Multiplex PCR in determination of Opiinae parasitoids of fruit flies, Bactrocera sp., infesting star fruit and guava

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Abstract

Malaysia is a tropical country that produces commercial fruits, including star fruits, Averrhoa carambola L. (Oxalidales: Oxalidaceae), and guavas, Psidium guajava L. (Myrtales: Myrtaceae). There is a high demand for these fruits, and they are planted for both local consumption and export purposes. Unfortunately, there has been a gradual reduction of these fruits, which has been shown to be related to fruit fly infestation, especially from the Bactrocera species. Most parasitic wasps (Hymenoptera: Braconidae: Opiinae) are known as parasitoids of fruit fly larvae. In this study, star fruits and guavas infested by fruit fly larvae were collected from the Malaysian Agricultural Research and Development Institute. The parasitized larvae were reared under laboratory conditions until the emergence of adult parasitoids. Multiplex PCR was performed to determine the braconid species using two mitochondrial DNA markers, namely cytochrome oxidase subunit I and cytochrome \(b\). Two benefits of using multiplex PCR are the targeted bands can be amplified simultaneously using the same reaction and the identification process of the braconid species can be done accurately and rapidly. The species of fruit flies were confirmed using the COI marker. The results obtained from our study show that Diachasmimorpha longicaudata (Ashmead) (Hymenoptera: Braconidae), Fopius arisanus (Sonan), and Pysttalia incisi (Silvestri) were parasitoids associated with Bactrocera carambolae (Drew and Hancock) (Diptera: Tephritidae) infested star fruits. Fopius arisanus was also the parasitoid associated with Bactrocera papayae (Drew and Hancock) infested guavas. Maximum parsimony was constructed in Opiinae species to compare tree resolution between these two genes in differentiating among closely related species. The confirmation of the relationship between braconids and fruit fly species is very important, recognized as preliminary data, and highly necessary in biological control programs.
Introduction

Malaysia is a tropical country that cultivates and produces a high amount of star fruits, *Averrhoa carambola* L. (Oxalidales: Oxalidaceae), and guavas, *Psidium guajava* L. (Myrtales: Myrtaceae). They are the most important commercial fruits because of their high demand for exportation purposes (Mokji and Abu Bakar 2007; Hii et al. 2011; Zakaria et al. 2011). According to the Department of Agriculture (2004), Malaysia’s star fruits have been exported to four major market countries: the Netherlands, France, Germany, and Singapore. A gradual reduction of these annual fruits has been shown to be associated with fruit fly infestation, especially from *Bactrocera* species. These species are highly diverse and broadly infest a wide variety of crops. The host records presented by Clarke et al. (2001) and Chua et al. (2010) determined that star fruits and guavas were mostly infested by *Bactrocera carambolae* (Drew and Hancock) (Diptera: Tephritidae) and *Bactrocera papayae* (Drew and Hancock), respectively. Infestation of star fruits and guavas by fruit fly species directly affects the quality of these fruits for domestic consumption and thus contributes to economic loss. Therefore, research on controlling targeted pests is valuable and significant for the maintenance and production of these fruits.

Parasitoids have been used as biological control in many countries in order to suppress the fruit fly species. Among the most commonly used biological control agents against fruit flies are members of Opiinae (Hymenoptera: Braconidae). They are known as solitary koinobiont endoparasitoids (Wharton et al. 2000; Wang et al. 2003) because the female wasps lay their eggs in their hosts, feed on the host tissues, and emerge as adults from the host puparia. For example, *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae), and *Psyttalia incisi* (Silvestri) were introduced into Hawaii and effectively reduced the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) (Harris et al. 1991; Vargas et al. 1993; Purcell 1998). *Bactrocera dorsalis* has become the most economically-important pest of edible fruits worldwide. Furthermore, it parasitizes the solanaceous fruit fly, *Bactrocera latifrons* (Hendel) (Diptera: Tephritidae) (Bokonon-Ganta et al. 2007; McQuate et al. 2007), and *Bactrocera cucurbitae* (Coquillett) (Bautista et al. 2004; Harris et al. 2010). In California, augmented releases of the parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae) have been used to suppression the olive fruit fly, *Bactrocera oleae* (Rossi) (Sime et al. 2006).

Several species of Opiinae have been recognized as potential parasitoids associated with *Bactrocera* species in Malaysia. The main
parasitoid species observed parasitizing *B. dorsalis* complex in star fruits at Selangor and Penang orchards include *F. arisanus, D. longicaudata*, and *P. incisi* (Ooi 1984; Vijaysegaran 1984; Serit et al. 1986; Ibrahim et al. 1995). According to Chua and Khoo (1995), it was concluded that *F. arisanus* were the main parasitoid associated with *B. carambolae*, which infested star fruits. Thus, they have been used in pest management programs. Moreover, a survey conducted by Chinajariyawong et al. (2000) showed that 13 Opiinae species have been recognized as parasitoids of fruit flies from a wide variety of host plant species. However, four Opiinae species have dominated this collection, including *D. longicaudata* and *F. arisanus*. These parasitoids were determined to be associated with *B. carambolae* and *B. papayae*, which infest star fruits and guavas. All the identifications of parasitoids were based on morphological characters.

