Isolation and Characterization of *Wolbachia* (Rickettsiales: Rickettsiaceace) from Several Economic Importance Parasitoids (Hymenoptera: Braconidae)

Muhamad Azmi Mohammed[1*], Ameyra Aman Zuki [1], Suhana Yusof[2], Salmah Yaakop[1]

[1] Centre for Insects Systematic, School of Environmental and Natural Resource Sciences, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, 43600 Bangi, Selangor, Malaysia.
[2] Horticulture Research Centre, Malaysian Agriculture for Research and Development Institute (MARDI), Persiaran MARDI-UPM, Ibu Pejabat MARDI, 43400 Serdang, Selangor, Malaysia.

* Corresponding author. Tel.: 60389215983; email: salmah78@ukm.edu.my
Manuscript submitted April 13, 2015; accepted May 22, 2015.
doi: 10.17706/ijbbb.2015.5.4.256-263

Abstract: Endosymbiotic relationship between *Wolbachia* (Rickettsiales: Rickettsiaceace) and braconids species is a new topic to be discussed among researchers due to its potential as biocontrol agents of fruit flies species. In this study, *Wolbachia Surface Protien* (*wsp*) marker has been used to detect the existence of *Wolbachia* molecularly that associated with braconids (Opiinae) collected from several localities throughout Peninsular Malaysia. Our results revealed that five species of parasitoids viz. *Fopius arisanus*, *F. vandensboschi*, *Psyttalia* sp.*, P. fletcheri* and *P. incisi* were symbiont positively with nine different strains of *Wolbachia*. A total of nine strains have been successfully sequenced and classified into three *Wolbachia* subgroups namely *Mel*, *Ri*, and *Inc* based on the results of clustering analysis. In this study, *Inc Wolbachia* group has been known as a novel group emerged of *Wolbachia*. These data should be useful in future *Wolbachia*-based biological control program that involving parasitoids and fruit flies.

Key words: Endosymbiont, *Wolbachia*, parasitoids, braconidae, opiinae.

1. Introduction

The using of insecticides to control crop pests in food production sector continuously be a controversial issue due to several health problems to the consumers [1]. Natural enemies consisting of parasitoids, predators, nematodes, and entomopathogenic microbes [2] are the common biocontrol agents to be used in replacing the insecticides [3]. For example, *Cotesia flavipes,* a braconid parasitoid species [4] has been acknowledged as the potential biocontrol agent in controlling pests stem borer, *Diatraea saccharalis* [4].

Symbiotic relationship between bacteria and insect species has been the popular topic to be discussed among the entomologists and bacteriologists, e.g *Wolbachia*. According to molecular phylogenetic analysis based on the 16S rRNA gene, *Wolbachia* belongs to α-Proteobacteria, whose evolutionary are related to intracellular bacterial species such as *Rickettsia*, *Anaplasma* and *Ehrlichia* [5]. It is highly associated with most of insects species by infecting almost 70% of them and importantly in evolutionary processes of insect species [6]. The relationships between *Wolbachia* and its host associated with the alteration of reproductive systems such as the induction of cytoplasmic incompatibility (CI), form of embryonic lethality in crosses between males and females infected with unalike *Wolbachia* strains or supergroups; induction of parthenogenesis in certain parasitic wasps; feminization of infected genetic males into functional females in

some isopod species; and male lethality [7]. Some insect species could not produce progeny without the infection of *Wolbachia*, viz. bed bug *Cimex lectularius* [8].

The well-discussed topic of the *Wolbachia* infections in endoparasitoids is the effects of *Wolbachia* towards the reproduction system of parasitoids (16). *Wolbachia* have been proven to infect several economic importance parasitoids such as *C. sesamiae* (15), *Asobara japonica* (17, 18) and others. In genus *Asobara*, *Wolbachia* is needed in oogenesis process, where females were dependent on *Wolbachia* to produce their oocytes (17). The infections of *Wolbachia* in *Asobara japonica* would also induce parthenogenesis, while other populations are not infected and reproduce sexually. *Wolbachia*-infected *A. japonica* females commonly produce small numbers of male offspring (18).

