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Phylogenetic Tree Construction in Reconfirmation of Parasitoid Species (Braconidae: Opiinae), Reared From Fruit Flies (*Bactrocera papayae*) Infesting Star Fruit (*Averrhoa carambola*) Based on Mitochondrial 16S rRNA Sequences

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ABSTRACT

Opiinae (Hymenoptera: Braconidae) is an important parasitoid of fruit flies (Diptera: Tephritidae). Accurate identification is needed for them to be effectively used as biological control agents. This study demonstrates the use of molecular phylogenetic analyses to reconfirm the species of Opiinae reared from tephritids infesting star fruit species. A total of 14 specimens were isolated and a 401 bp of mitochondrial 16S rRNA gene was amplified and sequenced from each of them. Three individual tephritids hosts (*Bactrocera papayae*) were also identified based on the mitochondrial ND1 gene. Maximum Parsimony (MP) tree was constructed using PAUP 4.0b10. Three species of Opiinae were successfully identified based on the mitochondrial 16S rRNA gene, namely *Diachasmimorpha longicaudata* (Ashmead), *Fopius arisanus* (Sonan), and *Psyttalia incisi* (Silvestri). These molecular-based findings reconfirmed the parasitoid species of *B. papayae* as recorded by previous studies based on morphology. In fact, the identification of Opiinae individuals have been reconfirmed based on topology and branching pattern of the phylogenetic tree as well as based on genetic distance analyses, which matched morphological-based identification.

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INTRODUCTION

Braconidae is a diverse family of Hymenopteran wasps, consisting of 22 genera (Yu *et al*., 2005) and more than 17000

species (Yu *et al*., 2004). Most of them are parasitoids of holometabolous insects, attacking the larval stage (Wang *et al*., 2004). However, braconids consist of subfamilies that are very specific when choosing their host to parasitize (Achterberg, 2002). For example, Microgastrinae preferentially parasitize Lepidopteran larvae, Aphidinae parasitize aphids, while Alysiinae and Opiinae parasitize cyclorraphous Dipteran larvae. These specificities represent the existence of host-parasitoid evolutionary relationships (Whitfield, 1992).

Because Braconidae is the second most important family of parasitoid wasps, a lot of studies were conducted in order to investigate their potential as biological control agents in controlling host pest populations (Greathead, 1986; Duan and Messing, 1997; Vargas *et al*,. 2007; Daane *et al*., 2008). In addition, this interest also arises from significant losses in economical value of commercial crops due to pest species (Clarke *et al*., 2005). The parasitoids include species in the subfamily Opiinae, which are among the important parasitoids of fruit fly species attacking star fruits (Chinajariyawong *et al*., 2000). Star fruit is one of the most preferred crops attacked by fruit fly pests (Tan & Serit, 1994). Aside from economic loss, the pest might also lead to increased costs due to farmers' disinfection and pest control from which might also result environmental pollution. Thus, biological control methods are safer, cheaper and more environmentally-friendly tools in controlling pest populations, with the objective of avoiding economic loss to star fruit crops (Yokomi & Tang, 1996; Daane & Johnson, 2010).

Among the subfamilies within the Braconidae family, Opiinae, the koinobiont endoparasitoid of cyclorraphous Dipterans, constitutes among the largest Braconidae subfamily (Wharton, 1997). All of their developmental stages are spent inside their hosts, where the male Opiinae emerges earlier than the female (Rungrojwanich & Walter, 2000). The genera *Biosteres* (Forester) and *Opius* (Wesmael), commonly used as parasitoids in controlling fruit fly species, belong to this subfamily. The fruit fly species being attacked in Malaysia include *Bactrocera carambolae* Drew & Hancock, *B. latifrons* (Hendel), and *B. papayae* Drew & Hancock (Chinajariyawong *et al*., 2000; Chua & Khoo, 1995). Host preferences are related to host suitabilities (Mohamed *et al*., 2003), which depend on certain factors namely sex ratio, generation interval, and fecundity of the parasitoid's progeny (Vargas *et al*., 2002; Rousse *et al*., 2006). In Malaysia, a total of seven species of fruit flies have been recorded as susceptible to parasitoid attacked, with six of them infesting star fruit (Chinajariyawong *et al*., 2000).

