Faculty of Resource Science and Technology

MOLECULAR CHARACTERIZATION OF FRUIT FLY, *BACTROCERA DORSALIS* COMPLEX FROM DIFFERENT FRUIT HOST

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Molecular Characterization of Fruit Fly, *Bactrocera dorsalis* complex From Different Fruit Host

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Declaration

I hereby declare that the thesis is based on my original work except for the citation which has been acknowledged. I also declared that it has not been previously or concurrently submitted for any other degrees at UNIMAS or any other institutions of higher learning.

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List of Abbreviations

COI	Cytochrome Oxidase I gene
CTAB	Cetyl Trimethyl Ammonium Bromide
ddH_2O	Double distilled water
DNA	Deoxyribonucleic Acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetate
H_2O	Water
$MgCl_2$	Magnesium Chloride
MEGA	Molecular Evolutionary Genetic Analysis
mtDNA	Mitochondria DNA
NaCl	Sodium chloride
PCR	Polymerase Chain Reaction
TAE buffer	Tris-acetate-EDTA buffer
Tris-HCL	Tris(hydroxymethyl) aminomethane
NJ	Neighbourjoing
MP	Maximum Parsimony
ML	Maximum Likelihood

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ABSTRACT

In this study, 27 adult samples of fruit flies were collected from Bau, Serian, Padawan and Kg. Pinang in Samarahan division. The identification method of *Bactrocera dorsalis* complex species based on the morphological characteristics of adult are sometimes difficult and got misidentified. The identification of *B. dorsalis* and *B. papayae* were done by using the morphological characters such as head, thorax, abdomen and wing pattern of species. A total of 656 base pairs within the mtDNA gene cytochrome oxidase I (COI) was analysed for two species of *B. dorsalis* complex: 27 individuals ingroup and one outgroup. Phylogenetic relationships among *B. dorsalis* complex were inferred using Neighbor-Joining, Maximum Parsimony, Maximum Likelihood (ML) and Bayesian analysis. The COI gene sequence of Kimura 2-parameter distance within 27 samples is ranging from 0.0% to 2.2% and between the outgroup, *Drosophila melanogaster* ranged from 0.0% to 18%. The result of COI suggested that *B. dorsalis* complex is monophyletic, showed that *B. dorsalis* and *B. papayae* are clustered together.

Key words: *Bactrocera*, cytochrome oxidase I, species complex, mitochondrial DNA, phylogeny.

ABSTRAK

Dalam kajian ini, 27 sampel lalat buah dewasa yelah dikumpul daripada Bau, Serian, Padawan dan Kg Pinang di Samarahan. Kaedah identifikasi Bactrocera dorsalis komplek berdasarkan ciri-ciri morfologi dewasa kadang-kadang mempunyai masalah dan tersalah identifikasi. Identifikasi B. dorsalis and B. papayae telah dijalankan dengan menggunakan ciri-ciri morfologi seperti kepala, 'thorax', abdomen dan jenis sayap. Sejumlah 656 pasangan bes gen mitokondrial DNA sitokrom oksida I telah dianalisis untuk 2 species B, dorsalis komplek : 27 individu 'ingroup' dan 1 'outgroup'. Hubungan filogenetik di antara B. dorsalis komplek telah diimplikasi dengan menggunakan 'Neighbor-Joining', 'Maximum Parsimony',' Maximum Likelihood' (ML) dan analisis 'Bayesian'. Jarak gen COI sekuen Kimura 2-parameter di dalam 27 sampel ialah daripada 0.0% ke 2.2 % dan julat di antara 'outgroup', Drosophila melanogaster ialah daripada 0.0 % ke 18 %. Hasil COI mencadangkan B. dorsalis komplek ialah monofiletik, menunjukkan B. dorsalis dan B. papayae ialah kelompok bersama.

Kata kunci: Bactrocera, sitokrom oksida I, spesies komplek, mitochondrial DNA, filogeni

1.0 INTRODUCTION

The genus *Bactrocera* was comprised of over 500 fruit fly species, of which many were considered serious pests that threaten the agricultural crops of countries in which they were found (Margosian *et al*., 2007). According to Drew and Hancock, (1994), the complex presumably includes more than 60 geographically diverse species. The *Bactrocera dorsalis* complex of tropical fruit flies (Diptera: Tephritidae: Dacinae) contains 75 described species, largely endemic to Southeast Asia (Clarke *et al*., 2005). The *B. dorsalis* complex of fruit flies comprises of 68 species with varying distributions in Asia, Australia and the Pacific islands (Steck, 2007). The complex consists of the species *B. dorsalis* itself, *B. papayae*, *B. carambolae* and *B. philippinensis*.

