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Identification of Genetic Diversity in *Bactrocera* spp. from Minahasa Regency Based on COI Barcode

M. Lengkong[1]*, J. Pelealu[2], M. Tulung[2], V. Mantiri[3], E.F. Lengkong[2]

[1]Doctoral student at the University of Sam Ratulangi, Manado, North Sulawesi, Indonesia
[2]Faculty of Agriculture, University of Sam Ratulangi, Manado, North Sulawesi, Indonesia
[3]Faculty of Mathematics and Natural Sciences, University of Sam Ratulangi, Manado, Indonesia

Abstract : Fruit flies are one of the most important insect pests in agricultural crops, especially in horticultural crops because they cause significant damage and losses to agricultural cultivation systems. To date, about 1400 species of fruit flies have been identified. The speed and accuracy of identification and characterization of fruit fly types is one of the keys to the success of controlling fruit flies in agricultural crops. DNA Barcode technology is one of the most widely used genetic diversification techniques currently available because it provides accurate analysis results and can distinguish organisms to the species level. This study aims to analyze the genetic diversity of mitochondrial COI genes in several types of *Bactrocera* associated with fruit and vegetable crops in Minahasa Regency and *Drosophila* sp. as an outgroups. The results showed that in the five species of *Bactrocera* there were an average difference of 75 nitrogen bases. The smallest amount of nitrogenous bases, as many as five nitrogen bases, were found in *B. albistrigata* and *B. fraunfeldi*, whereas the largest difference of 104 nitrogen bases were found in *B.umbrosa* and *B. tau*. Genetic diversity analysis revealed 12.4% genetic differences among *Bactrocera* spp insects, the smallest manifestation (0.8%) between *B. albistrigata* and *B. fraunfeldi* and the greatest diversity between *B.umbrosa* and *B. tau* (15.8%).
Keywords : Bactrocera spp, COI DNA Barcode, PCR

I. Introduction

Fruit fly (Diptera: Tephritidae) is one of the most important pests that attack fruits and vegetables worldwide[1,2]. Fruit flies are divided into four main groups: *Ceratitis*, *Bactrocera*, *Anastrepha* and *Rhagoletis*[3]. Up to now, fruit flies have been identified about 4000 species, and an estimated 1400 species of them attack soft fruits[4,1]. Specifically for *Bactrocera* has about 500 species arranged in 28 subgenus[5,6]. Although fruit flies result in the loss of billions of dollars in the cultivation of fruits, vegetables, flowers and a limiting factor in the development of agricultural crops in many countries, but some fruit flies are also useful as biological control agents for weeds[7].

The speed of identification accuracy and characterization of the fruit fly species is one of the keys to successful control of fruit flies in agricultural crops[8,9]. The large number of fruit fly species and the presence of unclear traits between species cause identification and the characteristics of these insects often encounter

difficulties[10,6]. Several identification methods that have been used to identify fruit flies include using morphological characters[11,12,13,14,6]. Using phenylpropanoid compounds[15], using PCR-RFLP molecular markers[16,17,18], and using RAPD molecular markers[19,20,21].

The use of morphological characters has provided many benefits to the identification of insects, including fruit flies[11,22,5,6]. Nevertheless, the use of morphological characters for the identification of adult fruit fly insects often results in identifcation errors resulting from homosplication in most morphological characters and the presence of unclear species at the insect family level[23,24].

Anticipating and complementing the limitations of identification morphologically, molecular identification has been developed, through barcode DNA technology that can analyze species genetic diversity by using a short piece of DNA[23,25,26].DNA Barcode is a short DNA sequence of a standard genetic locus suitable for identifying insect species[14].DNA Barcode for animal identification, including insects, is by using the mitochondrial gene encoding the formation of cytochrome oxidase I (COI)[23]. Of the several molecular markers available for genetic analysis, molecular DNA barcode markers are considered to be most advantageous for identifying fruit flies[8,27]. The COI barcode has succeeded in determining the genetic structure and phylogenetic relationship between species in the genus Bactrocera[28,29,30].

II. Research Methods

1. Time and Placeof Research

The researches were conducted in 2014, and located in Warembungan Village, Minahasa Regency, North Sulawesi Province, Indonesia, and surrounding areas, especially in areas covered by fruits and vegetables. The studies were conducted in the field and in the laboratory.