Correct identification is crucial for effective use of Opiinae as a biological control agent against fruit flies (Rugman-Jones *et al*., 2009). However, the classification and identification processes based on morphological characteristics alone are somewhat confusing when identifying Braconidae species (Quicke & Belshaw, 1999; Wharton, 2000; Wharton

& Achterberg, 2000). Identification based on molecular techniques has thus been used to overcome this deficiency (Rugman-Jones *et al*., 2009). Aside from the rapid characteristics of molecular identification, molecular based techniques are also useful in phylogenetic tree construction, which enables individuals of the same species to be grouped together in the same clade. This could also contribute to more accurate species identification based on the grouping of individuals (Rugman-Jones *et al*., 2009). Phylogenetic inferences have also been constructed on Braconidae based on morphological characteristics (Quicke & Achterberg, 1990; Achterberg & Quicke, 1992; Wharton *et al*., 1992). However, some of these studies cannot propose a meaningful reclassification, due to low support levels obtained for most clades in most analyses (Belokobylskij *et al*., 2004), as well as homoplasy brought by the morphological characteristics (Quicke & Belshaw, 1999). There is also incongruence between morphological and molecular data (Zaldivar-Riverón *et al*., 2005). Therefore, many studies have been done in order to construct the phylogeny of Braconidae based on molecular data (Sharanowski *et al*., 2011; Michel-Salzat & Whitfield, 2004) as well as a combination of molecular and morphological data (Yaakop *et al*., 2009; Zaldivar-Riverón *et al*., 2005; Shi *et al*., 2005; Whitfield, 2002; Dowton & Austin, 1998).

To date, a number of studies regarding phylogenetic relationships among Braconids have been successful. The use of mitochondrial genes as molecular genetic markers is very useful in resolving phylogenetic relationships among closely related species (Lunt *et al*., 1996; Xie *et al*., 2006). For example, the mitochondrial ND1 gene was successfully used in the separation of different species due to rapid mutation rate at every third codon of the sequence (Michel-Salzat & Whitfield, 2004; Segura *et al*., 2006; Wan *et al*., 2011; Yaakop, 2009). Where else, the usage of mitochondrial 16S rRNA gene was also successful in molecular identification (Tang *et al*., 2012) including the identification of insects (Li *et al*., 2010; Guo *et al*. 2012). There are also records regarding the usage of the mitochondrial 16S rRNA gene that successfully resolved the relationships among braconid species **(**Dowton & Austin, 1994; Belshaw & Quicke, 1997; Whitfield, 1997; Dowton & Austin, 1998; Dowton *et al*., 1998; Mardulyn & Whitfield, 1999; Whitfield *et al*., 2002). However, these types of studies are still scarce in Malaysia. Thus , the objective of the present study was to investigate the use of phylogenetic tree construction based on mitochondrial 16S rRNA gene in confirming the identification of braconid species reared from fruit flies (Diptera: Tephritidae) infesting star fruits. Aside from that, this study might also add to the current data on the type of species attacking fruit flies infesting star fruits.

MATERIALS AND METHODS

Specimens

A total of 14 taxa originating from Kluang, Johor, Malaysia were selected for this study.

Table 1 represents a list of the analyzed taxa, institutional numbers, locality as well as their accession numbers. The specimens were reared from tephritid larvae collected at some branch campuses of the Malaysian Agricultural Research and Development Institute (MARDI). Slices of star fruits together with the tephritid larvae were placed in transparent plastic containers (24.5 cm x 13.5 cm x 13.0 cm) covered with cloth netting, and the base was lined with a layer of saw dust to provide suitable conditions for the transition process of larval into pupal stages. The insect cultures were maintained at constant room temperature of 23°C and relative humidity of 69% (pers. comm. Mrs . Suhana Yusof). The samples were preserved in 98 % ethanol before being analysed. Species *Aspilota vaga* Belokobylskij (2007) was used as an outgroup in the phylogenetic analyses. *A. vaga* is a parasitoid of the leaf mining Dipteran larvae, instead of the Tephritidae species.