In Malaysia, information of *Wolbachia* in economic importance parasitoids (Hymenoptera: Braconidae) are very limited and not completely studied. Therefore, the isolation and characterization of *Wolbachia* using PCR-based detection of *Wolbachia Surface Protein* (*wsp*) region in several opiines species have been carried out. The information obtained are very useful and valuable as fundamental data towards in reducing of fruit fly populations from several localities in Peninsular Malaysia.

2. Materials and Methods

2.1. Opiines Collection

Adult of braconid parasitoids emerged from the infested larval stages of fruit flies, that infesting two host plant species, namely carambola (*Averrhoa carambola*) and luffa (*Luffa acutangula*) (Table 1). Samples of rotten fruits along with tephritids larvae were brought to laboratory and placed in plastic containers. To ensure the dryness of the container surface and to provide the optimum condition for metamorphosis process from larvae into pupa stage, layers of saw dust have been added to the containers. These steps of rearing process have been done in room temperature (24^0C to 25^0C) with relative humidity of 50% to 65%. A number of individual adult braconids were collected from the rearing process. The collected specimens were preserved in 90% ethanol before being used in laboratory work.

Table 1. List of Opiine Parasitoids Used in This Study and the Status of the *Wolbachia* Infection

Code	Insect species	Host plant species	Locality	Infection status
PKL01	*Fopius arisanus*	*Averrhoa carambola*	Kluang, Johor, MALAYSIA	Infected (+)
PKL02	*Fopius arisanus*	*Averrhoa carambola*	Kluang, Johor, MALAYSIA	Infected (+)
PKL03	*Fopius vandensboschi*	*Averrhoa carambola*	Kluang, Johor, MALAYSIA	Infected (+)
PSR01	*Fopius arisanus*	*Averrhoa carambola*	Serdang, MALAYSIA	Infected (+)
PSR02	*Fopius arisanus*	*Averrhoa carambola*	Serdang, MALAYSIA	Infected (+)
PSR03	*Fopius vandensboschi*	*Averrhoa carambola*	Serdang, MALAYSIA	Infected (+)
PSR04	*Psyttalia* sp.	*Averrhoa carambola*	Serdang, MALAYSIA	Infected (+)
PLC01	*Psyttalia incisi*	*Averrhoa carambola*	Lanchang, Pahang, MALAYSIA	Infected (+)
PST01	*Psyttalia fletcheri*	*Averrhoa carambola*	Setiu, Terengganu, MALAYSIA	Infected (+)

2.2. Final Braconids Species Identification

Opiine braconids have been identified morphologically up to species level based on taxonomic key by Fischer [9] and Wharton [10]-[12], while Chen & Wu [13] and Fischer [14] for *Fopius* and *Psyttalia,* respectively.

2.3. Bacterial DNA Isolation and PCR Amplification

A DNA sample has been extracted [15] from each individual of braconids by using freezing method with Qiagen kit (DNeasy® Blood & Tissue). The *Wolbachia Surface Protein* (*wsp*) *Wolbachia* has been used for PCR amplification using general *Wolbachia* primers consists of: *wsp* 81F (5'-TGGTCCAATAAGTGATGAAGAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTACTCCA-3'). These primers amplify a DNA fragment ranging from 590 to 632 bp depending on the individual *Wolbachia* strain. PCR amplification has been done in 25-µl reaction volumes: 6.5 µl dd H_2O, 12.5 µl MyTaq™ Red Mix, 1.0 µl forward and reverse primers (20 pmol/µl), and 4.0 µl DNA template. Thermocycling conditions are as follows: initial denaturation at 95^{0}C for 1 min, followed by 25-35 cycles with denaturation step at 95^{0}C for 15 s, annealing at 55^{0}C for 15 s, extension at 72^{0}C for 10 s and a final extension at 72^{0}C for 10 min. *Wolbachia*-infected on *Fopius arisanus* has been used as positive control, while substituting deionised distilled water for template DNA as negative control. A total of 3 µl of each amplified PCR product has been run for electrophoresis on 1.5% agarose gel to ensure the presence of amplified DNA. The PCR product has been purified using QIAquick® PCR Purification Kit by following the manufacturer's instructions. The purified products have been sent to First Base Sdn. Bhd, Petaling Jaya, Selangor, Malaysia for sequencing analysis.