2. Sampling Method

Insect samples were obtained by taking the fruits of star fruit, balsam-pear, tropical almond, guava and jackfruit, which had been ripe that had allegedly infested fruit flies. Each fruit is placed on a plastic container containing sand (will be the place where the larva grows into a pupa). The plastic container is placed in a gauze cage. After about 10 days the pupae can be harvested from the sand medium and placed on a circular plastic container measuring 15 cm in diameter and 10 cm high which remains sandy and placed in a 30 x 30 cm gauze cage. Pupaes are kept to adulthood, and when adult insects appear, are fed from a mixture of hydrolysate and sugar proteins with a ratio of 1: 1. In the cage, also placed water in a plastic cup container which on the lid is made a hole, then inserted axis of cotton covered with tissue paper to be ensured moisture. After about a week of maintenance, the adult fruit fly population was harvested for morphological identification and partially brought to the laboratory for DNA analysis. For the needs of comparative insects are *Drosophila* sp. was done in the same way but only using mature belimbing fruit and placed on a gauze walled cage whose upper part is left open and placed in an area of star fruit plant area allowing Drosophila insects to enter and be caught.

3. Laboratory Research

a. DNA extraction

The DNA samples were extracted manually from intact body of fruit fly as many as 25 individuals / species using CTAB method with some modification. 2 µL of CTAB extract buffer was included in an existing mortal of fruit fly specimens, after crushing, extractants were transferred into two 1.5 µL eppendorf tubes and incubated in water at 65ºC for 2 hours. After cooling, centrifuged at 13,000 rpm for 10 minutes. The supernatant was transferred to a new eppendorf tube and added with chloroform - isoamyl alcohol, homogenized by the way the tube was reversed slowly and then centrifuged again for 10 minutes at 13,000 rpm. The supernatant was again transferred to a new eppendorf tube and added 1 x of ethanol absolute volume, after homogenization, the DNA pellet was collected with 13,000 rpm centrifugation for 10 min. Dispose of all fluids in the tube, rinse the pellet DNA with 70% alcohol and centrifugate 10,000 rpm for 5 minutes. Dry the DNA pellet at room temperature, then resuspension with 25 µL sterile aquades. The extracted DNA is ready for use or stored in a frezer (-20 ºC).

b. Amplification of Polymerase Chain Reaction (PCR)

The PCR reaction is performed in a total volume of 50 µl. The work stages are as follows: COI gene primer diluted with water (nuclease free) to 100 pmol / µl and then aliquoted to 10 pmol / µl inserted into Eppendorf tube. 10 µl 5x FirePol Master Mix, 1 µl COI-specific primer, LCO1490 (5'GGTCAACA AATCATAAAGATATTG-3') and HCO-2198 (5'TAAACTTCAGGGTGACCAAAAA AT CA-3'), 3 µl template DNA, and 35 µl water (ion-free and nuclease) Were mixed, so that every 50 µL PCR reaction contains 1.25 *Taq* DNA polymerase units, 0.2 µM of each dNTPs, 1.5 mM MgCl2, and 0.2 µM of each sample DNA template primer.

The PCR (Biometra T-Personal) machine temperature was adjusted for amplification processes which include: (i) DNA predenaturation at 94ºC for 5 minutes, (ii) denaturation at 94ºC for 30 seconds, (iii) primary annealing at temperature 50ºC for 30 seconds, and (iv) DNA extension at 72ºC for 50 seconds. The three stages (i, ii, iii) take place in a cycle of 40 times and the final stage of final extension at 72ºC for 5 minutes.

c. Electrophoresis

PCR amplification results for electrophoresis use 1% agarose gel. 10 µl of PCR products that already have a loading dye are fed into agarose gel wells. Used 100 bp Ladder DNA to estimate the length of the DNA band by 10 µl Ladder DNA mixed with 2 µl of 6x dye loading dipped into agarose gel. The electrophoresis process was carried out for 30 minutes at a voltage of 70 Volts with electrophoresis devices and visualized with UV-Transluminator.

d. PCR Product Sequencing

PCR products obtained, before the sequencing is done, PCR clean up first. The sequencing process is done by sequencing cycle method using BigDye® Terminator v3.1 Cycle Sequencing Kit with ABI PRISM® 3700 DNA Analyzer instrument. The sequencing process is done twice in different directions (forward and reverse) according to the available primary. The sequencing process is carried out at First Base Laboratories Sdn Bhd, Malaysia.