B. dorsalis is known as the Oriental fruit fly and it is a member of *B. dorsalis* complex. Drew and Hancock (1994) stated that the distribution of the true *B. dorsalis* is restricted to mainland Asia (except the peninsular of southern Thailand and West Malaysia). *B. papayae* is an abundant species which can be found in tropical rainforests and it is endemic to Malaysia. This species also inhabits the secondary forest and cultivated area. *B. papayae* has very wide host range such as banana (*Musa*), starfruit (*Averrhoa carambola*), guava (*Psidium guajava*), mango (*Mangifera indica*), papaya (*Carica papaya*) (White and Elson-Harris, 1994). *B. papayae* is genetically similar to several other members of *B. dorsalis* complex (Mahmood, 2004; Nakahara *et al*., 2000). Wee and Tan (2000) mentioned that this species is known to be hybridised with the *B. carambolae*, however Mahmood (2004) concluded there was no gene flow between these two species in Malaysia.

Fruit flies can become the pest if their population number are increasing and causing a certain level of damage. Fruit flies of the genus *Bactrocera*, particularly *B. dorsalis* species are known to be one of the major pest of tropical fruits (Drew and Romig, 1997). They

were commonly called fruit flies due to their close association with fruit and vegetables (Kapoor, 1993). In Malaysia fruit flies incur severe damage to certain potential fruit crops like starfruit and guava (Mahmood, 2004). Nearly 35 per cent of the known fruit fly species attack soft fruits of which mango, guava, citrus, ber, peach and several cucurbitaceous vegetables are important (Ravikumar, 2006). Mango and guava are two important fruit crops which are severely damaged by the most common species of fruit flies, *B. dorsalis* complex (Ravikumar, 2006). Most of fruit flies are phytophagous, with larvae developing in the seed-bearing organs of plants (Meyer *et al*., 2010). As they are phytophagous pest, they may cause damage to the crops if not properly protected besides they provide losses to the owner. *B. dorsalis* has been a serious problem as far as control is concerned, mainly because it is a complex (probably of 8-10 species) in India (Kapoor, 2005).

The identification method of Tephritidae species based on the morphological characteristics of adult sometimes got misidentification. Therefore, the misidentification process nowadays can be overcomed through the molecular method as it is more effective in identifying the species especially when DNA barcoding was proposed as a tool for identification of species (Hebert *et al*., 2003). According to Liu *et al*. (2011), DNA barcodes were short and standardised DNA sequences suitable to identify species. The mitochondrial gene and cytochrome c oxidase subunit I (COI) were often used for animal identification (Hebert *et al*., 2003).

In this study, there were a total of 27 samples of adult fruit flies collected from Bau, Serian and Padawan in Sarawak, including three museum samples collected from Kg. Pinang which collected at the Samarahan division. The samples were collected by using the

pheromones trap as the attractant. Although *B. dorsalis* complex is known as a phytophagous pest, however, little work has been done on the genetic relationships among the members of this species complex. Some species of the *B. dorsalis* complex could not be easily distinguished morphologically. In the present study, the variable region of the mitochondrial cytochrome oxidase subunit I (COI) gene was used to obtain better estimates of divergence for the species complex. The phylogenetic relationships among the *B. dorsalis* complex and between the outgroup were determined.

1.1 Problem Statement

Species belonging to the *B. dorsalis* complex are morphologically very similar with diagnostic characters found in relatively minor variation in the colour of the wing patterns, thorax, legs and abdominal structure. Sometimes misidentification based on the morphological characteristics happened to identify the species. Hence, the molecular method is more effective way to reveal the problem of the species identification.

1.2 Objectives

1. To identify the morphology of fruit fly *B. dorsalis* complex from different fruit host.
2. To infer the phylogenetic relationship among the members of the *B, dorsalis* complex.