2.4. Clustering Analysis

The sequences obtained were checked via BLAST (Basic Local Alignment Search Tool) to confirm no contamination occurs to the samples. The clustering analysis has been implemented on nine *wsp* sequences and several *wsp* sequences obtained from the GenBank (Braig *et al*. [16]) (Table 2). *Wsp* sequences dataset has been aligned using ClustalW [17] for multiple alignments. PAUP 4.0 version b10 [18] has been used to cluster the *Wolbachia* species using Maximum Parsimony (MP) and Neighbor-Joining (NJ) analyses. The clustering analyses were set to 1000 replications for both trees. *Wuchereria bancrofti* (DQ093846) and *Aprostocetus* sp. (HQ121415) have been used as outgroups in this study.

Table 2. Reference *wsp* Strains Used in This Study Including *Wolbachia* Group Nomenclature, Host Species and GenBank Accession Number [16]

Wolbachia group	Host species	Associated *Wolbachia* strain	GenBank Accession number
Des	*Dacus destillatoria*	*wDes*	AF295344
Aus	*Glossina austeni*	*wAus*	AF020077
Haw	*Drosophila simulans* (Hawaii)	*wHa*	AF020068
Pap	*Phlebotomus papatasi*	*wPap*	AF020082
Uni	*Muscidifurax uniraptor*	*wUni*	AF020071
Mors	*Glossina morsitans*	*wMors*	AF020079

3. Results and Discussion

In this study, we have successfully isolated and characterized nine sequences of *wsp* (*Wolbachia Suface Protien*) from five opiine species (*Fopius arisanus*, *F. vandensboschi*, *Psyttalia* sp., *P. fletcheri* and *P. incisi*) that collected from four localities (Johor, Selangor, Pahang, and Terengganu) in Peninsular Malaysia. The *wsp*

gene has been chosen as the marker for *Wolbachia* detection due to high variability, which makes possible for a precise result using on phylogenetic analyses [16], [19], [20]. The results showed that *Wolbachia* presented in all opiine braconid samples in this study, which positively associated in several species from several studies, namely *Asobara tabida* [20], *Cotesia sesamiae* [21] and *Fopius arisanus* [22]. Furthermore, our results presented several additional new data on the association between *Wolbachia* and Opiines.

Clustering analysis by implementation of *wsp* sequences using Neighbor-Joining (NJ) and Maximum Parsimony (MP) analyses have constructed congruent topologies in this study (Fig. 1-Fig. 2). A total of nine *wsp* sequences have been clustered at three different clades (Fig. 1-Fig. 2). Four of the *wsp* sequences (*Fopius arisanus* PKL01, *F. arisanus* PKL01, *F. vandensbochi* PKL03, and *Psyttalia* sp. PSR04) were clustered under *Mel* subgroup; one *wsp* sequence (*P. fletcheri* PST01) was clustered under *Ri* subgroup; and the remaining four *wsp* sequences (*P. incisi* PLC01, *F. vandensboschi* PSR03, *F. arisanus* PSR01, and *F. arisanus* PSR02) were clustered together in a new emerging novel group, *Wolbachia* subgroup, *Inc*.

From this data, *Wolbachia* of *F. arisanus* have been clustered into three different subgroups, namely *Mel*, *Ri*, and *Inc*. Three different *Wolbachia* strains in *F. arisanus* localized at three different subgroups (Fig. 1 and Fig. 2) are divergent, suggesting that each strain was independently acquired [23]. Besides that, two strains of *F. vandensboschi* were infected and clustered into two different subgroups—*Mel* and *Inc*. It indicated that one species of host can harbor more than one *Wolbachia* strain which are congruent with a previous study by Parvizi *et al*. [24]. One species of *Paraphlebotomus* sandflies from Iran were believed to be infected by two *Wolbachia* strains, namely *Turk 54* (*wPap*) and *Turk 07* (novel strain) [24]. Another previous study congruent with our result is the study of *Wolbachia* infection in tephritid fruit flies from Thailand [23]. It have been proven that *Bactrocera ascita* sp. B was simultaneously infected by five independent *Wolbachia* strains which clustered into five subgroups namely *Mel*, *Des*, *Cuc*, *Con*, and *Asc* groups.

