MORPHOLOGY AND HISTOLOGY OF REPRODUCTIVE ORGAN AND FIRST SCREENING OF *Wolbachia* IN THE OVARY OF RED PALM WEEVIL, *Rhynchophorus ferrugineus* (COLEOPTERA: DRYOPHTHORIDAE)

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ABSTRACT

This study was conducted to assess intracellular bacteria *Wolbachia* in the ovaries of female Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* from three populations in Malaysia by means of PCR using *wsp*- specific primer. The morphology and histology of ovary of RPW were also studied and examined by stereo microscope LEICA EZ4 HD that was equipped with LAS EZ software and light microscope Zeiss Axio Scope with iSolutionLite software respectively. We found that the adult female had two pairs of ovaries, lateral oviduct, common oviduct, bursa copulatrix, spermatheca and vagina. Histological study of the ovariole revealed that it is categorised under polytrophic ovariole. The ovariole is divided into four regions, the terminal filament, the germarium, the vitellarium and the stalk or calyx. Besides, the infection status showed that all three populations were not infected with *Wolbachia*. Our result suggests that no infection of *Wolbachia* in RPW reproductive system.

Keywords: *Wolbachia*, ovary, *Rhynchophorus ferrugineus*, biopesticide.

ABSTRAK

Kajian ini dilakukan untuk mengesan kehadiran bakteria intrasel *Wolbachia* di dalam ovari Kumbang Merah Palma (RPW), *Rhynchophorus ferrugineus* (RPW) daripada tiga populasi di Malaysia dengan menggunakan kaedah tindakbalas rantaian polimerase (PCR) yang menggunakan pencetus khusus, *wsp*. Morfologi dan histologi ovari RPW juga telah dikaji dan dicerap dengan menggunakan streomikrokskop LEICA EZ4 HD yang dilengkapi dengan perisian LAS EZ dan mikroskop cahaya Zeiss Axio Scope yang dilengkapi dengan perisian iSolutionLite. Kami telah mendapati bahawa RPW betina dewasa mempunyai dua pasang ovari, oviduktus lateral, oviduktus sepunya, *bursa copulatrix*, spermateka dan vagina. Secara histologinya pula, ovariol kumbang ini dikategorikan sebagai ovariol politrofik. Ovariol terbahagi kepada empat bahagian iaitu filamen penghujung, germarium, vitelarium dan kaliks. Selain itu, status jangkitan menunjukkan ketiga-tiga populasi tidak dijangkiti oleh *Wolbachia*. Kajian kami menunjukkan bahawa tiada jangkitan *Wolbachia* terhadap sistem pembiakan RPW.

Kata Kunci: *Wolbachia*, ovari, *Rhynchophorus ferrugineus*, pestisid biologi.

INTRODUCTION

Red Palm Weevil (RPW), *Rhynchophorus ferrugineus* is one of 60, 000 beetle species that had been identified. It belongs to the family of Dryophthoridae, subfamily Rhynchophorinae and tribe Rhynchophorini (Bouchard et al. 2011). *Rhynchophorus ferrugineus* was firstly detected in India in the year of 1891 and in 1906 it was firstly reported as serious coconut plant pest (Lefroy 1906). Later, in 1917 it started to attack date palm, *Phoenix dactylifera* and became the major pest of this palm (Mohan 1917). In Malaysia, the infestation of this weevil was identified by Department of Agriculture in 2007 (Yong et al. 2015). The infestation towards coconut plantation, *Cocos nucifera* in Terengganu, Malaysia was increased rapidly from 58 localities in 2007 to 858 localities in 2011 (DOA 2011; Wahizatul Afzan et al. 2013). Damage to the date palm is mainly caused by the feeding of larvae inside the trunk. This conceal feeding behaviour of the RPW larvae makes the early detection of infestations more challenging.