DNA Extraction

DNA samples were extracted from three host Tephritidae by using the Qiagen kit (DNeasy® Blood & Tissue), based on the original guidelines. Individual braconids were also extracted for their DNA using the Qiagen kit (DNeasy® Blood & Tissue), but with modifications of steps one to three. The modified method known as the freezing method, allowed the isolation of DNA from the samples without damaging them so that they were still morphologically identifiable (Yaakop, 2009). 180 µl ATL buffer and 20 µl K proteinase were put into the centrifuge tube along with the sample. The samples were then incubated in a water bath at 55°C for 10 minutes before being mixed. They were then stored in a freezer at -20°C for two days. The DNA extraction then followed the extraction kit's original instructions.

PCR Amplification

Polymerase chain reaction (PCR) amplification was done by amplifying about 600 bp of the NADH1 dehydrogenase (ND1) and 400 bp of mitochondrial 16S rRNA genes from all genomic DNA for tephritids and braconids respectively. The oligonucleotide primers for amplifying tephritids sequences include foward Nad1-F5'-TAGTTGCTTGGTTGTGTATTCC-3' Nardi *et al*. (2005) and reverse Nad1-R5'-AGGTAAAAAACTCTTTCAAGC-3' Nardi *et al*. (2005), while for amplifying braconids sequences were 16S Wb5'-CACCTGTTTATCAAAAACAT-3' Dowton & Austin (1994) 16S outer5'-CTTATTCAACATCGAGGTC-3' Whitfield (1997).

Polymerase Chain Reaction (PCR) amplification for tephritid (ND1 region) was performed in 25 µl reaction volumes: 16.45 µl dd H_20, 2.5 µl PCR buffer 10X (Vivantis), 1.25 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl foward and reverse primers (20 pmol/µl), 0.3 µl *Taq* DNA polymerase (5 U/µl) (Vivantis), and 3 µl DNA template. The PCR's temperature profile consisted of an initial denaturation period of 5 min at 94°C, followed by 35 cycles of 94°C for 30s, 54°C for 1 min, and 72°C for 1 min 30s. A final extension step was added at 72 °C for 10

TABLE 1
List of braconids and tephritids taxa examined with UKM institution numbers, locality and accession numbers.

Label	UKM Institution No.	Species	Locality	Gene	Accession no.
34.1	UKM000087	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233524
34.2.2	UKM000089	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233531
34.3	UKM000090	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233530
34.4	UKM000091	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233525
34.5	UKM000092	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233526
34.6.2	UKM000094	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233527
34.7	UKM000095	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233528
34.8	UKM000096	*Diachasmimorpha longicaudata*	Malaysia: Johor, Kluang, MARDI	16S	JX233529
34.9.2	UKM000098	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233532
34.9.3	UKM000099	*Psyttalia incisi*	Malaysia: Johor, Kluang, MARDI	16S	JX233533
34.10.2	UKM000101	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233534
34.12.2	UKM000103	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233535
34.13.2	UKM000105	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233536
34.14.2	UKM000107	*Fopius arisanus*	Malaysia: Johor, Kluang, MARDI	16S	JX233537
FF 34.6	UKM000110	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233538
FF 34.8	UKM000114	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233539
FF 34.10	UKM000115	*Bactrocera papayae*	Malaysia: Johor, Kluang, MARDI	ND1	JX233540

min. PCR amplification was carried out for braconid (16S region) in 25 µl reaction volumes composed of 15.2 µl dd H_20, 2.5 µl PCR buffer 10X (Vivantis), 1.30 µl 50 mM $MgCl_2$, 0.5 µl 10 mM dNTPs, 0.5 µl foward and reverse primers (20 pmol/µl), 0.5 µl *Taq* DNA polymerase (5 U/µl) (Vivantis), and 4 µl DNA template. The double stranded DNA was amplified in an Eppendorf Mastercycler using initial denaturation period of 3 min at 94°C, followed by 35 cycles of 94°C for 1m, 45°C for 1 min, and 72°C for 1 min. A final extension step of 72°C for 5 min was added. Three microlitres of each amplified PCR product was run for electrophoresis on 1.5% agarose gel, stained with ethidium bromide (ETBR), and visualized under ultraviolet light for the detection of the amplified DNA. The PCR product with the correct amplified DNA fragment size was purified using a purification kit (GeneAid) following the manufacturer's instructions. The purified products were sent to First Base Sdn. Bhd. for sequencing.