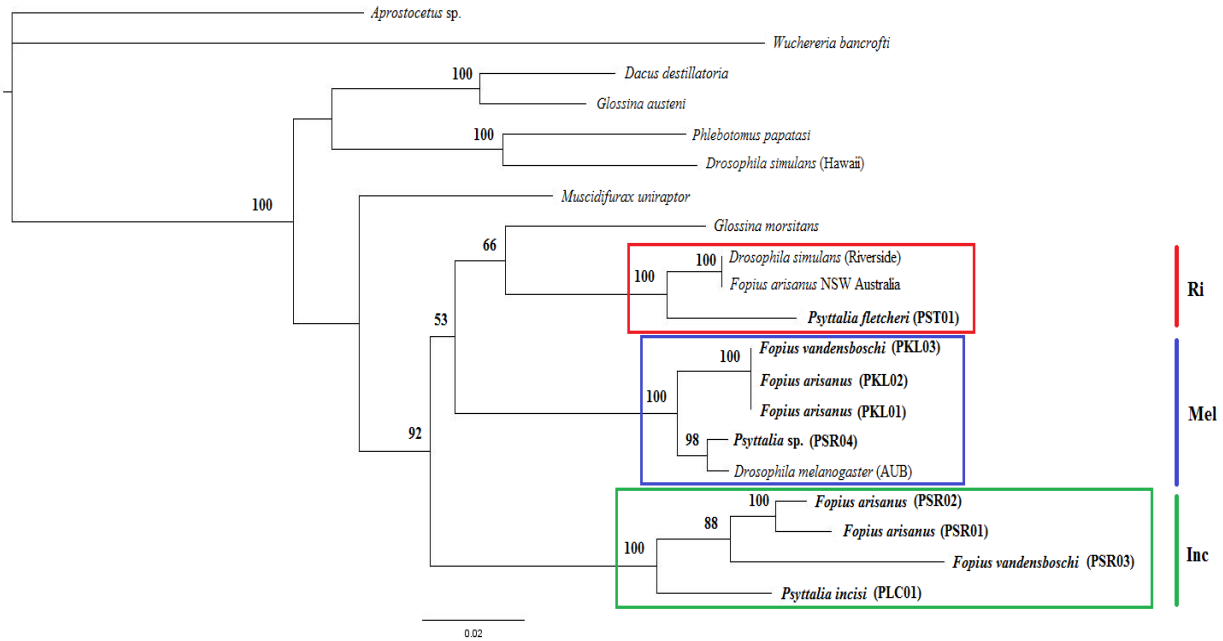

Fig. 1. Neighbour-Joining tree based on *wsp* gene sequences using NJ analysis. Number above branches are bootstrap values (1000 replications).

The clustering analysis showed significant differences between *Wolbachia* supergroups. The most common *Wolbachia* endosymbionts found in arthropods are classified into supergroup A and B [19], [25], [26], [27]. The outgroups in this study were *Aprostocetus* sp. which have been infected with *Wolbachia*

supergroup B and *Wuchereria bancrofti* which belong to *Wolbachia* supergroup D. The clustering analysis showed that *Wolbachia* supergroup A distinctively separated from outgroups that belong to supergroup B and D. All *wsp* sequences of this study were groups together in supergroup A clade with bootstrap support of 100% in both NJ tree and MP tree.