Current methods recommended in order to control *Rhynchophorus* species have fixated on Integrated Pest Management (IPM) involving pheromone lures, surveillance, chemical treatments and cultural control (Abraham et al. 1998). However, the recent concern of Gulf countries about the side - effects of chemical pesticides on the environment has resulted in the constraint of these products usage. Thus, interest now has turned to biological control that examining the potential of developing a biopesticide, based on nematodes, viruses, bacteria (Gush 1997) and fungi (Tiago et al. 2014). Recent study by Farah Nadiah et al. (2018) have found the effect of different diets towards gut bacterial abundance in RPW. Six most abundant group of bacteria that are known to have significant roles toward host's gut such as aiding in digestion, synthesizing hormone, and protecting from pathogenic bacteria growth were recorded. There are Enterobacteriaceae, *Leminorella grimontii*, Entomoplasmatales, *Erysipelothrix*, *Lactobacillus* and *Leuconostoc*. These data on the microbes study could also have potential to be used in exploring new dimension of RPW pest management.

Internal genitalia of female insects generally consists of a pair of ovaries and oviductus, common oviduct with accessory sex gland and spermatheca (Engelmann 1970). Insect ovaries consists of functional units, the ovarioles. There are two types of ovarioles, panoistic and meroistic. Meroistic ovariole can be divided into two which are polytrophic meroistic ovariole and telotrophic meroistic ovariole (Buning 2006).

Wolbachia is a cytoplasmically inherited rickettsia and can be found in a wide range of arthropods (Hilgenboecker et al. 2008; Jeyaprakash & Hoy 2000; Werren & Windsor 2000) including Coleoptera, Diptera, Lepidoptera, Orthoptera, Hymenoptera and Hemiptera/Homoptera (Werren & Windsor 2000). *Wolbachia* can regulate host reproduction via feminization, cytoplasmic incompatibility (CI), male killing and parthenogenesis (Blagrove et al. 2012; Werren et al. 2008). Besides reproductive parasitism, *Wolbachia* also involves in mutualistic relationships with nematode hosts (Werren et al. 2008) and also plays important role in the ovary development of silkworm (Zha et al. 2014). The ubiquitous behaviour of *Wolbachia* as well as the manipulation of host's reproductive system makes this symbiont among the most auspicious targets for disease or pest control.

This study aims to describe the morphology and histology of the *R. ferrugineus* ovary and to determine the presence or absence of *Wolbachia* in *R. ferrugineus* ovary based on PCR analysis. The findings would help in finding a potential biopesticide for RPW control.

MATERIALS & METHODS

RPW Sampling

Females Red Palm Weevil, *R. ferrugineus* were collected from three locations in Malaysia, Seberang Takir, Terengganu, Kuala Krai, Kelantan and Yan, Kedah, where the locations were known to be infested. Only females were used because *Wolbachia* are maternally inherited bacteria (Ali et al. 2016). The sampling was conducted by using traps that were comprised of pheromone and sugarcane as a bait. The samples collected were brought to the laboratory for rearing purpose and further analysis.

RPW Rearing

Samples were placed in the good air ventilation plastic container along with the sugarcanes as their food sources. The samples were reared at room temperature 30 ± 2°C with humidity between 60% to 80% (Norzainih et al. 2015). The photoperiod was 12:12 (L: D).

Gross Morphology of Ovary

The samples were surface sterilized with 70% alcohol. Next, the dissection was done by cutting open the dorsal part of the abdomen from posterior to the anterior in order to expose the internal organs. Dissections were conducted in phosphate-buffered saline (PBS) with an autoclaved dissecting kit. Image of reproductive organ in-situ and ex-situ were captured using stereo microscope LEICA EZ4 HD that was equipped with LAS EZ software before the organ was isolated from the insect. Some parts of the isolated reproductive organ were placed in sterile 1.5 mL microcentrifuge tube in the presence of formalin to proceed with the histological procedure. Another parts were placed in 100% acetone and stored under -20 °C for the screening procedure.

Tissue Sections

The ovaries were fixed in formalin for two to four hours. Next, the tissues were undergone tissue processing for 10 hours which firstly, the formalin was removed by washing in 95% ethanol for an hour, and the ovary dehydrated three times in 100% ethanol for an hour each. Tissue was left in the mixture of 100% ethanol and sub-Xylene for an hour and three times in sub-Xylene for an hour each. Lastly the tissues were infiltrated with paraffin wax two times and embedded. Tissues were sectioned (3-5µm) using Leica RM2245 microtome. The slides containing tissues were stained using Hematoxylin and Eosin (H & E) staining. Images of the stained sections were observed under light microscope (Zeiss Axio Scope) with iSolutionLite software.