2.0 LITERATURE REVIEW

2.1 Biology of Tephritid fruit fly

On the basis of the body form, structure and biology, the fruit flies may be divided into two groups. The first group infests almost all kinds of vegetables and fruit, which members of the subfamily Dacinae. The second group belongs to subfamilies Trypetinae and Schistopterinae usually breed in flower heads (Kapoor, 1993). The difference between male and female of Tephritid fruit fly is the female has long extendible ovipositor (White and Elson-Harris, 1992). *B. dorsalis* female adults locate their hosts through volatile compounds released by host plant species (Shi *et al*., 2003). The ovipositor is used to deposit about a dozen of white banana-shaped eggs in a cluster into a pulp or seed of fruit.

The life cycle of the fruit flies are short reproductive cycle which a new generation of adults develops in only two weeks. The tephritid life cycle include the stages of egg, three larval instars, pupa and adults (Isabel, 2009). Fruit fly eggs are laid into unripe and ripening fruit where the larvae develop and feed on the pulp of fruits. Larvae have three instars that feed on the fruit pulp, and this can result in complete destruction of the fruit. Mature larvae drop from the fruits onto the ground where they pupate in the soil at 2 to 5 cm depth (Liu *et al*., 2011). Fruit flies develop by complete metamorphosis. Complete metamorphosis usually differs from the adults in terms of structure, behavior, and habitat, and it transforms into passive pupal stage before becoming an adult. The adults are flower loving but their larvae are herbivorous and continue to plague humankind (Kapoor, 1993). Larvae feed on fleshy fruits and vegetables of plants in many families. Immature adults are able to disperse over at least 60 km to find fresh food resources and breeding substrates (Liu *et al*., 2011).

2.2 Habitat Distribution

The fruit flies are well distributed all over the world and they infest a large number of host plants. Such species may become widespread when their host plants are widespread either naturally or spread by man (Kapoor, 1993). Most species in Asia and the South Pacific occur in rain forest habitat where they oviposit in the fruit of many different plant families (Drew and Hancock, 2000). Climate, particularly temperature and rainfall, is the main factor influencing the distribution of the fly (Liu *et al*., 2011). *B. dorsalis* distributed from Pakistan and India east to southern China, Taiwan and Southeast Asia. *B. papayae* is found in Malaysia and the great island groups of Sumatra, Java and Borneo and the distribution is not overlap with the *B. dorsalis*. While the *B. philippinensis* occurs in the Philippines and the native distribution of the *B. carambolae* overlaps overlaps extensively with the *B. papayae* (Steck, 2007).

2.3 Pheromone as attractant

The attractant used in the pheromones traps was the sex pheromones. The main chemical attractants presently in use were methyl eugenol, cue-lure and trimedlure (Kapoor, 1993). Methyl eugenol was an ether which was the most powerful male lure and quite long lasting under suitable conditions. It had being widely used for male annihilation technique for management of *Bactrocera* spp (White and Elson Harris, 1992). Previous study done by Zuriani (2008) showed that, *B. dorsalis* was the common fruit fly in Samarahan Division which got 81 percent out of 493 individuals of fruit fly species. The effectiveness of methyl eugenol was successfully demonstrated in isolated islands of Rota islands in Marianas, followed by Saipan and Titian islands. The demonstration was successfully eradicating the population of *B. dorsalis* (Vijaysegaran, 1996).

2.4 Fruit fly traps

There were various types of traps used in the collection of fruit flies in the orchads. The efficiency of traps depends on climatic factors including temperature, rainfall, air movements and sunlight (Kapoor, 1993). When handle the pheromone traps, there were some factors to be considered like the attractiveness of the pheromones, pheromones concentration release rate, designing of trap including color, trap placement, trap durability and the areas of trap influence. Even though they can attract both sexes, however the pheromones traps were generally species specific (McEwen, 1997).

The common types of traps used in capturing Indian fruit flies are Steiner Type, G-K collapsable trap, sticky trap, and McPhail trap (Kapoor, 1993). Steiner type can catch a large number of fruit flies in a short period of time because it has a large area for evaporation and a large open area at each end which can be helped in wider distribution of attractant vapour. G-K collapsable trap is cheaper and easier to build and transport and this trap was used for population studies of *B. zonata*. Sticky trap is not useful for permanent collection as the collected specimens will damage due to the adhesive but it is useful for detection purpose in a small area especially around town and airports.

2.5 Host Plants Association

The host plants were utilised by many *Bactrocera* species for courtship and mating and specific larval food sites (Drew *et al*., 2008). The oriental fruit fly has been recorded from more than 150 kinds of fruit and vegetables, including citrus, guava, mango, papaya, avocado, banana, loquat, tomato, surinam cherry, rose-apple, passion fruit, persimmon, pineapple, peach, pear, apricot, fig, and coffee (Weems and Heppner, 2010). Mango (*Mangifera indica*) and guava (*Psidium* spp.) were infested by most species of the *B. dorsalis* complex collected from various localities (Iwaizumi, 2004).