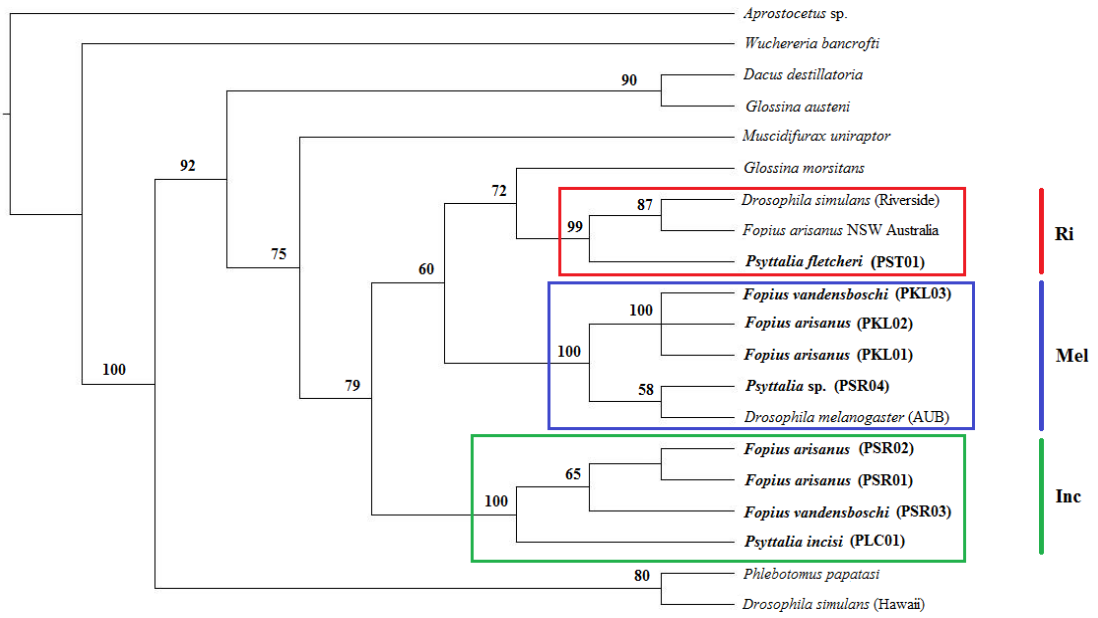

Fig. 2. Maximum Parsimony tree based on *wsp* gene sequences using MP analysis. Numbers above branches are bootstrap values (1000 replications).

Results obtained in this study are considered as preliminary data in measuring the infection status of endosysmbiont *Wolbachia* in several opiine species. Consequently, our results would provide more effective biological control programs in relation to alteration of reproductive system that involving parasitoids and fruit flies. Different habitats and ecology [28] and speciation agent by generating reproductive isolation [29] are two main factors that contribute to diversity of *Wolbachia.* More new strains would be discovered in the future studies of *Wolbachia* infection in parasitoids.

Acknowledgment

The first author thanks Mrs. Norsiah Mohamad Jani and Mr. Samsudin Yusof from Malaysian Agriculture for Research and Development Institute (MARDI) for collecting and rearing the specimens. This research was funded by the research grants FRGS/1/2014/SG03/UKM/02/1 and GUP-2014-029.

References

[1] Coats, J. R. (1994). Risks from natural versus synthetic insecticides. *Annual Review of Entomology, 39(1)*, 489-515.

[2] Brown, S. L., & Balch, G. T. (1987). *Private Pesticide Applicator Training Manual*. USA: Circular ANR.

[3] Bale, J. S., Van Lenteren, J. C., & Bigler, F. (2008). Biological control and sustainable food production. *Philosophical Transactions of the Royal Society B, 363,* 761-776.

[4] Lv, J., Wilson, L. T., Beuzelin, J. M., White, W. H., Reagan, T. E., & Way, M. O. (2011). Impact of *Cotesia flavipes* (Hymenoptera: Braconidae) as an augmentative biocontrol agent for the sugarcane borer (Lepidoptera: Crambidae) on rice. *Biological Control, 2,* 159-169.

[5] Breeuwer, J. A., Stouthamer, R. A., Barns, S. M., Pelletier, D. A., Weisburg, W. G., & Werren, J. H. (1992).

Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. *Insect Molecular Biology*, *1,* 25-36.

[6] Breeuwer, H., & Werren, J. H. (1990). Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. *Nature*, 346, 558-560.

[7] Stouthamer, R., Breeuwer, J. A. J., & Hurst, G. D. D. (1999). *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. *Annual Review Microbiology, 53,* 71-102.

