang District. Knowing the species of fruit flies are needed to do the proper control. In addition to morphological identification, it is necessary to carry out molecular characterization to obtain accurate results in characterizing species differences. This study aims to identify the fruit fly based on morphology and molecularly PCR-RAPD for mapping the genetic closeness of the relationship between individual fruit flies. Bactrocera morphologically identified at LIPI, namely *B. carambolae, B. papayae, B. caudata, B. albistrigata, B. umbrosa, B. curcubitae, B. tau,* and *B. kinabalu*. For molecular identification PCR-RAPD, shows the dendrogram results from the Neighbor-Joining analysis based on RAPD markers of DNA band characters showing the genetic proximity of eight species divided into three groups of Bactrocera based on their location. The closeness of genetic relationships between individuals was analyzed using Pairwise Distance Calculation which describes the genetic distance between species. The results of Pairwise Distance Calculation ranged from 0.13-0.42. By knowing what species there fruit flies are in red guava plantations in the Deli Serdang district, it can make it easier for farmers to monitor and control fruit flies in red guava plantations.

Keywords: Fruit fly; Molecular; Morphology; Species

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INTRODUCTION

Fruit flies (Diptera: Tephritidae) are polyphagous with a broad host range. Some are known to be oligophagous and monophagous (Suputa et al. 2010). (Drew dan Romig 2013) stated that identifying fruit fly species is very important because some taxa of fruit flies have almost the same variation in morphological characters. *B. papayae* and *B. carambolae* are difficult to distinguish due to their close relationship, so the body size and wings look the same. Unlike other species, it can be distinguished directly by looking at the wings and abdomen (Pramudi et al. 2013).

Fruit flies generally have essential features on the head, thorax, wings, and abdomen (Faria et al. 2014). The head consists of an antenna, eyes, and spots. In the thorax region is the presence of medial post-sutural vittae and lateral post-sutural vittae (Manurung et al. 2020). On the wing, there are basal costal, costal, microtrichia, costal band, anal streak, and wing patterns. In the abdomen, there is the presence or absence of the T pattern in the terga, whether or not it integrates between the second terga and the color pattern in the terga (Pramudi et al. 2013).

The diversity of fruit flies is strongly influenced by the availability of host plants and the preference of fruit flies for their hosts (Manurung et al. 2020). In an area, fruit flies will move if the feed source has been reduced (Hidayat 2015). The size of the population of fruit flies in the environment is influenced by air temperature, while the abundance of fruit fly populations in the tropics is affected by rainfall (Adnyana et al. 2019). Precipitation is closely related to humidity, especially soil moisture which correlates with the chance of the appearance of fruit fly imago. This is because the final instar larvae will come out of the host tissue, then pupae in the ground (Suputa et al. 2010).

To determine genetic variation among the species investigated, using genetic markers. By using Polymerase Chain Reaction (PCR) and Random Amplified Polymorphic DNA (RAPD) methods, DNA polymorphisms can be identified, which are used as genetic markers to identify the characteristics of fruit fly populations that have been collected from different geographic locations (Jenkins et al. 2012). In addition, RAPD analysis can quickly and effectively identify genetic markers to differentiate closely related species (Kumar dan Gurusubramanian 2011).

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This RAPD technique is used to determine the geographic origin of pest insects, map the local and global genomes of a species, know the genetic diversity of an organism, and construct an organism's genetic map (Singh et al. 2011). Different species can show different levels of polymorphism, comparable to variations in the RAPD locus and the number of the amplified locus (Jiang et al. 2014). The basis of RAPD analysis is the use of the Polymerase Chain Reaction (PCR) tool which is an in vitro method for multiplying DNA sequences and is very useful for genotypic identification, kinship analysis, phylogenetic and genetic mapping (Muladno 2021).

According to Zhang et al. (2010), *Bactrocera dorsalis* complex that occupies the *Bactrocera subgenus* is monophyletic. *B. dorsalis* complexes have different shapes and characteristics but share the same ancestor. According to Dharmayanti (2011), phylogenetic analysis is used to determine how the family descended during the evolutionary process. Species with a lower genetic distance have a closer kinship, and vice versa (Kumar et al. 2020). Several factors, such as isolation by distance, geography, ecology, and reproduction, can cause significant differences in genetic distances in populations. When this happens, a new type will emerge that can adapt to its environment naturally in the long run (Schmitt dan Haubrich 2008). Lucic et al. (2011) stated that kinship between individuals shown by dendrogram correlates with individual genetic distance. Close kinship shows low genetic distance, and far kinship shows high genetic distance (Noroozi et al. 2022).