Morphological characters can sometimes be limited when it comes to differentiating between closely related parasitoid species. Therefore, molecular methods are more suitable for identification of distinguished parasitoid species (Stouthamer et al. 1999; MacDonald and Loxdale 2004; Greenstone 2006; Gariepy et al. 2007; Bon et al. 2008). Accurate identifications of these parasitoids are highly significant and useful because they can be applied in biological control agents of fruit flies species (Vargas et al. 2007; Harris et al. 2010).

Recently, multiplex PCR, has been widely used to detect parasitoid species in a single reaction. This innovative tool is particularly very time saving because of the ability to amplify different fragment sizes of DNA targets simultaneously (Bej et al. 1991; Erlandson 2003; Traugott et al. 2006; Gariepy et al. 2007). Additionally, the sensitivity and specificity of the primers can be increased, thus preventing the formation of nonspecific products. Therefore, identification of parasitoid species can be determined accurately and rapidly. Generally, multiplex PCR has been implemented in parasitoid studies to identify multiple parasitoid species and host-parasitoid interactions. For example, Gariepy et al. (2005) developed species-specific PCR primers for three *Peristenus* species that parasitized *Lygus* nymphs. The results indicated that the PCR products with different amplification lengths determined the *Peristenus* species in a single reaction. Multiplex PCR has also been developed by Traugott et al. (2006) in detecting three parasitoid species of lepidopteran pests. Correct identification of these parasitoids has been used to successfully apply the parasitoids to suppress lepidopteran cabbage pests. Although this approach is widely used in applied entomology, it is also useful in the diagnosis of medical and veterinary diseases (Xu et al. 2012). Due to its high sensitivity and ability to detect different DNA targets simultaneously, this approach is commonly used in pathogen diagnosis. For instance, in insect parasitology, multiplex PCR has the ability to identify different mosquito species and strains that vector malaria (Saiwichai et al. 2007; Grafeo et al. 2008; Swain et al. 2009) and detect plant pathogens in insect vectors (Roy et al. 2005; Ravikumar et al. 2011). These molecular applications have provided information that allows rapid and accurate identification of species.

The aim of this study was to detect and identify the parasitoid species based on the cytochrome oxidase 1 (COI) and cytochrome *b* (Cyt *b*) gene sequences simultaneously. The benefits of using these genes are that they are relatively conserved regions and show a high
degree of nucleotide variations to distinguish among the species (Cann et al. 1984; Irwin et al. 1991; Hebert et al. 2004; Boehme et al. 2011). The COI gene is commonly known as DNA barcode and has been standardized for the molecular identification of all animals, including insects. To give better identification of these species, phylogeny has been constructed to differentiate between closely related species. Similarly, tephritid species are also confirmed by using the COI gene. Our study aimed to provide more information in recording potential parasitoids associated with fruit flies that infest star fruits and guavas. Accurate determination of these species would contribute to more effective pest management programs and directly increase production of these fruits.

Materials and Methods

Sample collection and DNA isolation
The larvae of tephritids that infested star fruits and guava were collected from Malaysian Agricultural Research and Development Institute (MARDI) of Kluang, Johor and Lanchang, Pahang, Malaysia (Table 1). Several of these larvae were reared for adult emergence. The tephritid larvae and emerged braconids were stored in 98% ethanol for preservation. DNA was isolated from three tephritid larvae to show the replications using the standard extraction kit and protocol provided by DNeasy Blood and Tissue Kit (Qiagen, www.qiagen.com). However, the first three steps from this kit were modified in order to develop a freezing method for the DNA isolation of adult braconid species (Yaakop et al. 2009). The first step of the freezing method was adding 180 µL of buffer ATL and 20 µL of proteinase K into the samples. Then, these samples were incubated at 55°C and kept in a freezer at -22°C overnight. The steps afterwards remained the same. This method was used so that the voucher specimens would still exist and the samples for morphological re-examination would be retained.

PCR amplification
A partial mitochondrial DNA COI gene was amplified by PCR from the tephritid species using the primer designed by Han and Ro (2005). However, for braconid species, multiplex PCR was performed using two pairs of primers designed by Folmer et al. (1994) and Simon et al. (1994) to amplify COI and Cyt b genes, respectively (Table 2).