Sequence Alignment

The sequencing results underwent pairwise alignment using the BioEdit version 7.0.2. software (Hall, 2005) to produce the final sequence. Species names of the samples were then identified by comparing the final sequence with GenBank data through the internet software Basic Local Alignment Search Tool (BLAST) (Altschul *et al*., 1997, Tatusova *et al*., 1999).

Phylogenetic Analysis

The 16S gene sequences of the 14 samples were aligned using Clustal W under default parameter settings, together with the sequence of *Aspilota vaga* (JX233523). The sequences were then adjusted visually by referring to the secondary structures present. All sequences were analyzed using PAUP 4.0b10 for phylogeny reconstruction. The methods of analysis in PAUP included maximum parsimony (MP) with 1000 stepwise addition replicates in a heuristic search (Swofford, 2002). The MP tree was subjected to bootstrap analysis with 1000 replicates to obtain bootstrap value support and topology stability estimates. MEGA 3.1 was used to obtain transition and transversion ratio (Kumar *et al*., 2004), pairwise genetic distances as well as other characteristics of the DNA sequences.

RESULTS AND DISCUSSION

DNA sequences from 14 braconid samples were amplified, producing 401 bp each of 14 sequences. The results from the BLAST search of the tephritid sequences showed that the host species were *Bactrocera papayae* (Table 1). The BLAST results for braconids consisted of three species, namely *Diachasmimorpha longicaudata* (Ashmead), *Fopius arisanus* (Sonan) and *Psyttalia incisi* (Silvestri). Therefore, this present study based on 16S genetic markers and analyses of 14 specimens suggested that *P. incisi*, *F. arisanus*, and *D. longicaudata* appeared to be parasitoids of *B. papayae* infesting star fruits in Kluang Johor. Our molecular results supported the

rearing and morphology based findings of Chinajariyawong *et al*. (2000) but they reported more parasitoids attacking tephritids infesting star fruits namely *F. vandenboschi, P. makii,* and *P. fletcheri* which were not detected in the present study. The fact that these braconids are parasitoids of tephritid fruit flies was also supported by the studies of Stuhl *et al*. (2011), Bautista *et al*. (1999), and Guang-Hong *et al*. (2006). But, these researches done outside of Malaysia found *B. dorsalis* to be the host which was not the case here. In addition, there is a scarcity of records of braconids-tephritid relationships in Malaysia.

Characteristics of DNA Sequences

A total of 113 out of 385 sites were represented as variable sites. Of these, 91 of them were parsimoniously informative sites (80.5%), while the rest of them were parsimoniously uninformative (19.5%). Only 23% out of 400 characters were informative, indicating that the 16S gene is indeed a conserved mitochondrial gene. Among all of the sequences, 67.6 % of the characters represented conserved sites, indicating that these sequences were highly conserved. The nucleotide composition averages among all the sequences were 38.5% (T), 6.6% (C), 47.1% (A), and 7.8% (G). The highest nucleotide pair average was for AA (155), followed by TT (122), while the lowest was for GA (1). Additionally, no GT and GC pairs existed. Among these pairs, the average values of transitional (ti) pairs and transversional (tv) pairs are 9 and 37 respectively, resulting in a ti/tv ratio of 0.24.

Distance Analysis

Pairwise genetic distance was ascertained based on the Kimura-2-Parameter test (Pevsner, 2009). All positions containing gaps and missing data were eliminated from the dataset. The genetic distance between specimens of the same species was zero, indicating that there was no difference in their genetic compositions. For the outgroup, the genetic distance was the highest between the groups of *D. longicaudata* and *F. arisanus* with a value of 0.202, while the lowest was between *F. arisanus* and *P. incisi* which was 0.174 (Table 2).

The presence of variations among species could be detected even at the smallest genetic distance value. However, the distance analyses conducted showed that no genetic distances existed between individuals of the same species. Thus it was proven that there was no species variation

TABLE 2
Pairwise genetic distance between species; *D. longicaudata, P. incisi,* and *F. arisanus* based on the Kimura two-parameter method.