There is a need for information about existing fruit fly species with new fruit fly species so that the source of origin of these pests can be identified, and fruit fly pest control programs can be emphasized in the source areas of fruit fly attacks (Pramudi et al. 2013). However, in North Sumatra, especially Deli Serdang Regency, information about the molecular characteristics of fruit flies is still rare, so it is necessary to carry out morphological and molecular identification to determine fruit fly species more clearly (Di Francesco et al. 2018).

MATERIALS AND METHODS

Insect Provision. *Bactrocera* sp. was obtained from the collection of fruit flies trapped by using the attractant mixture of Methyl Eugenol with Processed Cocoa Waste.

Morphological identification. At the Biology Research Center Laboratory of the Indonesian Institute of Sciences (LIPI), fruit flies are identified by looking at parts of the head, thorax, abdomen, and wings using a microscope and using a fruit fly identification book namely: Biological characters of fruit flies *Bactrocera umbrosa* (Fabricius) from North Sumatera, Indonesia (Manurung et al. 2020); The Australian Handbook for Identification of Fruit Flies (Plant Health Australia 2011); Automatic identification of fruit flies (Diptera: Tephritidae) (Faria et al. 2014).

DNA isolation. Fruit fly DNA extraction techniques are based on the method stated in the Genomic DNA Wizard, namely: fruit flies are crushed in cold Nuclei lysis solution using mortar until smooth. Insert into the tube and add Nuclei Lysis Solution and it Shaked by use vortex 10 seconds. It was then incubated at 650C for 15-30 minutes. A 3 µl RNase Solution volume was added to the nuclei lysate tissue, and proteinase K was 17.5 µl. It was mixed by flipping the tubes 2-5 times, then incubating at 370C for 15-30 minutes. The sample is left to cool at room temperature. A protein precipitation Solution of 200 µl was added and vortexed for 20 seconds. Then centrifuged for 4 minutes at a speed of 14,000 * g. The supernatant was removed, and the pellet solution was transferred into a tube containing 600 µl of isopropanol at room temperature. The solution is mixed slowly. Then centrifuged for 3 minutes at a speed of 14,000 * g. The supernatant was removed, added 600 µl 70% ethanol at room temperature, and mixed slowly. Then centrifuged for 1 minute at 14,000 * g. Ethanol is removed and the pellet is dried for 15 minutes. DNA rehydration solution 100 µl is added and incubated at 650C for 1 hour, and DNA is stored at pri (Pramudi et al. 2013).

PCR amplification. The primer used is based on previous research by (Pramudi et al. 2013) derived from Macrogen, namely OPC-01 (TTCGAGCCAG), OPI-17 (GGTGGTGATG), OPL-07 (AGGCGGGAAC), OPL-08 (AGCAGGTGGA), OPL-16 (AGGTTGCAGG). For the mixture, namely: 12.5 µl Go Taq Green PCR master mix, 8 µl Nuclei Free Water, 2.5 µl DNA of fruit fly species, 1 µl forward mtD7 primer, and 1 µl reverse mtD9 primer into PCR tube. The 35-cycle PCR program settings used can be seen in Table 1.

Electrophoresis. As many as 4 µl of fruit fly DNA from PCR were put into 1.5% agarose gel stained with ethidium bromide in TAE 1X buffer. Also included were 2 µl 100 bp of DNA ladder as a marker. Then electrophoresis at 80 Volt for 60 minutes. Then visualized in the UV-illuminator to make the DNA band produced.

Table 1. Setting PCR for amplification

Proceed	Temperature	Time
Pre-Denaturation	94	3 minutes
Denaturation	94	15 second
Annealing	53	15 second
Extension	70	1 minute
Extension	72	1 minute

RESULTS AND DISCUSSION

From the results of identification at LIPI there were eight species of fruit flies in the locations of red guava crops in Deli Serdang District, namely *Bactrocera papayae*, *B. carambolae*, *B. caudata*, *B. albistrigata*, *B. curcubitae*, *B. umbrosa*, *B. tau*, and *B. kinabalu*. In general, Bactrocera can be distinguished from its wing and abdominal patterns, namely: 1). *B. albistrigata*, on the wing with a very thin costal band to the apex, transverse black bands reaching r-m and dm-cu, and black bands on the anal line; The abdomen is brownish yellow with a medial longitudinal dark band that extends from terga III to terga V. 2). *B. curcubitae*, on the wing, there is a dark brown band with a costal band that extends to meet a large spot on the wing tip, and there is also a dark brown band on the dm-cu vein line; In the abdomen, brownish yellow with a black T pattern, the medial longitudinal dark band is of medium size. 3). *B.*

umbrosa, on the wing of the costal band, three additional bands extend from the costal wing to the underside of the wing; the abdomen is brownish yellow, and the medial longitudinal dark band is in terga III-IV.