PCR was carried out using MyGene MG96G Thermalcycler (Hangzhou LongGene Scientific Instruments Co, www.longgene.com) with the following cycles for COI of tephritids: 94°C for 3 min as initial denaturation, followed by 40 cycles of 93°C for 1 min, 56°C for 1 min, 72°C for 2 min, and the final extension step of 72°C for 15 min. For braconids, multiplex PCR was performed in order to amplify two DNA fragments simultaneously. The PCR conditions were: initial denaturation at 94°C for 3 min, followed by 40 cycles of 94°C for 1 min, 47–50°C for 1 min, and 72°C for 2 min. The final extension was at 72°C for 10 min. PCR amplification for COI tephritid was performed in a 25 µL reaction volume containing 16.50 µL dd H2O, 2.5 µL PCR buffer 10X (Vivantis, www.vivantechnologies.com), 1.30 µL 50 mM MgCl2, 0.5 µL 10 mM dNTPs, 0.5 µL forward and reverse primers (10 pmol/µL), 0.2 µL Taq DNA polymerase (5 U/µL) (Vivantis), and 3 µL DNA template (10–15 ng/µL). Meanwhile, for braconids, multiplex PCR was done in 50 µL reaction volume containing 33.0 µL dd H2O, 5.0 µL PCR buffer 10X (Vivantis), 2.5 µL 50 mM MgCl2, 1.0 µL 10 mM dNTPs, 0.5 µL forward and reverse primers (10 pmol/µL), 0.5 µL Taq DNA polymerase (5 U/µL) (Vivantis), and 6 µL DNA template.
(1–5 ng/µL). Amplified products were separated by gel electrophoresis and purified using the Geneaid purification kit (www.geneaid.com). Then, they were directly sent to the sequencing service company First Base (www.base-asia.com) for sequencing.

**Results**

**Multiplex PCR assay**

The COI and Cyt b genes were successfully amplified under the specific conditions described. Using the protocol described above, the COI and Cyt b genes produced fragments of 710 bp and 440 bp, respectively. For negative control, only chemical reagents were loaded for PCR amplification without DNA. Therefore, no PCR products were generated (Figure 1). All the reagents remained constant, except for changes in annealing temperature. Optimization was done in annealing temperature to enhance the amplification of specific products. It is a crucial parameter in multiplex PCR to achieve distinct bands for each primer set (Elizaquivel and Aznar 2008). The result showed each species of braconids has their own annealing temperature to produce higher amplification of COI and Cyt b genes simultaneously.

**Phylogenetic inference for braconids**

A total of 707 bp and 438 bp sequences were obtained from multiple alignments of COI and Cyt b genes, respectively. Out of 707 charac-
ters from COI fragments, 162 (22.9%) were parsimony informative. However, Cyt b sequences were more parsimony informative, with the value of 119 (27.2%). Additionally, the conserved sites constituted 522 (73.8%) characters, showing that the COI gene was highly conserved compared to the Cyt b gene. The conserved sites of Cyt b showed 307 (70.1%) characters. The pairwise genetic distance was estimated between four braconid species based on a Kimura-two-parameter test. This algorithm distinguishes between two types of substitutions (transitions and transversions). It is useful to estimate genetic divergence (Zhou et al. 2007). The highest nucleotide distance estimated from COI sequences was between D. longicaudata and P. incisi, followed by the distance between D. longicaudata and F. arisanus. Meanwhile, the highest nucleotide distance estimated from Cyt b sequences was between D. longicaudata and Fopius sp., followed by the distance between D. longicaudata and P. incisi (Table 3).

For the COI gene, the MP analysis based on equally-weighted characters produced two parsimonious trees (Figure 2). The best tree had a minimum evolution of 269 steps, with a consistency index of 0.8104, a homoplasy index of 0.1896, and a retention index of 0.9006. However, MP analysis for the Cyt b gene produced three parsimonious trees with a tree length of 195 (Figure 3). The best tree had a consistency index of 0.8104, a homoplasy index of 0.1896, and a retention index of 0.9006. The MP bootstrap trees produced for both genes were highly identical after bootstrap analysis with 1000 replications. Both tree topologies of MP analysis indicated D. longicaudata was highly distanced from other species. This species formed its own monophyletic clade, supported by a 100% bootstrap value. Otherwise, F. arisanus and Fopius sp. were indicated to be closely related species, highly supported by 96% and 67% bootstrap values of Cyt b and COI MP trees. Additionally, the lowest genetic distance shown between these species proved that they were genetical-
ly related to each other.

**BLAST results of tephritids**

For the tephritid species infesting star fruits, the result of BLAST showed that all three samples were referred to the *B. carambolae*. Otherwise, in guavas, the tephritid species determined from BLAST analysis were most identical to *B. papayae*. They were chosen based on higher value of maximum score, query coverage, maximum identical, and E-value (equal to zero) in BLAST analysis.