[8] Hosokawa, T., Koga, R., Kikuchi, Y., Meng, X. Y., & Fukatsu, T. (2010). *Wolbachia* as a bacteriocyte associated nutritional mutualist. *Proceedings of the National Academy of Sciences of the United States of America*, *Vol. 107,* pp. 769-774.

[9] Fischer, M. (1972). Hymenoptera: Braconidae (Opiinae I). *Das Tierreich, 91, 1-620.*

[10] Wharton, R. A. (1987). Changes in nomenclature and classification of some opiinae Braconidae (Hymenoptera). *Proceedings of the Entomological Society of Washington, Vol. 89*, pp. 61-67.

[11] Wharton, R. A. (1988). Classification of the Braconid subfamily Opiinae (Hymenoptera). *The Canadian Entomologist, 120,* 333-360.

[12] Wharton, R. A. (1997). Generic relationships of opiinae Braconidae (Hymenoptera) parasitic fruit infesting Tephritidae (Diptera). *Contributions of the American Entomological Institute, 30*, 1-53.

[13] Chen, J. H., & Wu, Z. S. (1994). *The Alysiini of China (Hymenoptera: Braconidae: Alysiinae)*. Beijing: China Agriculture Press.

[14] Fischer, M. (1987). Hymenoptera: Opiinae III — Athiopische, orientalische, australische und ozeanische Region. *Das Tierreich, 104,* 1-734.

[15] Yaakop, S., Van Achterberg, C., & Ghani, I. A. (2009). *Heratemis* Walker (Hymenoptera: Braconidae: Alysiinae: Alysiini): Revision and reconstruction of the phylogeny combining molecular data and morphology. *Tijdschrift voor Entomologie, 152,* 3-64.

[16] Braig, H. R., Zhou, W., Dobson, S. L., & O'Neill, S. L. (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. *Journal of Bacteriology, 180(9),* 2373-2378.

[17] Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F., & Higgins, D. G. (1997). The ClustalX windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Research, 25,* 4876-4882.

[18] Swofford, D. L. (2002). *PAUP*. Phylogenetic Analysis Using Parsimony (*and Other Methods)*. Massachusetts: Sinauer Associates.

[19] Zhou, W., Rousset, F., & O'Neil, S. L. (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. *Proceedings of the Royal Society of London Series B Biological Science, Vol. 265, No. 1395,* pp. 509-515.

[20] Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., & Bouletreau, M. (1999). Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. *Molecular Biology and Evolution, 16,* 1711-1723.

[21] Mochiah, M. B., Ngi-Song, A. J., Overholt, W. A., & Stouthamer, R. (2002). *Wolbachia* infection in *Cotesia sesamiae* (Hymenoptera: Braconidae) causes cytoplasmic incompatibility: Implications for biological control. *Biological Control*, *25,* 74-80.

[22] Morrow, J. L., Frommer, M., Shearman, D. C. A., & Riegler, M. (2014). Tropical Tephritid fruit fly community with high incidence of shared *Wolbachia* strains as a platform for horizontal transmission of endosymbionts. *Environmental Microbiology, 16(12)*, 3622-3637.

[23] Jamnongluk, W., Kittayapong, P., Baimai, V., & O'Neill, S. L. (2002). *Wolbachia* infections of Tephritid fruit

flies: Molecular evidence for five distinct strains in a single host species. *Current Microbiology, 45,* 255-260.

[24] Parvizi, P., Bordbar, A., & Najafzadeh, N. (2013). Detection of *Wolbachia pipientis*, including a new strain containing the *wsp* gene, in two sister species of *Paraphlebotomus* sandflies, potential vectors of zoonotic cutaneous leishmaniasis. *Memorias do Insitutot Oswaldo Cruz, 108(4),* 414-420.

[25] Ravikumar, H., Ramachandraswamy, N., & Sampathkumar, S. (2010). A preliminary survey for *Wolbachia* and bacteriophage WO infections in Indian mosquitoes (Diptera: Culicidae). *Tropical Biomedicine, 27,* 384-393.