Sometimes there is a black color widening laterally. 4). *B*. caudate, on the wing, there is a black ribbon on the rib line that extends to the spot at the apex of the wing. In the abdomen, pale yellow with a black T pattern. 5). *B*. *carambolae*, on the wings with black bands on the costa line and the anal line. The apex wing is shaped like a fishing line; In the abdomen, there is a clear T pattern, and there is a black rectangular shape in IV. 6). *B*. *papayae*, on the wings, there is a black band on the costa line and the anal line that is clearly visible; The brownish-yellow abdomen is visible with a T pattern, terga III in males with pecten (bristles) on each side, and there is a pair of ceromata (spots) on terga V. 7). *B*. *tau*, wings with black bands on the line of ribs that extend to the spot at the apex of the wings; The abdomen is pale yellow with a black T pattern. The anterolateral angle in terga IV and V is black and wide. Ceromae with bright color in terga V. 8). *B*. *kinabalu*, the wing of the costal band passes through very thin at R2 + 3, extends and does not extend to the wing tip (apex); On the abdomen that is brownish yellow, the vertebrae are visible with the T pattern, there is a black pattern that extends to terga III-IV. The head, thorax, abdomen, and wing patterns can be seen in Figure 1.

This study also shows that polymorphism can be seen between individuals in a population and between populations. Based on the band profile shows that the polymorphic DNA band size produced from each primer is generally different. From the DNA band that was scored, there was not one primer that showed all individuals from the five populations. In other words, RAPD using these five primers produced 91.18% polymorphic DNA bands (Al-Khayri et al. 2022). The total ribbon patterns produced were 211 ribbon patterns with a total number of polymorphic bands, as many as 138 ribbon patterns. This shows the potential ability of the RAPD method to detect genetic differences between individuals, meaning that each individual has a different RAPD profile or fingerprint based on the primer. Different species can show different levels of polymorphism, comparable to variations in the RAPD locus and the number of amplified loci (Jiang et al. 2014).

Some amplified fruit fly DNA samples also contained bands that did not appear, in the OPL-08 primers with ribbon numbers 3, 10, 12, 14, and 15 did not show the presence of DNA bands. This is due to the absence of amplification because the primers used are not in accordance with printed DNA. (Simbolon et al. 2017) states that primers that are not specific or appropriate can lead to the amplification of other regions in the genome that are not targeted or otherwise there is no amplified genome area. Some experimental evidence shows that the difference of just one base pair is enough to cause a mismatch of the primary mold which then prevents amplification (Faria et al. 2014). According to Pramudi et al. (2013) that the slightest change in the reaction can change the amount and intensity of the amplification product so repeatability is difficult to maintain. (Muladno 2021) also added that RAPD cannot distinguish homozygous and heterozygous individuals because it is a dominant marker and difficult to detect small changes in the structure of DNA (Boomibalagan et al. 2021; Sabit et al. 2021).

Based on the results of amplification using 5 primers, data were obtained in the form of scoring to determine genetic variation between individuals in 28 fruit fly samples. The grouping of the results of the analysis through the phylogenetic tree (dendrogram) can determine the relationship between fruit flies in the locations of red guava crops in Deli Serdang Regency based on RAPD molecular markers. The phylogenetic tree can be seen in Figure 2.

Based on the phylogenetic tree analysis showed a genetic closeness relationship between species from the five populations based on RAPD markers resulting in 3 clusters namely *B. caudata* (Kolam and Namoriam) and *B. umbrosa* (Kolam, Namoriam, and Sei Mencirim) because they were in the same group (Cluster 1); *B. curcubitae* (Sei Beras Sekata), *B. caudata* (Sei Mencirim and Sei Beras Sekata), *B. umbrosa* (Sei Beras Sekata), *B. carambolae* (Sei Mencirim and Sei Beras Sekata), and *B. papayae* (Kolam, Namoriam, Sei Mencirim, and Sei Beras Sekata) because they are in the same group (Cluster 2); *B. kinabalu* (Kolam), *B. carambolae* (Sawit Rejo, Kolam and Namoriam), *B. tau* (Pond), *B. papayae* (Sawit Rejo), *B. curcubitae* (Sawit Rejo, Kolam, Namoriam and Sei Mencirim), *B caudata* (Sawit Rejo), *B.umbrosa* (Sawit Rejo) and *B. albistrigata* (Kolam) because they are in the same group (Cluster 3).