Distance	*D. longicaudata*	*P. incisi*	*F. arisanus*
D. longicaudata	-	-	-
P. incisi	0.181	-	-
F. arisanus	0.202	0.174	-

among the examined specimens of Opiinae.

Phylogenetic Inferences

A total of 136 most parsimonious trees were constructed by maximum parsimony (MP) analysis based on equally weighted total substitution. The best 136 lengths of MP tree constructed (Fig. 1) had consistency index (CI), homoplasy index (HI), and retention index (RI) values of 0.9044, 0.0956, and 0.9645 respectively. All of the *D. longicaudata* fell into clade A, while the rest were grouped into clade B. The *P. incisi* and *F. arisanus* lineages fell into different subclades. The phylogenetic bootstrap tree produced based on 1000 replicates was identical to the MP tree. The MP tree indicated that *D. longicaudata, P. incisi* and *F. arisanus* formed a monophyletic group. In addition, *P. incisi* and *F. arisanus* were closely related to each other. This statement was also supported by the low genetic distance value between them. They formed sister groups with high 74% bootstrap support. *D. longicaudata* was a sister group to both *P. incisi* and *F. arisanus* with 100% bootstrap value.

Instead of referring only to genetic distance analysis, the absence of species variation of individuals within the same genus emerging from the 16S sequences was further proven based on the constructed

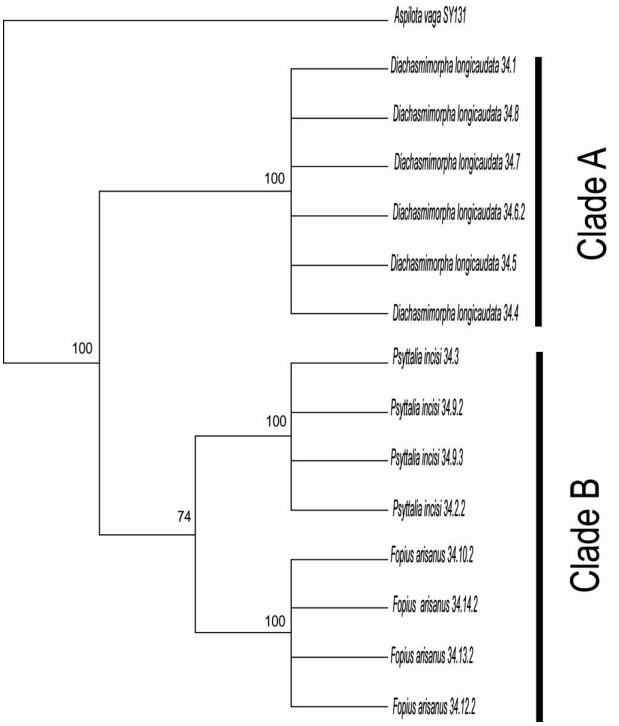

Fig. 1: The Maximum Parsimony tree for 14 species of Opiinae and rooted by outgroup *Aspilota sp.* (Hymenoptera: Braconidae: Alysiinae) based on 16S sequences. Numbers above branches indicate bootstrap values (%).

MP tree. The topology of this tree confirmed that there was no species variation as well as no mistake in the identification of the Opiinae parasitoids based on the molecular methods. The voucher specimens were also re-identified using a stereomicroscope StemiD4 and referring to a morphological genus key (Achterberg, unpublished) to confirm the species taxonomy, and this morphological-based identification matched the identification based on molecular methods using 16S rRNA marker.

CONCLUSIONS

The mitochondrial 16S gene was successfully used to group the Opiinae genus. Molecular approaches are useful for improving the identification of specimens identified initially based on morphological characteristics alone (Chinajariyawong *et al*., 2000; Belokobylskij & Ku, 1998; Aydogyu, 2008; Yilmaz & Beyarsian, 2009). Reconfirmation of the identity of parasitoids was successful, and it was found that *D. longicaudata* (Ashmead), *F. arisanus* (Sonan) and *P. incisi* (Silvestri) appeared to be potential biological control agents of *B. papayae*. Our findings although based on star fruits from a single locality are significant enough to confirm the identities of parasitoids of star fruit pests. However, future studies using star fruits from different geographical origins in Peninsular Malaysia as well as getting more emerging braconids from additional fruit species and using more genes should be done.

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