[26] Ros, V. I., Fleming, V. M., Feil, E. J., & Breeuwer, J. A. (2009). How diverse is the genus *Wolbachia*? Multiple-gene sequencing reveals a putatively new *Wolbachia* supergroup recovered from spider mites (Acari: Tetranychidae). *Applied Environmental Microbiology, 75,* 1036-1043.

[27] Mingchay, P., Sai-Ngam, A., Phumee, A., Bhakdeenuan, P., Lorlertthum, K., Thavara, U., *et al*. (2014). *Wolbachia* supergroups A and B in natural populations of medically important filth flies (Diptera: Muscidae, Calliphoridae, and Sarcophagidae) in Thailand. *The Southeast Asian Journal of Tropical Medicine and Public Health, 45(2),* 309-318.

[28] Kittayapong, P., Milne, J. H., Tigvattananont, S., & Baimai, V. (2000). Distribution of the reproduction-modifying bacteria, *Wolbachia*, in natural populations of tephritid fruit fly hosts in Thailand. *Science Asia, 26,* 93-103.

[29] Werren, J. H. (1997). Biology of *Wolbachia*. *Annual Review of Entomology*, *42(1),* 587-609.

Muhamad Azmi Bin Mohammed was born at Pahang, Malaysia on 19th October 1989. He received his pre-university education at Pahang Matriculation College in 2007 before pursuing his first degree in biology (animal) at Universiti Kebangsaan Malaysia (UKM) and had received the dean list award in 2012. During his first degree, he had conducted research on morphology and histology aspects of the salivary glands of *Oxya japonica* (Orthoptera: Acrididae). He then received his master degree in 2014 at the same university majoring in entomology and conducted research on the stored grain pests in Malaysia.

He works as a research assistant for several months before pursuing his PhD. Now, he is a PhD candidate in Universiti Kebangsaan Malaysia (UKM) doing research on *Wolbachia*-infected Braconidae in Malaysia.

Ameyra Aman Zuki was born at Kelantan on 7th October 1988. She received her first degree in biology (genetics) at Universiti Kebangsaan Malaysia (UKM) on phylogenetics study of genus *Vatica* (Dipterocarpaceae: Malvales) in Malaysia. After her first degree, she continued her master degree also at Universiti Kebangsaan Malaysia (UKM) majoring in genetics and study on phylogenetics of Braconidae in Malaysia.

She received her master degree in 2013 and worked as research assistant for a year before continuing her PhD. Now, she is a PhD candidate at Universiti Kebangsaan Malaysia (UKM) doing research on endosymbiont *Bracovirus* in Malaysia.

Suhana Yusof received her first degree in biology at Universiti Putra Malaysia (UPM). She then furthered her master study majoring in entomology at Universiti Kebangsaan Malaysia (UKM). Now, she is a research officer at Horticulture Research Centre, Malaysian Agriculture for Research and Development Institute (MARDI).

Salmah Yaakop was born at Melaka on 13th May 1978. She got her first degree in Universiti Kebangsaan Malaysia majoring in zoology (entomology) before pursuing her master degree in zoology (entomology) also in Universiti Kebangsaan Malaysia. She got the acceptance to continue her PhD at State University of Groningen, the Netherlands doing research on taxonomy and phylogeny of Alysiinae and Opiinae (Hymenoptera: Braconidae).

She worked as a research assistant and teaching assistant at School of Environmental & Natural Resource Sciences, Faculty of Science and Technology, University Kebangsaan Malaysia. She then worked as a researcher at Zoological Museum, Department of Entomology, Universitiet van Amsterdam, the Netherlands. After that she worked as a research officer at Biotechnology and Breeding Centre, Malaysian Palm Oil Board (MPOB). Now she is a senior lecturer at School of Environmental & Natural Resource Sciences, Faculty of Science and Technology, University Kebangsaan Malaysia, Malaysia.

She has received several awards from Ministry of Sciences and Technology, Malaysia for her studies in master and PhD. The Netherland government awarded her jan tinbergen scholarships in 2001. She also was recognized by University Kebangsaan Malaysia when the university awarded her the service award in 2013.