Based on the dendrogram, *B. caudata* 4 and *B. caudata* 5 fruit flies is in the same cluster and have the closest genetic distance range of 0.13 (Table 2), which means the two samples have more immediate kinship when compared to individuals other. According to (Lucic et al. 2011) that the relationships between individuals shown by dendrogram correlate with individual genetic distances. Close kinship shows a low genetic distance, and close kinship shows a high genetic distance (Tomazi et al. 2018).

The closeness of genetic relationships between individuals was analyzed using Pairwise Distance Calculation which described the genetic distance between species. Pairwise Distance Calculation results ranged from 0.13-0.42 (Table 2), indicating that the species found were still closely related (Tomazi et al. 2018). Species with lower genetic distance values have closer kinship relationships and vice versa. Species with far genetic distance values have far kinship relationships (Dharmayanti 2011). The genetic distance difference in the population can be caused by several factors, such as isolation by distance, geography, ecology, and reproduction. If this happens, new types will emerge that can adapt to their environment naturally in the long term (Schmitt dan Haubrich 2008).

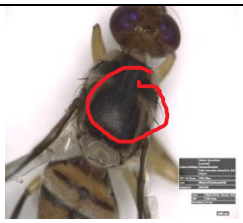
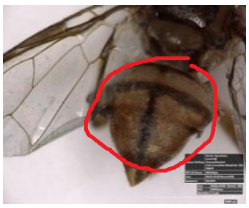
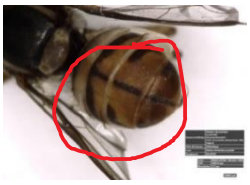
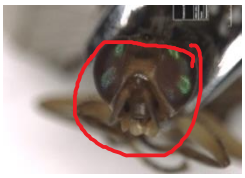
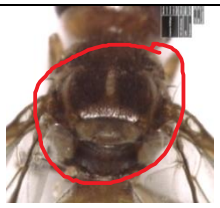
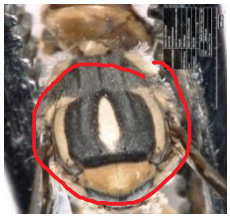
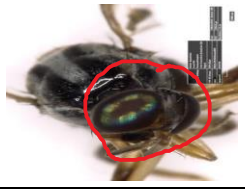
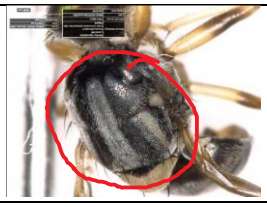
Character	Caput	Thorax	Abdomen	Wing
B. papayae				
B. carambolae				
B. kinabalu				
B. tau				
B. umbrosa				
B. curcubitae				
B. caudata				
B. albistrigata				

Figure 1. Fruit fly found in the locations of red guava crops in Deli Serdang District

Table 2. Genetic distance fruit flies red guava crops in Deli Serdang Regency based on RAPD