4. Data Analysis

The DNA sequencing in the form of chromatogram was edited using Geneious v5.6 software[31]with the following steps: the beginning and end of the DNA sequence was removed approximately 30 bp (primary sequence), then reverse and complement using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) integrated in Genious v5.6. COI gene sequences are identified through the Barcode of Life Database (BOLD) System[32]. The alignment of the DNA sequences performed by MUSCLE aims to determine the difference of nucleotides between each sample of fruit flies.

The sequences were converted into FASTA format (Fast Alignment) to compare with the closest relative sequences contained in the Gen Bank using Basic Local Alignment Search Tool (BLAST) (http://blast.ncbi.nlm.nih.gov). The phylogenetic tree was reconstructed using the Neighbor-joining method and the genetic distance was analyzed using the Tamura-Nei method integrated in Geneious v5.6.

Iii. Results and Discussion

DNA samples that have been isolated from adult insects intact manually using CTAB method provide good enough DNA results, although using simple methods without strict purification treatment, this is evident from the total DNA electrophoresis results seen intact in all samples (Figure 1). Isolation of DNA by using intact adult insects for molecular analysis purposes was also performed[14,24], although some also use only certain body parts of insects[28,33].

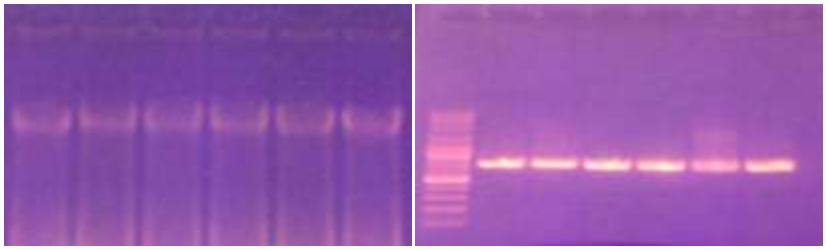

Figure 1. Left, electrophoresis of total DNA samples; and right, electrophoresis of PCR amplification results

PCR amplification of mitochondrial genes using COI-specific primers LCO1490 (5'GGTCAACAA ATCATAAAGATATTG-3') and HCO-2198 (5'TAAACTTCAGGGTGACCAAAAAATCA-3') produced a single DNA fragment with 661 base pairs. Based on the chromatogram from the sequenced PCR product sequence each sample was aligned using MUSCLE (Multiple Sequence Comparison by Log-Expectation)[34](Figure 2) indicating that all sample DNA sequences do not have stop codons so ascertained the sequence readings are accurate, and there is no frame shiftsor amplification error in pseudogene genes.

Chromatogram data showed that of the six insect samples observed showed the diversity of the nucleotide base of COI, there were also three insertion and deletion events (indels) in the sequence of bases 533-535, 536-538, and 542-544. The insertion and deletion event involves 2 (two) adjacent codons that automatically affect the type of amino acid produced by affecting the open resding frame of the COI gene. The deletion effect on the base sequence of 533-535 causes the loss of isoleucine, and threoninebecomes serine in *B. tau*. Deletions on bases 536-538 cause the loss of threonine, and phenylalanine to isoleucine in *Drosophila* sp. While the insertion events in the base sequence 542-544 cause the formation of threonine, and isoleucine into serin in *B. tau*, and on *Drosophila* sp. Besides the formation of treonin, isoleusin becomes phenylalanine.