DNA Extraction

Fifteen to twenty weevils were used from each population for DNA extraction. DNA was extracted from the isolated reproductive organ from a single individual. Dneasy Tissue Kit (Qiagen Inc., Valencia, CA) was used for DNA extraction essentially according to manufacturer's instruction.

Polymerase Chain Reaction

Wolbachia infection was detected by Polymerase Chain Reaction (PCR) using the following wsp-specific primers: 20 nm forward primer (81F) 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and 20 nm reverse primer (691R) 5'-AAAAATTAAACGCTACTCCA-3' in 25 µl reactions containing 12.5 µL GoTaq Green Mastermix, 7.5 µL nuclease free water (ddH2O) (Promega, WI, USA), 1 µL primer 81F and

691R and 3 µL DNA template. PCRs were run under the following cycling conditions by PCR Eppendorf Mastercycler Nexus system: 95 °C for 3 min, followed by 35 cycles of 15 s at 95 °C, 15s at 55 °C, 10 s at 72 °C and a final extension step at 72 °C for 10 min. The PCR products were electrophoresed on a 1.5 % agarose gel, stained with DNA florosafe stain and visualized under ultraviolet illumination to determine the presence and general size of the amplified DNA. Identical reactions with template DNA from parasitoid wasp genus *Psyttalia* sp. that was previously identified as *Wolbachia*-infected was used as positive control to monitor PCR conditions and contaminations.

RESULTS

The internal reproductive organ of female *R. ferrugineus* consists of two pairs of ovaries, lateral oviduct, common oviduct, bursa copulatrix, spermatheca and vagina (Figure 1a and b). The ovaries located on both sides of the alimentary canal. Each ovary has two polytrophic type ovarioles and each ovariole ends in a terminal filament, which unites with the filament from the other ovariole to form a short ligament. This joins the ligament from the other ovary to form a common median ligament, which runs posteriorly and attaches to the posterior flexure of the hindgut. The ovarioles of each side are long and open into the calyx of the lateral oviduct.

Histologically, the ovariole is of the polytrophic type and is divided into four regions, the terminal filament, the germarium, the vitellarium and the stalk or calyx. There is no sheath of enclosing the ovary as a whole. However, each ovariole has a wall which is made up of two layers, an outer ovariole sheath and an inner tunica propria. The terminal filament is a short tube filled with connective tissue (Figure 2a). Germarium is the anterior part of the egg tube and contains trophocytes (nurse cells) (Figure 2a). Vitellarium is the region of the ovariole immediately beyond the germarium and it consists of the follicle with an oocyte enveloped by the follicular epithelium and germinal vesicle (Figure 2b). The ovarioles open together into an expansion of the oviduct known as the calyx (Figure 2c). The calyx is lined with a longitudinal fold of intact columnar epithelial cells with round nuclei in different positions. Besides that, the lumen is filled with a liquefied secretion containing cellular remnants. These cells are surrounded by inner circular muscle layer and an outer band of longitudinal muscle fibres.

By means of a PCR approach using the general *wsp*-specific primers, a total of 55 samples of RPW, *R. ferrugineus* from three different states in Malaysia were screened for the presence of *Wolbachia*. All tested weevils of 20 samples from Terengganu (not shown), 20 samples from Kedah (not shown) and 15 samples from Kelantan (Figure 3) were negative for *Wolbachia* infection, as the PCR amplification products with expected size about 600 bp (arrow) were not present.

DISCUSSION

The result for the gross morphology of RPW ovary is consistent with that mentioned by El-Naggar et al. (2010). However, another Coleopteran like *Cephalodesmius armiger* Westwood is reported to have a reproductive system that is reduced to a single ovariole on the left side (Lopez-Guerrero 1995).

Histologically, most of the insects are described to have four regions of ovariole such as *Callosobruchus maculatus* (Mohamed et al. 2015), *Anopheles pharoensis* (Yamany 2012), and *Graphosoma lineatum* (Ozyurt et al. 2013). Unlike *Cephalodesmius armiger* Westwood,

they are lacking of a terminal filament and the germarium attached to the abdominal tissue by trachea (Lopez-Guerrero 1995).