**Discussion**

*Bactrocera carambolae* has been recorded as the primary pest infesting star fruits. The parasitoids that have been recognized based on COI and Cyt b genes were *D. longicaudata*, *P. incisi*, and *F. arisanus*. Previous research on star fruits also found that these Opiinae species were the main parasitoids associated with *B. dorsalis* complex, including *B. carambolae* (Chua and Khoo 1995; Ibrahim et al. 1995; Chinajariyawong 2000). Otherwise, damage on guavas was caused by *B. papayae*, which is associated with the parasitoids of *Fopius* sp. A large survey conducted by Chinajariyawong et al. (2000) listed *D. longicaudata*, *F. arisanus*, and *P. incisi* as the most common parasitoids species detected in guavas and star fruits originating from Malaysia and Thailand. However, *Fopius vandenboschi* (Fullaway) has been recorded parasitizing *B. papayae* and *B. carambolae* in guavas. Additionally, star fruits were also the preferred host fruit for *F. arisanus* associated with *B. carambolae* (Chua and Khoo 1995). Besides *F. arisanus* and *F. vandenboschi*, no record has been made regarding *Fopius* sp. attacking *Bactrocera* sp. in Malaysia. Sometimes, several parasitoid species have restricted fruit flies species in certain crops. Therefore, accurate identification of braconid or tephritid species was very significant because the species were widely used in biological control programs (Vargas et al. 2007; Harris et al. 2010).

*Diachasmimorpha longicaudata* was shown to be highly different from other species based on phylogenetic analysis, which produced the highest genetic distance between this species and others. *Fopius arisanus* and *Fopius* sp. were closely related species, with the lowest genetic distance of 0.113 and 0.139 (COI and Cyt b gene). The slight differences between them are due to difficulties in distinguishing them properly. This study successfully identified both species by forming two distinct clades in both topologies. To improve identification of these species, further taxonomical work should be done, especially in 47.1.1 and 47.2 individuals to determine the *Fopius* sp. This information is very important, as it can be applied to the biological control of *Fopius* sp. and *F. arisanus* tephritids, parasitoids for guavas and starfruits, respectively. Only *F. arisanus* and *F. vandenboschi* were recorded infesting star fruits and guavas in Malaysia (Chinajariyawong et al. 2000). In our study, *Aspilota* sp. was selected as the outgroup because it belongs to subfamily Alysiinae, shown to be similar to the subfamily Opiinae that attacked Diptera species. However, *Aspilota* sp. were indicated parasitizing specific hosts largely from the families Phoridae and Platypezidae (Wharton et al. 1997).

Therefore, detection of parasitoids species using multiplex PCR was essential in recognizing the species. Amplification of COI and Cyt b genes simultaneously in multiplex PCR directly gives accurate and precise information in determining species. The sensitivity of the primer also increased because two different primers were applied in same the reaction (Traugott et al. 2006; Bon et al. 2008). Comparison information between
these two markers can be made easily, and a phylogenetic tree was constructed to reconfi rm these fi ndings. Additionally, multiplex PCR considerably reduces the lengthy process of singleplex PCR (Traugott et al. 2006) and is cost effective. All the samples of braconids were produced from a rearing method. Although this method required more time for braconid emergence, determination of these species was done accurately and rapidly using multiplex PCR by screening each sample with multiple target genes.

Previously, there was no record regarding utilizing COI and Cyt b genes simultaneously in multiplex PCR. Only the use of species-specific markers of COI genes was studied in detecting multi-parasitism species within their hosts (Tilmon et al. 2000; Erlandson et al. 2003; Traugott et al. 2006; Bon et al. 2008). More interspecifi c variability, but moderate intraspecifi c variability, shown in COI genes caused it to be used often in molecular studies in Hymenoptera (Simon et al. 1994; Dowton and Austin 2001). Furthermore, it was widely used as DNA barcoding due to the high degree of nucleotide variations for rapid identifi cation of the species (Herbet et al. 2004; Foottit et al. 2009; Asokan et al. 2011; Boehme et al. 2011). These advantages are especially useful in detecting and determining immature developmental stages of the species because they often lack morphological characters. Bactrocera carambolae and B. papayae are closely related species (Chua et al. 2010; Liu et al. 2011). The COI gene successfully differentiates between these tephritid species in larvae by showing the highest maximum identical during the BLAST analysis. On the other hand, detection of species using the Cyt b gene was still limited, even though this gene also showed nucleotide variations (Irwin et al. 1991). Additionally, it contains both slowly and rapidly evolving codon positions, as well as more conservative regions (Boakye et al. 1999; Farias et al. 2001; Meece et al. 2005). It shows that the Cyt b genetic differences within the insects are high enough to differentiate at the species level. However, there was no available information about Cyt b sequences in the GenBank regarding Opiinae parasitoids species, except studies by Gimeno et al. (1997). Therefore, Cyt b sequences of F. arisanus, P. incisi, and Fopius sp. will be new submissions to the database. Similar topology trees produced in this study clearly resolved and indicated that these selected genes were suitable for species level classification.