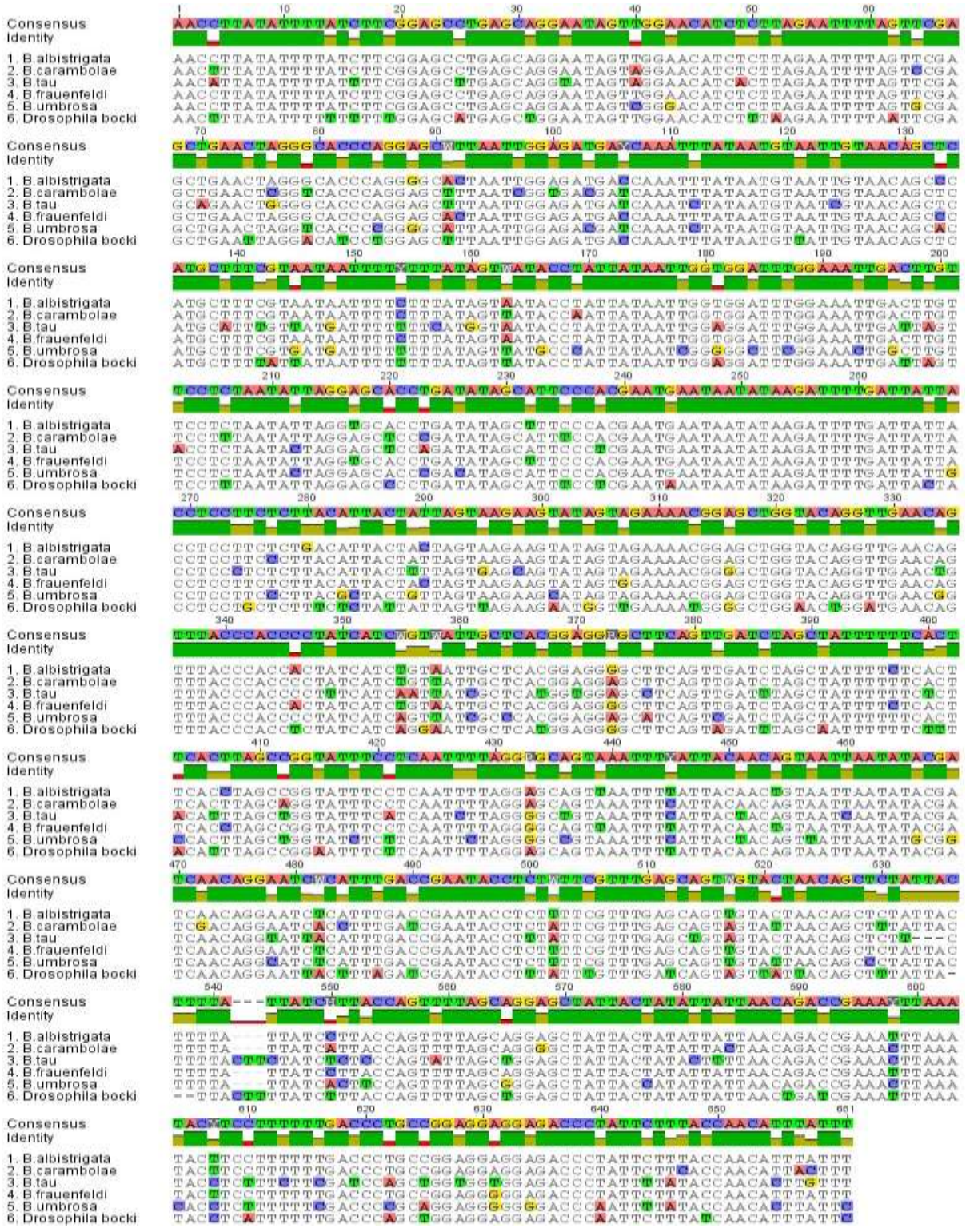

Figure 2. Chromatogram of the COI sequence alignment of the insect sample.

The occurrence of changes in DNA sequences in organisms is the result of a process of mutation and the response of organisms to natural selection. The indel event on the sample insects, According to [31],the potential for 4,924 indels in *Bactrocera* sp, and generally occurs in non-coding regions. Indels substitution can be used to investigate physiological functions in mammals[36], genes and proteins[37], and are an important source of phylogetic information[38].

Table 1.Matrix of differences in the number of nitrogen bases of DNA samples

	B.albistrigata	**B.carambolae**	**B.frauenfeldi**	**B.tau**	**B.umbrosa**	**D.bocki**
B.albistrigata		48	5	102	82	98
B.carambolae	48		49	98	81	98
B.frauenfeldi	5	49		100	81	98
B.tau	102	98	100		104	113
B.umbrosa	82	81	81	104		130
D.bocki	96	98	98	113	130	

Overall the difference in the number of nitrogen bases of *Bactrocera* spp DNA (Table 1) were 75. The smallest difference (only 5 bases) were between *B. albistrigata* and *B.frauenfeldi*, whereas the largest difference (104 bases) were between *B.umbrosa* and *B. tau* .

Based on the reconstruction of phylogenic trees (Figure 3), it was seen that all insects of *Bactrocera* spp. was a group of insects that are monophyletic, the group of organisms originating from the same ancestor and all descendants of these organisms have a very close kinship relationship. The results of this study were consistent with those reported[28,39,40,41]who explored *Bactrocera* insects based on COI genes and DNA sequences of 16 S. They claimed a very close genetic relationship between *Bactrocera* spp. and members of the subgenus*Batrocera* to form monophyletic phylogenic trees.