The ovariole of RPW has two layers of wall. It is different from other Coleopteran for example *Crioceris asparagi* that each of their ovariole is lined externally by an epithelial sheath, which extends from the apex of the terminal filament to the pedicel (Gupta & Riley 1967). The germarium is a cylindrical tube with more or less uniform diameter through most of its length. The trophocytes can be divided into two types which are the distal part of the trophic chamber and cysts of trophocytes. Further growth of oocytes occurs in the vitellarium. Developing oocytes are arranged in a single row along the vitellarium and grow successively larger towards the posterior end of each vitellarium. The anterior part is the youngest while the posterior part is the oldest oocyte. As the young oocyte reaches the vitellarium, its nucleus undergoes a series of transformations that resulting in the germinal vesicle. The folded epithelium of calyx is to allow for expansion during the process of ovulation (Gupta & Riley 1967).

For the screening of *Wolbachia* infection, the results obtained are the first to demonstrate zero infection of *Wolbachia* in *R. ferrugineus* although *Wolbachia* infections have been reported for several Coleopterans (Kondo et al. 1999; Vega et al. 2002; Werren & O'Neill 1997), including a variety of weevils (Hsiao & Hsiao 1985; Jeyaprakash & Hoy 2000; Werren et al. 1995) two of which are the parthenogenetic species *Naupactus tesselatus* (Werren et al. 1995) and *Cathormiocerus britannicus* (Piper et al. 2001). *Wolbachia* have been discovered in more than 30 beetles species so far (Vega et al. 2002) and all belong to supergroups A and B except *Rhynocyllus conicus* (Froehlich) that belongs to supergroup F (Lo et al. 2002).

Current screening of *Wolbachia* on beetle was done on seven selected beetle families that are Buprestidae, Hydraenidae, Dytiscidae, Hydrophilidae, Gyrinidae, Haliplidae, and Noteridae by Sontowski et al. (2015). All of the beetles are aquatic beetles except Buprestidae. Hydraenidae that are considered to be "true water beetles" as most of the adult stage found submerged in freshwater (Jach 1998) showed the highest infection of *Wolbachia* in which it was found in a proportion of 63% of the tested species. It proves that *Wolbachia* not only can be found in terrestrial arthropods but also can be found in aquatic arthropods.

Therefore, it is crucial to know the presence of *Wolbachia* in RPW in order to know the role of this bacteria in this weevil. In *Aedes aegypti*, *Wolbachia* has been successfully optimised as a bio control agent by reducing the replication of the dengue virus in infected mosquitoes (Frentiu et al. 2014).

In addition, the only record of infection RPW by a bacterial pathogen was reported by Banerjee and Dangar (1995) who isolated *Pseudomonas aeruginosa* (Schroeter) Migula (Pseudomonadacae) from naturally infected specimens collected in Kerala, India. This bacterium was pathogenic to weevils when ingested through force feeding or when insects were forced to wade through a suspension of bacterial cells. The consequent mortality occurs eight days after inoculation with the bacteria. However, the information on the potential of *Wolbachia* as natural enemies of RPW still scarce.

CONCLUSION

Understanding the reproductive morphology and histology of this insect can be useful as it provides basic information about the reproductive behaviour of RPW. This information can be an initial reference for other researches in order to develop an efficient IPM. Besides that, this study is also the first to report on the infection status of *Wolbachia* in *R. ferrugineus*.