2.6 Molecular studies of fruit flies

2.6.1 Cyctochrome Oxidase I (COI)

In this study, the variable region of the mitochondrial cytochrome oxidase subunit I (COI) gene was used to obtain better estimates of divergence for two species of the *B. dorsalis* complex. Many previous studies have been used the COI gene for obtaining the divergences among the species (Liu *et al*., 2011; Zhang *et al*., 2010; Jamnongluk *et al*., 2003.

The COI gene is used in this study because it appears to be among the most conservative protein-coding genes in the mitochondrial genome of animals (Brown, 1985). The COI gene was the slow-evolving gene in the mitochondrial protein coding gene (Simon *et al*., 1994). The conserved sequence of COI gene allow researchers to use it as a 'universal' primers, and it has been widely used to investigate multiple different taxa and for interspecific analysis.

According to Hebert *et al*., (2003), in terms of the degree of variation, it was expected to be low in intraspecific variation such that through a given cluster analysis, the sequences from polymorphic species would cluster together in a genetic distance.

2.6.2 DNA Extraction and COI gene Amplification

For the DNA extraction, total DNA was extracted from individual fruit fly adults by the crude boiling methods (O'Neill *et al*., 1992). Thoracic tissue was homogenized with a sterilized pestle in a 1.5-mL microcentrifuge tube filled with 100 μL of STE buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0)), and 1 mM EDTA (pH 8.0). The homogenate was heated at 95°C for 10 min before being centrifuged at 4000 rpm for 1 min at room temperature. Two microliters of supernatant were used as the DNA template for the polymerase chain reaction (PCR) (Zhang *et al*., 2010.

According to the previous study done by Liu *et al*., (2011) they extracted the DNA from the whole adult fruit fly of *Bactrocera invadens* by using the commercial tissue/cell DNA mini kit (Tiangen, China). The universal COI gene primers used were LCO1490 and HCO-2198 with the following sequences (5'GGTCAACAAATCATAAAGATATTG-3') and (5'TAAACTTCAGGGTGACCAAAAAATCA-3'), Liu *et al*. (2011). The reaction condition used in the polymerase chain reaction as follows: 94°C for 3 min, followed by 39 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min and then a final incubation at 72°C for 10 min.

Through the study of the molecular identification and phylogeny of *Bactrocera* species done by Asokan *et al*. (2011), they had extracted the total of DNA from individual fruit fly by using the CTAB method. The samples then was homogenised with the liquid nitrogen as the cuticle do not lysis when incubate. The PCR was carried out in a thermal cycler with the following cycles: 94 °C for 4 min as initial denaturation followed by 35 cycles of 94 °C 40 s, 47 °C for 45 s 72 °C for 45 s and 72 °C for 20 min as final extension.

2.6.3 Phylogenetic study of *B. dorsalis* complex

Molecular diagnostics have also been used by insect researchers to infer phylogenetic relationships and for identification to species level (Chua *et al*., 2009; Smith and Bush 1997; Jamnongluk *et al*., 2003). A previous study done Liu *et al*. (2011) showed that the mitochondrial COI gene based identification method was effective for *B. invadens*. In this study, 570 bp of mitochondrial COI gene were successfully sequenced for 14 samples of fruit fly from two different localities in Burundi, China. The genetic distances among all 14 samples of *B. dorsalis*, *B. papayae* and *B. philippinensis* were very small (0.006 to 0.021). The NJ tree showed that all the samples were in same cluster within *B. invadens*, which revealed that they showed the closest relationships with *B. invadens*.