**Conclusions**

Multiplex PCR is an innovative tool for determining Opiinae species by amplifying multiplex targets of genes simultaneously. In our study, COI and Cyt b genes were selected because they showed higher nucleotide variations for rapid identifi cation of Opiinae. This advantage would give accurate and precise information about this species and can be confi rmed by constructing the phylogeny. The MP tree successfully identifi ed and differentiated between closely related species of Opiinae. Based on the results, D. longicaudata, P. incisi, and F. arisanus were parasitoids associated with B. carambolae that infested star fruits. However, in guavas, Fopius sp. was the parasitoid associated with B. papayae. This information is useful because the braconid’s hosts were specifi c for certain species of fruit flies and crops. Furthermore, this information can be applied in biological control programs against fruit flies that damage star fruits and guavas.

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References

Allwood AJ, Chinajariyawong A, Drew RA, Hamacek EL, Hancock DL, Hengsawad C, Jipanin JC, Jirasurat M, Kong Krong C, Kritsanaepaiboon S, Leong, CTS, Vijasegaran S. 1999. Host plant records for fruit flies (Diptera: Tephritidae) in Southeast Asia. The Raffles Bulletin of Zoology 7: 1–92.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. Journal of Molecular Biology 215(3): 403–410.

Asokan AR, Rebijith KB, Singh SK, Sidhu AS, Siddharthan S, Karanth PK, Ellango R, Ramamurthy VV. 2011. Molecular Identification and Phylogeny of Bactrocera Species (Diptera: Tephritidae). Florida Entomologist Society 94(4): 1026–1035.

Bautista RC, Harris EJ, Vargas RI, Jang EB. 2004. Parasitization of melon fly (Diptera: Tephritidae) by Fopius arisanus and Psyttalia fletcheri (Hymenoptera: Braconidae) and the effect of fruit substrates on host preference by parasitoids. Biological Control 30: 156–164.

Bej AK, McCarty SC, Atlas RM. 1991. Detection of coliform bacteria and Escherichia coli by multiplex polymerase chain reaction, comparison with defined substrate and plating methods for water quality monitoring. Applied Environmental Microbiology 57: 2429–2432.

Boakye DA, Tang J, Truc P, Merriweather A, Unnasch TR. 1999. Identification of bloodmeals in haematophagous Diptera by cytochrome b heteroduplex analysis. Medical and Veterinary Entomology 13: 282–287.

Boehme P, Amendt J, Zehner R. 2011. The use of COI barcodes of molecular identification of forensically important fly species in Germany. Parasitology Research 110(6): 2325–2332.

Bokonon-Ganta AH, MacQuate GT, Messing RH. 2007. Natural establishment of a parasitoid complex on Bactrocera (Diptera: Tephritidae) in Hawaii. Biological Control 42: 365–373.

Bon MC, Hoelmer K, Coutinot D, Ramualde N. 2008. PCR-based, rapid diagnosis of parasitism of Lygus spp. (Hemiptera: Miridae) by Peristenus relictus (Hymenoptera: Braconidae). Biocontrol Science and Technology 18(5): 505–516.

Cann PL, Brown WM, Wilson AC. 1984. Polymorphic sites and the mechanism of evolution in human mitochondrial DNA. Genetics 106: 479–499.

Chinajariyawong A, Clark AR, Jirasurat M, Kritsanaepiboon S, Labey HA, Vijaysegaran S, Walter GH. 2000. Survey of Opine parasitoids of fruit flies (Diptera: Tephritidae) in Thailand and Malaysia. The Raffles Bulletin of Zoology 48(1): 71–101.

Chua TH, Chong YV, Lim SH. 2010. Species determination of Malaysian Bactrocera pests using PCR-RFLP analyses (Diptera: Tephritidae). Pest Management Sciences 66(4): 379–84.
Chua TH, Khoo SG. 1995. Variations in carambola infestations by *Bactrocera carambolae* Drew and Hancock (Diptera: Tephritidae) with fruit availability in a carambola orchard. *Researches on Population Ecology* 37: 151–157.

Clarke AR, Allwood A, Chinajariyawong A, Drew RAI, Hngsa-wad C, Krong CK, Kritsaneepiboon S, Vijaysegaran S. 2001. Seasonal abundance and host use patterns of seven *Bactrocera* Macquart species (Diptera: Tephritidae) in Thailand and Peninsular Malaysia. *Raffles Bulletin of Zoology* 49: 207–20.

Department of Agriculture, Malaysia. 2004. *Technical Document for Market Access on Star fruit (Carambola).* Department of Agriculture, Malaysia.