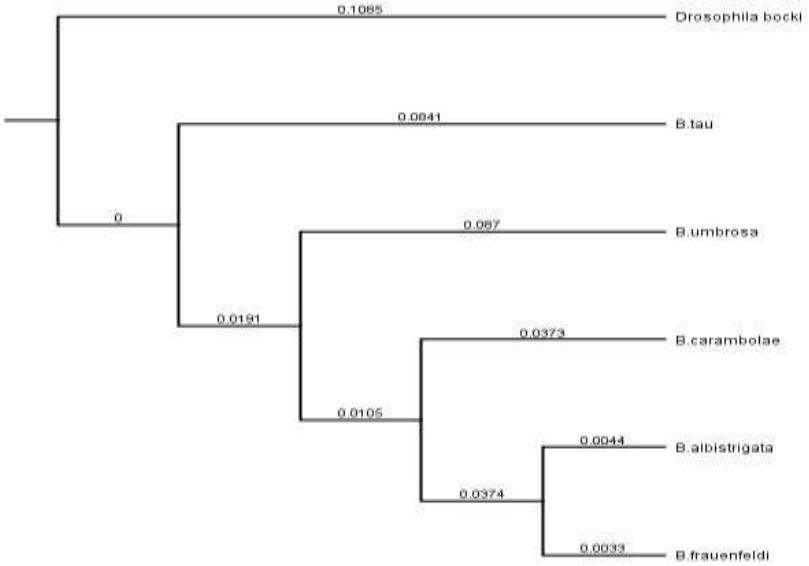

Figure 3. Phylogenetic Bactrocera spp from Minahasa Regency Based on COI Barcode

The matrix of genetic similarity (Table 2) shows that the genetic similarity between *Bactocera* spp was 88.6%, or diversity was 12.4%. *B. albistrigata* and *B. frauenfeldi* have the closest genetic similarities (99.2%). This very close genetic similarity is possible because between the two species is only distinguished by 5 nitrogenous bases. The smallest genetic similarity between *B.umbrosa* and *B. tau* is 84.2%. Other Researher [39]used the same method to obtain 8% genetic diversity between three fruit flies namely *B. dorsalis*, *B. Zonata* and *B. correcta*. According to [40]who studied phylogenetic from *Bactrocera* spp. found genetic diversity in *Bactrocera* subgenus ranged from 9-19%.

Table 2. Genetic similarity between fruit fruits based on nucleotide sequences

	B.albistrigata	**B.carambolae**	**B.frauenfeldi**	**B.tau**	**B.umbrosa**	**D.bocki**
B.albistrigata	100					
B.carambolae	92,6	100				
B.frauenfeldi	99,2	92,7	100			
B.tau	84,8	85,1	84,5	100		
B.umbrosa	87,7	87,7	87,5	84,2	100	
D.bocki	85,4	85,4	85,4	83,4	80,5	100

At the beginning of this study, outgroup sample insects originated from the genus *Drosophila* with unknown species, but after the alignment of the nucleotide sequence found in GenBank it was closer to *Drosophila bocki*. This species according to[41] spread in Taiwan and Thailand, so this information is an interesting phenomenon to be examined later. The genetic equivalence matrix (Table 2) on the comparison of the genetic similarity of *Drosophila bocki* to the *Bactrocera* types has shown the smallest genetic equivalence value compared to the genetic similarities in the *Bactrocera* subgenus, this is understandable because although both are Diptera but derived from different families (Tephritidae And Drosophilidae).

Iv. Conclusion

Analysis of *Bactrocera* spp diversity found in Minahasa Regency based on molecular DNA marker, COI barcode, shows an average difference of 75 nitrogen bases in the five*Bactrocera* species. The smallest nitrogenous base diversity (five nitrogenous bases) occurs between *B. albistrigata* and *B.frauenfeldi*, while the largest difference (104 nitrogenous bases) occurs between *B. umbrosa* and *B. tau*. Genetic diversity analysis showed 12.4% genetic diversity among *Bactrocera* spp. insects. The smallest diversity (0.8%) occurred between *B.albistrigata* and *B. frauenfeldi*, and the greatest diversity (15.8%) occurred between *B.umbrosa* and *B. tau*. A follow-up study is needed to ascertain whether *Drosophila* is really *Drosophila bocki*.

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