Apart from that, another study done by Smith *et al*. (2003) on the phylogenetic relationships among 24 *Bactrocera* species belonging to 9 subgenera were inferred from DNA sequence of portions of the mitochondrial 16S rRNA, cytochrome oxidase II, tRNALys, and tRNAAsp genes. The maximum parsimony analyses showed that the the subgenus of *Bactrocera* is monophyletic. The subgenus *Bactrocera* in this study included the four members of *B. dorsalis* complex (as defined by Drew and Hancock, 1994). Zhang *et al*. (2010) also done the study on the molecular phylogeny of *Bactrocera* species by using the two mtDNA fragments are a 689-bp segment of the COI gene and a 345-bp segment of the16S rDNA gene. Thirty-five individuals representing seven *Bactrocera* species found in the Chongqing region in China were sequenced for both fragments. The results of phylogeny showed that seven species of the *Bactrocera (B. dorsalis* complex) (as defined by Drew & Hancock 1994) included in this study formed a monophyletic clade. The subgenus *Daculus* is one lineage by itself, which does not fall into the *Bactrocera* group or *Zeugodacus* group.

3.0 MATERIALS AND METHODS

3.1 Study Site

The sampling was conducted at the Bau, Serian, and Padawan area in Sarawak. The fruit flies were collected at every 1 kilometre along the road of Bau, Serian and Padawan. The Bau, Serian and Padawan were a sub-district of the city of Kuching, in the state of Sarawak, Malaysia. The figure below showed the map of the study sites.

Figure 1: Study sites that were done in Bau, Serian and Padawan area in Sarawak. Map from the http://maps.google.com.

▲ Indicate the study sites.

3.2 Sampling Methods

Pheromones traps were used as the trap for catching fruit flies. A plastic bottle was one type of modified pheromones traps. A 500 mL of plastic bottle was used and it had two entrance holes. A few drops of methyl eugenol were put at the tissue and placed inside the bottle. To hang the trap, a small wire was fixed on the top of the bottle. The traps were hanged on the fruit trees like guava, mango, papaya, melon, and banana. It was 1.5m above the ground. The traps were left for one hour which consider as sufficient time to attract the fruit flies. The traps then were collected after one hour and fruit flies from each trap were counted, recorded, identified and preserved.

3.3 Laboratory Methods

3.3.1 Morphological Identification

The collected specimens were identified by using the compound microscope. According to (Kapoor, 1993), earlier classification of fruit flies were based mostly on trivial characters. A gradual increase of fruit flies taxa based on such characters, a situation had developed that it became difficult to identify the specimens. Now, taxonomic characters include important morphological features like head, thorax, abdomen and also including genitalia. Identification followed Drew and Hancock (1994).

3.3.2 Preservation

According to the previous study by Wu *et al*. (2011), the specimens were preserved in 95% ethanol and then stored at -20 ºC until process for DNA extraction. The specimen bottles were used to keep the specimens. For labelling, the locality, the collector's name, date, the specimen's name and also the host plants were required. The voucher specimens of the species were kept for the experiment.

3.3.3 DNA Extraction

DNA samples were extracted from the whole adult of fruit fly by using a manual extraction of CTAB method (Standard hexadecyl trimetyl ammonium bromide). According to Grewe *et al*. (1993) firstly, a total of 1-2 cubic milimetres grinded samples were added into 1.5 ml microcentrifuge tube that containing 700 µL of CTAB buffer and 15 µL Proteinase K (20 mg/ml). The tube was incubated in the water bath at 65ºC for 2-3 hours until the tissue completely dissolved. Then, 600 µL of chloroform-isoamyl alcohol (CIA) was added before the tube can be centrifuged at 13000 rpm for 10 minutes.

After that, the upper layer of the supernatant was taken and transferred into new labelled tubes. The equal volume of cold absolute ethanol was added into the tubes and the tubes were centrifuged again at 13000 rpm for 10 minutes. Then, the ethanol was carefully discarded so that the DNA pellet was still intact in the bottom of the tube. Equal volume of cold 70% ethanol and 25 µL of 3M of NaCl were added into the tube. The tube then was spin at 13000 rpm for 10 minutes before discarding the ethanol again. The pellet was dried at the room temperature before it can be re-dissolved in 25 µL of water (ddH_2O). The samples of DNA extraction was kept in the freezer (-20ºC).

3.3.4 Gel Electrophoresis

By using 1% of agarose gel, the electrophoresis was done to visualise the product of the DNA extraction. 0.5 g of agarose powder was weighted out and 50 ml of 1X TAE buffer solution was added into the agarose powder. They were mixed well before heating in the microwave oven for 2 minutes. Casting tray was set on with the comb inserted in it. Then, 1 µL of of ethidium bromide was added into the agarose. After the solution mixed, pour them into the gel tray. The gel got cooled about 30 minutes before taking out the comb. Gel was inserted into the tank which consists of TAE buffer. 2 µL of sample was pipetted