Dowton M, Austin AD. 2001. Simultaneous analysis of 16S, 28S, COI and morphology in the Hymenoptera: Apocrita: Evolutionary transitions among parasitic wasps. *Biological Journal of the Linnaean Society* 74: 87–111.

Elizaquivel P, Aznar R. 2008. A Multiplex Rti-PCR reaction for simultaneous detection of *Escherichia coli* 0157:H7, *Salmonella* spp. and *Staphylococcus aureus* on fresh, minimal processed vegetables. *Food Microbiology* 25: 705–713.

Erlandson M, Braun L, Baldwin D, Soroka J, Ashfaq M, Hegedus D. 2003. Molecular markers for *Peristenus* spp. (Hymenoptera: Braconidae), parasitoids associated with *Lygus* spp. (Hemiptera: Miridae). *Canadian Entomologist* 5: 71–83.

Farias IP, Orti G, Sampaio I, Schneider H, Meyer A. 2001. The Cytochrome b gene as a phylogenetic marker: The limits of resolution for analyzing relationships among Cichlid fishes. *Journal of Molecular Evolution* 53: 89–103.

Felsenstein J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783–791.

Folmer O, Black M, Hoeh W, Lutz R, Vrijenhoek R. 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. *Molecular Marine Biology and Biotechnology* 3: 294–299.

Foottit RG, Maw HEL, Havill NP, Ahern RG, Montgomery ME. 2009. DNA barcodes to identify species and explore diversity in the Adelgidae (Insecta: Hemiptera: Aphidoidea). *Molecular Ecology Resources* 9: 188–195.

Gariepy TD, Kuhlmann U, Haye T, Gillott C, Erlandson M. 2005. A single-step multiplex PCR assay for the detection of European *Peristenus* spp., parasitoids of *Lygus* spp. *Biocontrol Science and Technology* 15: 481–495.

Gariepy TD, Kuhlmann U, Gillott C, Erlandson M. 2007. Parasitoids, predators and PCR: the use of diagnostic molecular markers in biological control of arthropods. *Journal of Applied Entomology* 131: 225–240.

Gimeno C, Belshaw RD, Quicke DLJ. 1997. Phylogenetic relationships of the Alysiinae/Opiinae (Hymenoptera: Braconidae) and the utility of cytochrome b, 16S and 28S D2 rRNA. *Insect Molecular Biology* 6(3): 273–284.

Graffeo R, Masucci L, Bugli F, Damiano F, Pinnetti C, Federico G, Fadda G. 2008. Multiple malaria infections in a pregnant
woman from Nigeria: detection by multiplex PCR. New Microbiologica 31: 565–567.

Greenstone MH. 2006. Molecular methods for assessing insect parasitism. Bulletin of Entomological Research 96: 1–13.

Hall T. 2005. Bioedit version 7.0.4. Department of Microbiology, North Carolina State University.

Han HY, Ro KE. 2005. Molecular phylogeny of the superfamily Tephritoidea (Insecta: Diptera): New evidence from the mitochondrial 12S, 16S and COII genes. Molecular Phylogenetic Evolution 34: 416–430.

Harris EJ, Okamoto RY, Lee CYL, Nishida T. 1991. Suitability of Dacus dorsalis and Ceratitis capitata (Diptera: Tephritidae) as hosts of the parasitoid Biosteres arisanus (Hymenoptera: Braconidae). Entomophaga 36:425–430.

Harris EJ, Bautista RC, Vargas RI, Jang EB, Eitam A, Leblanc L. 2010. Suppression of melon fly (Diptera: Tephritidae) populations with releases of Fopius arisanus and Psyttalia fletcheri (Hymenoptera: Braconidae) in North Shore Oahu, HI, U.S.A. Biological Control 55: 593–599.

Hebert PDN, Penton EH, Burns JM, Janzen DH, Hallwachs W. 2004. Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly Astraptes fulgerator. Proceedings of the National Academy of Sciences USA 101: 14812–14817.

Hii CL, Ong SP, Law CL. 2011. Drying studies of tropical fruits cultivated in Malaysia: A Review. Journal of Applied Sciences 11(24): 3815–3820.
MULTBLAST: A web application for multiple BLAST searches. Bioinformation 5(5): 224–226.

Mokji MM, Abu Bakar SAR. 2007. Starfruit classification based on linear hue computation. Elekrika 9: 14–19.

Ooi PA. 1984. A fruit fly survey in a starfruit orchard in Serdang, Selangor. Journal of Plant Protection in the tropics 1(1): 63–65.

Purcell MF. 1998. Contribution of biological control to integrated pest management of tephritid fruit flies in the tropics and subtropics. Integrated Pest Management Reviews 3: 63–83.