Units	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27
2	0.33																										
3	0.34	0.25																									
4	0.35	0.20	0.28																								
5	0.34	0.35	0.36	0.37																							
6	0.26	0.36	0.37	0.38	0.37																						
7	0.23	0.33	0.33	0.35	0.33	0.16																					
8	0.28	0.33	0.33	0.35	0.33	0.31	0.28																				
9	0.30	0.35	0.35	0.37	0.35	0.33	0.30	0.27																			
10	0.30	0.31	0.32	0.34	0.29	0.33	0.30	0.29	0.32																		
11	0.32	0.36	0.37	0.39	0.37	0.35	0.32	0.22	0.31	0.33																	
12	0.33	0.30	0.31	0.32	0.35	0.36	0.32	0.32	0.34	0.31	0.36																
13	0.34	0.31	0.32	0.33	0.35	0.36	0.33	0.33	0.35	0.32	0.37	0.22															
14	0.27	0.28	0.29	0.31	0.26	0.30	0.27	0.26	0.29	0.20	0.30	0.28	0.29														
15	0.26	0.27	0.28	0.30	0.26	0.29	0.26	0.25	0.28	0.19	0.29	0.27	0.28	0.13													
16	0.36	0.37	0.38	0.40	0.38	0.39	0.36	0.35	0.38	0.34	0.39	0.37	0.38	0.31	0.30												
17	0.33	0.34	0.35	0.36	0.35	0.35	0.32	0.32	0.34	0.31	0.36	0.34	0.35	0.28	0.27	0.18											
18	0.27	0.29	0.30	0.32	0.30	0.30	0.26	0.26	0.28	0.26	0.30	0.29	0.30	0.23	0.22	0.32	0.29										
19	0.41	0.42	0.42	0.44	0.31	0.43	0.40	0.40	0.42	0.36	0.44	0.41	0.42	0.33	0.32	0.45	0.41	0.37									
20	0.31	0.32	0.32	0.34	0.21	0.33	0.30	0.30	0.32	0.26	0.34	0.31	0.32	0.23	0.22	0.35	0.31	0.27	0.20								
21	0.35	0.36	0.37	0.39	0.37	0.38	0.34	0.34	0.37	0.33	0.38	0.36	0.37	0.30	0.29	0.35	0.31	0.31	0.44	0.34							
22	0.33	0.34	0.35	0.36	0.30	0.36	0.32	0.32	0.34	0.28	0.36	0.34	0.34	0.26	0.25	0.37	0.34	0.29	0.37	0.27	0.36						
23	0.34	0.35	0.36	0.37	0.31	0.37	0.33	0.33	0.35	0.29	0.37	0.35	0.35	0.27	0.26	0.38	0.35	0.30	0.38	0.28	0.37	0.27					
24	0.32	0.34	0.34	0.36	0.30	0.35	0.32	0.32	0.34	0.28	0.36	0.33	0.34	0.25	0.24	0.37	0.33	0.29	0.37	0.27	0.36	0.20	0.26				
25	0.30	0.31	0.32	0.34	0.28	0.33	0.30	0.29	0.32	0.26	0.33	0.31	0.32	0.23	0.22	0.34	0.31	0.26	0.34	0.24	0.33	0.18	0.24	0.16			
26	0.27	0.33	0.34	0.36	0.34	0.30	0.27	0.28	0.31	0.30	0.32	0.33	0.34	0.27	0.26	0.36	0.33	0.27	0.41	0.31	0.35	0.33	0.34	0.33	0.30		
27	0.32	0.34	0.34	0.36	0.34	0.35	0.32	0.31	0.34	0.30	0.35	0.33	0.34	0.28	0.27	0.32	0.29	0.28	0.41	0.31	0.28	0.33	0.34	0.33	0.31	0.32	
28	0.33	0.34	0.35	0.37	0.35	0.36	0.32	0.32	0.34	0.31	0.36	0.34	0.35	0.28	0.27	0.27	0.24	0.29	0.42	0.32	0.31	0.34	0.35	0.34	0.31	0.33	0.29

Note: (1) *B.umbrosa* Sawit Rejo; (2) *B. umbrosa* Kolam; (3) *B. umbrosa* Namoriam; (4) *B. umbrosa* Sei Mencirim; (5) *B. umbrosa* Sei Beras Sekata; (6) *B.curcubitae* Sawit Rejo; (7) *B.curcubitae* Kolam; (8) *B.curcubitae* Namoriam; (9) *B.curcubitae* Sei Mencirim; (10) *B.curcubitae* Sei Beras Sekata; (11) *B.caudata* Sawit Rejo; (12) *B.caudata* Kolam; (13) *B.caudata* Namoriam; (14) *B.caudata* Sei Mencirim; (15) *B.caudata* Sei Beras Sekata; (16) *B.carambolae* Sawit Rejo; (17) *B.carambolae* Kolam; (18) *B.carambolae* Namoriam; (19) *B.carambolae* Sei Mencirim; (20) *B.carambolae* Sei Beras Sekata; (21) *B.papayae* Sawit Rejo; (22) *B.papayae* Kolam; (23) *B.papayae* Namoriam; (24) *B.papayae* Sei Mencirim; (25) *B.papayae* Sei Beras Sekata; (26) *B.tau* Kolam; (27) *B.albistrigata* Kolam; (28)*B.Kinabalu* Kolam