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APPENDICES

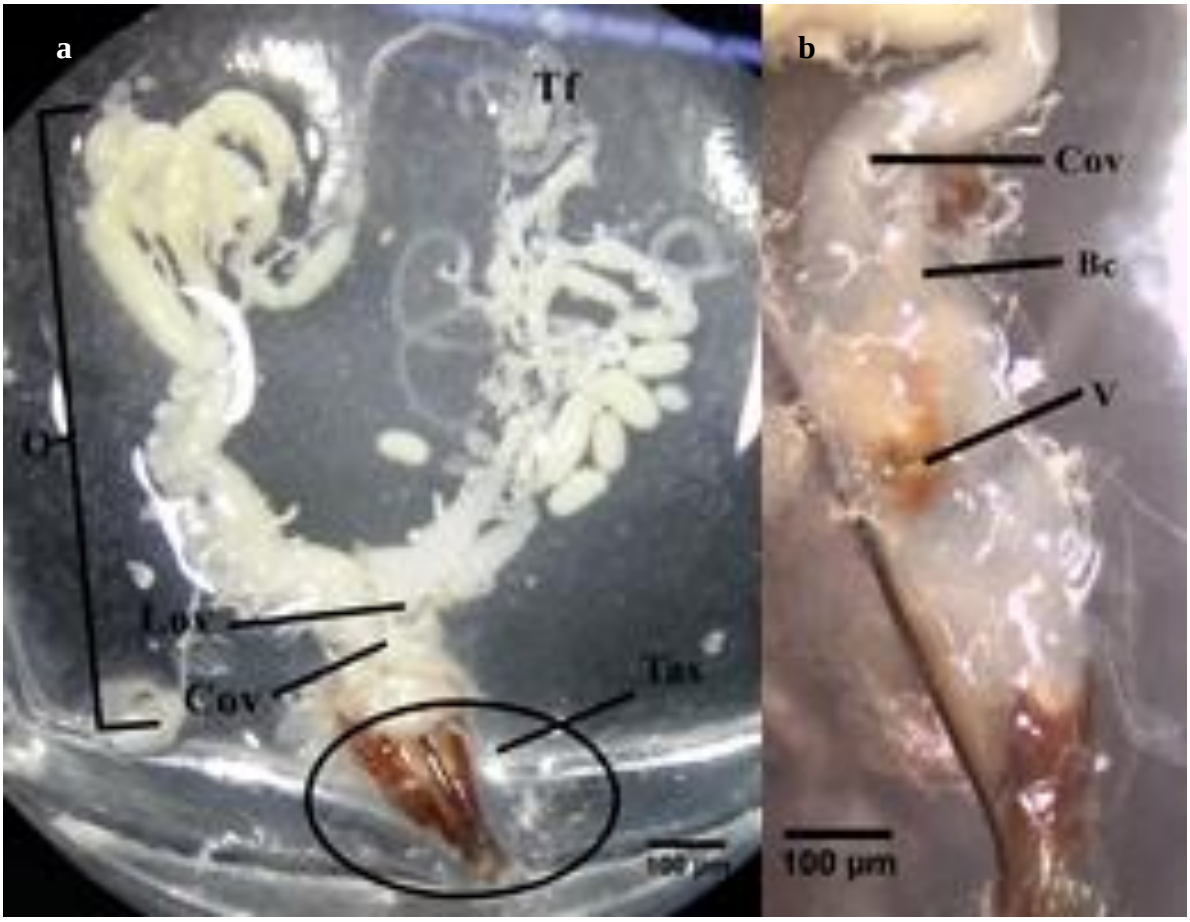

Figure 1 Ex-situ of female reproductive system of red palm weevil, *Rhynchophorus ferrugineus*. (a) Morphology of female RPW reproductive organ, (b) Terminal abdominal sterna. Tf: terminal filament, O: paired of ovaries, Lov: lateral oviduct, Cov: Common oviduct, Tas: Terminal abdominal sterna, Bc: Bursa copulatrix, V: Vagina.