Ravikumar G, Raje Urs S, Vijaya Prakash NB, Rao CGP, Vardhana KV. 2011. Development of a multiplex polymerase chain reaction for the simultaneous detection of microsporidians, nucleopolyhedrovirus, and densovirus infecting silkworms. Journal of Invertebrate Pathology 107: 193–197.

Roy A, Fayad A, Barthe G, Brlansky RH. 2005. A multiplex polymerase chain reaction method for reliable, sensitive and simultaneous detection of multiple viruses in citrus trees. Journal of Virological Methods 129: 47–55.

Saiwichai T, Harnyuttanakorn P, Sukhumavasi W, Buddhhirakkul P, Bhumiratana A, Rojana-premsuk J, Nithiuthai S. 2007. Diagnosis of Plasmodium gallinaceum in infected mosquitoes by multiplex PCR. Journal of Tropical Medicine and Parasitology 30: 76–80.

Serit M, Jaal Z, Tan KH. 1986. Parasitism of Dacus dorsalis (Hendel) in a village ecosystem in Tanjung Bungah, Penang, Malaysia. Fruit Flies: Proceedings of the 2nd International Symposium: 441–448.

Sime KR, Daane KM, Nadel H, Funk CS, Messing RH, Andrews JW, Johnson MW, Pickett, CH. 2006. Diachasmimorpha longicaudata and D. kraussii (Hymenoptera: Braconidae), potential parasitoids of the olive fruit fly. Biocontrol Science and Technology 16: 169–179.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P. 1994. Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. Annals of the Entomological Society of America 87: 651–701.

Stouthamer R, Hu J, van Kan FJPM, Platner GR, Pinto JD. 1999. The utility of internally transcribed spacer 2 DNA sequences of the nuclear ribosomal gene for distinguishing sibling species of Trichogramma. BioControl 43: 421–440.

Swain S, Mohanty A, Mahapatra,N, Parida SK, Marai NS, Tripathy HK, Kar SK, Hazra RK. 2009. The development and evaluation of a single step multiplex PCR for simultaneous detection of Anopheles annularis group mosquitoes, human host preference and Plasmodium falciparum sporozoite presence. Transactions of the Royal Society of Tropical Medicine and Hygiene 103: 1146–1152.

Swofford DL. 2002. Phylogenetic analysis using parsimony and other methods, 4.0 beta version. Sinuauer Associates.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting,
position-specific gap penalties and weight matrix choice. *Nucleic Acids Research* 22(22): 4673–4680.

Tilmon KJ, Danforth BN, Day WH, Hoffmann MP. 2000. Determining parasitoid species composition in a host population: A molecular approach. *Annals of the Entomological Society of America* 93: 640–647.

Traugott M, Zangerl P, Juen A, Schallhart N, Pfiffner L. 2006. Detecting key parasitoids of lepidopteran pests by multiplex PCR. *Biological Control* 39: 39–46.

Vargas RI, Leblanc L, Putoa R, Eitam A. 2007. Impact of Introduction of *Bactrocera dorsalis* (Diptera: Tephritidae) and classical biological control releases of *Fopius arisanus* (Hymenoptera: Braconidae) on economically important fruit flies in French Polynesia. *Journal of Economic Entomology* 100: 670–679.

Vargas RI, Stark JD, Uchida GK, Purcell M. 1993. Opiine parasitoids (Hymenoptera: Braconidae) of oriental fruit fly (Diptera: Tephritidae) on Kauai island, Hawaii: islandwide relative abundance and parasitism rates in wild and orchard guava habitats. *Environmental Entomology* 21: 246–253.

Vijaysegaran S. 1984. The Occurrence of Oriental Fruit Fly on starfruit in Serdang and the status of its parasitoids. *Journal of Plant Protection in the tropics* 1(2): 93–98.

Wang XG, Messing RH, Bautista RC. 2003. Competitive superiority of early acting species: a case study of opiine fruit fly parasitoids. *Biocontrol Science and Technology* 16: 391–402.

Wharton, RA, Marsh PM, Sharkey MJ. 1997. Manual of the New World Genera of the Family Braconidae (Hymenoptera). *Special Publication of the International Society of Hymenopterists* 1: 439.

Wharton RA, Trostle MK, Messing RH, Copeland RS, Kimani-Njogu SW, Lux S, Overholt WA, Mohamed S, Sivinski J. 2000. Parasitoids of medfly, *Ceratitis capitata* and related tephritids in Kenyan coffee: a predominantly koinobiont assemblage. *Bulletin of Entomological Research* 90: 517–526.

Xu XG, Chen GD, Huang Y, Ding L, Li ZC, Chang CD, Wang CY, Tong DW, Liu HJ. 2012. Development of multiplex PCR for simultaneous detection of six swine DNA and RNA viruses. *Journal of Virological Methods* 183: 69–74.