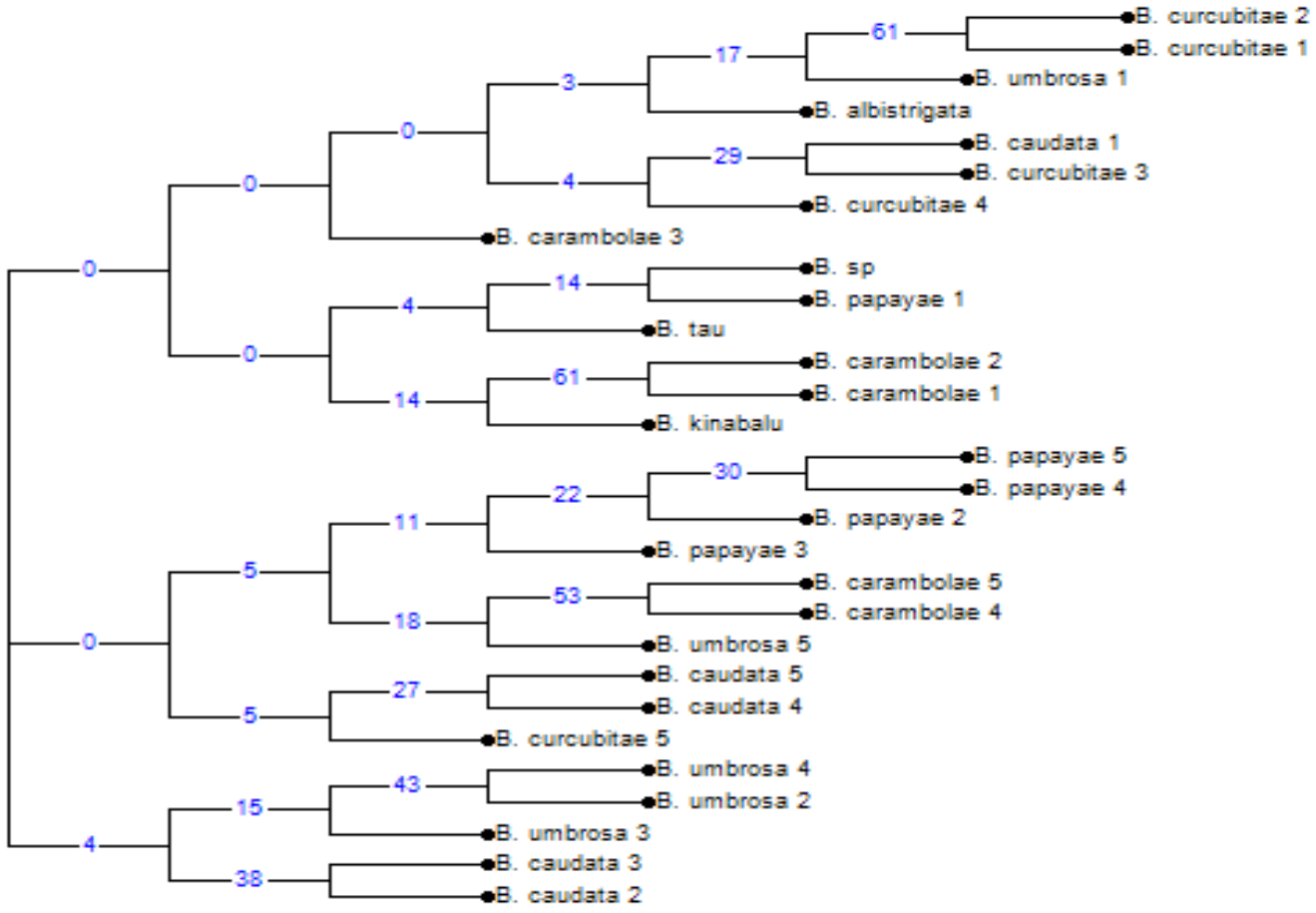


Figure 2. Dendrogram of fruit fly relationship in the locations of red guava crops based on RAPD markers. The number above the branch is the bootstrap value (%)

Note: (1) Sawit Rejo; (2) Kolam; (3) Namoriam; (4) Sei Mencirim; (5) Sei Beras Sekata

CONCLUSIONS

Based on the morphology, in the locations of red guava crops in Deli Serdang District, eight fruit fly species were found, namely *B. caudata, B. papayae, B. tau, B. carambolae, B. albistrigata, B. curcubitae, B. umbrosa, and B. kinabalu* were identified. The results of molecular analysis on the PCR-RAPD method based on the dendrogram there are 3 clusters, which showed a close kinship between Bactrocera species in 5 populations in Deli Serdang District. By knowing what species there fruit flies are in red guava plantations in the Deli Serdang district, it can make it easier for farmers to monitor and control fruit flies in red guava plantations.

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