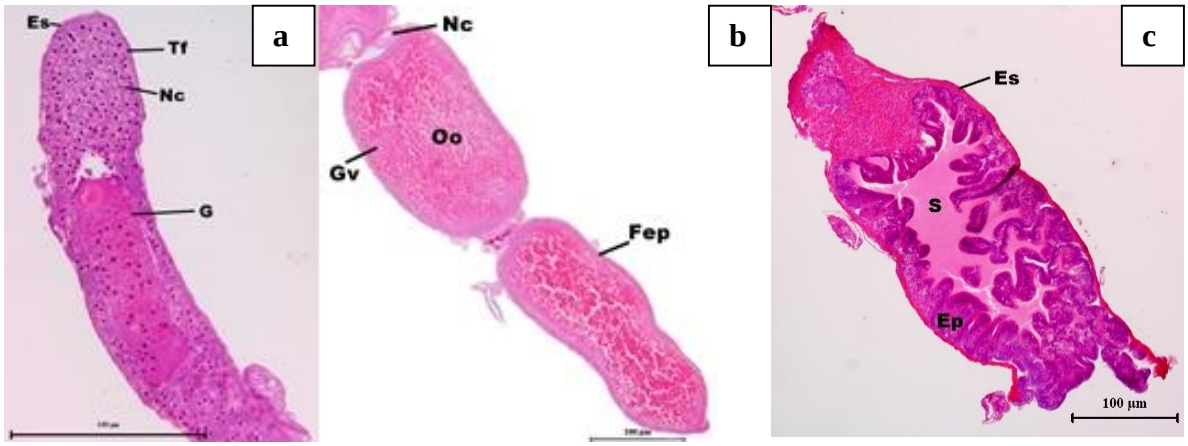

Figure 2 Longitudinal sections of the ovariole. (a) Parts of ovariole, (b) Vitellarium part of ovariole, (c) the ovary calyx of female RPW. Tf: Terminal filament, Nc: Nurse cell, G: Germarium, Gv: Germinal vesicle, Oo: Oocyte, Fep: Follicular epithelium, Es: External sheath Ep: Epithelial cells, S: Secretions of the epithelial cells.

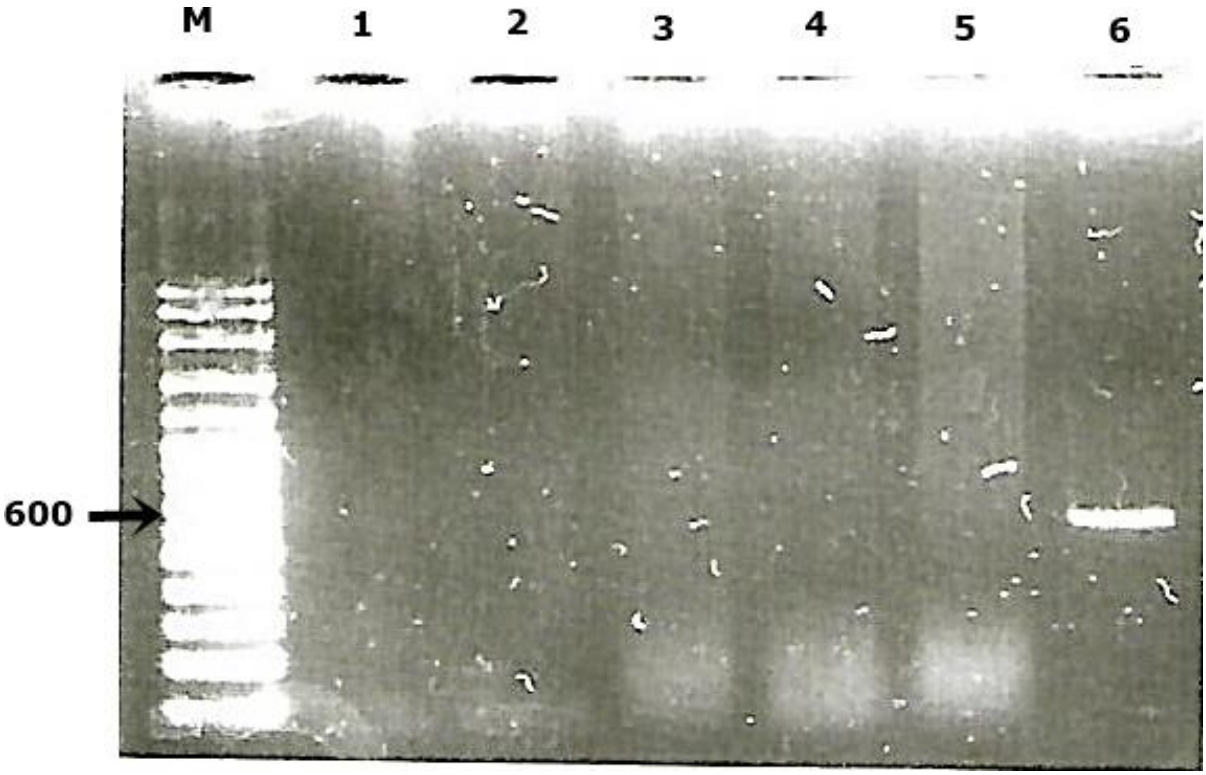

Figure 3 PCR screening for the presence of *Wolbachia* in *R. ferrugineus* from Kelantan. Lane M represents 100bp plus DNA ladder. Lanes 1 – 5 represent RPW samples. Lane 6 represent *Psyttalia* sp.. with *Wolbachia* sp. for positive control.