Yaakop S, Achterberg CV, Idris AB. 2009. *Heratemis* Walker (Hymenoptera: Braconidae: Alysiinae: Alysiini): Revision and reconstruction of the phylogeny combining molecular data and morphology. *Tijdschrift voor Entomologie* 152: 3–64.

Zakaria L, Wan Chik M, Heng KW, Salleh B. 2012. *Fusarium* species associated with fruit rot of banana (*Musa* spp.), papaya (*Carica papaya*) and guava (*Psidium guajava*). *Malaysian Journal of Microbiology* 8(2): 127–130.

Zhou R, Zeng K, Chen X, Yang Z, Shi S, Wu Cl. 2007. Population genetics of speciation in nonmodel organisms: I. Ancestral polymorphism in mangroves. *Molecular Biology and Evolution* 24(12): 2746–2754.
### Table 1. Details of braconid and tephritid samples used in analyses.

| Code | Institution no. | Species          | Crop species / Hosts | Locality            | Accession no. COI | Accession no. Cyt b |
|------|-----------------|------------------|----------------------|---------------------|--------------------|---------------------|
| 34.1 | UKM000087       | *D. longicaudata* | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240376           | JX240364            |
| 34.2.1| UKM000088       | *Psyllia incisi*  | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240380           | JX240368            |
| 34.5 | UKM000092       | *D. longicaudata* | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240377           | JX240365            |
| 34.6.1| UKM000093       | *D. longicaudata* | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240378           | JX240366            |
| 34.7 | UKM000095       | *D. longicaudata* | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240379           | JX240367            |
| 34.9.1| UKM000097       | *P. incisi*      | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240381           | JX240369            |
| 34.10 | UKM000100       | *F. arisanus*    | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240382           | JX240370            |
| 34.12 | UKM000102       | *F. arisanus*    | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240383           | JX240371            |
| 34.13 | UKM000104       | *F. arisanus*    | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240384           | JX240372            |
| 34.14 | UKM000106       | *F. arisanus*    | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240385           | JX240373            |
| 47.1.1| UKM000108       | Fopius sp.       | *P. guava*           | Malaysia: Lanchang, Pahang, MARDI | JX240386         | JX240374            |
| 47.2 | UKM000109       | Fopius sp.       | *P. guava*           | Malaysia: Lanchang, Pahang, MARDI | JX240387         | JX240375            |
| FF34.1| UKM000110       | -                | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240388          | -                   |
| FF34.2| UKM000111       | -                | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240389          | -                   |
| FF34.3| UKM000112       | -                | *A. carambola*       | Malaysia: Kulang, Johor, MARDI | JX240390          | -                   |
| FF47.1| UKM000116       | -                | *P. guava*           | Malaysia: Lanchang, Pahang, MARDI | JX240391         | -                   |
| FF47.2| UKM000117       | -                | *P. guava*           | Malaysia: Lanchang, Pahang, MARDI | JX240392         | -                   |
| FF47.3| UKM000118       | -                | *P. guava*           | Malaysia: Lanchang, Pahang, MARDI | JX240393         | -                   |

### Table 2. List of the primers used for PCR amplifications.

| Gene | Primer name | Sequences (5’-3’) | References |
|------|-------------|-------------------|------------|
| COI  | COI F       | 5-TAC AAT TTA TCG CCT AAA CTT CAG CC-3 (Forward) | Han & Ro (2005) |
| COI  | COI R       | 5-CAT TTC AAG TTG TGT AAG CAT C-3 (Reverse) | Han & Ro (2005) |
| LCO2198 |              | 5-GGT CAA CAA ATC ATA AAG ATA TTG G-3 (Forward) | Folmer et al. (1994) |
| HCO1490 |              | 5-TAA ACT TCA GGG TGA CCA AAA AAT CA-3 (Reverse) | Folmer et al. (1994) |
| Cyt b | CB-J-10933  | 5-TCT TTT TGA GGA GCW ACW GTW ATT AC-3 (Forward) | Simon et al. (1994) |
| Cyt b | CB-N-11367  | 5-AAT TGA ACG TAA AAT WGT RTA AGC AA-3 (Reverse) | Simon et al. (1994) |

### Table 3. The pairwise genetic distance between the COI gene (below diagonal) and Cyt b gene (above diagonal) of braconid species.

|              | *D. longicaudata* | *P. incisi* | *F. arisanus* | *Fopius sp.* |
|--------------|------------------|-------------|---------------|--------------|
| *D. longicaudata* | -               | 0.233       | 0.21          | 0.247        |
| *P. incisi*    | 0.191           | -           | 0.195         | 0.223        |
| *F. arisanus*   | 0.169           | 0.142       | -             | 0.139        |
| *Fopius sp.*   | 0.155           | 0.165       | 0.113         | -            |