

ABSENCE OF *WOLBACHIA* IN RED PALM WEEVIL, *RYNCHOPHORUS FERRUGINEUS* OLIVIER (COLEOPTERA: CURCULIONIDAE): A PCR-BASED APPROACH

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(Received 8th Nov 2017; accepted 27th Feb 2018)

Abstract. Among the intracellular bacterial symbionts, the genus *Wolbachia* (Rickettsiaceae: Rickettsiales) is one of the most abundant taxa associated with reproductive systems of various insects. It is cytoplasmic inherited endosymbiont that induce various reproductive alterations. Characterization analysis has revealed that a number of arthropods harbour *Wolbachia* sp. estimates from 20-80%, with emphasize on Coleopterous insects (41 species have been reported so far). Therefore, in the context of broad existence and unique phenotypic actions to alter reproductive systems of various insect, we aimed to determine the intriguing possibility of this endosymbiont from one of the deadliest palm pest, i.e. red palm weevil (RPW), *Rynchophorus ferrugineus* (Curculionidae) from different geographical locations using polymerase chain reactions (PCR) with four sets of *Wolbachia*-specific primers (SPs) along with one pair of universal bacterial primer (BP). Parallel analysis was also carried out with *Wolbachia* strain isolated from whitebacked plant hopper, *Sogatella furcifera* (Homoptera: Delphacidae), as a positive control. Our analysis confirmed the absence of *Wolbachia* sp. across the various life stages of RPW reared in laboratory or captured from the field. Moreover, the phylogenetic analysis of all closely related *Wolbachia*-mediated weevils were compiled and retrieved from NCBI database indicates the extent of transfection of this bacterium into RPW for the future work on biological control of RPW. This study may facilitate to understand further evolutionary consequences of *Wolbachia* infection in weevils.

Keywords: RPW, biological control, endosymbiotic bacteria, *Wolbachia*, PCR

Introduction

A number of symbiotic bacteria associated with insects profoundly influence their host physiology. Among these endosymbionts, *Wolbachia* sp. belongs to order Rickettsiales, is widely distributed in invertebrates (Arthropods). The evidences are mounting that *Wolbachia* can infect a number of insect species (20-80%) (Werren and Windsor, 2000; Jiggins et al., 2001; Tagami and Miura, 2004; Hilgenboecker et al., 2008; Zug and Hammerstein, 2012) from different orders including Diptera, Hymenoptera, Coleoptera, Lepidoptera, Orthoptera, and Hemiptera/Homopter (Werren and Windsor, 2000). Extrapolations of this percentage among the total number of insects and nematode species make *Wolbachia* one of the most abundant endosymbiont. This genus has also been reported from filarial nematodes, the causative agents of river blindness and elephantiasis in human (Fenn et al., 2006). *Wolbachia* has the potential to distort the host reproductive system by various phenotypic effects (Blagrove et al., 2012; Ali et al., 2016) such as cytoplasmic incompatibility (Poinot et al., 2003) (CI), parthenogenesis induction (Stouthamer et al., 1999) (PI), male killing (MK) (Jiggins et

al., 2001) and feminization (Negri et al., 2006). Among these phenotypes, MK, PI, and feminization are highly selective advantageous to female-biased sex ratio while the CI is related to decreasing the number of offspring by reducing the egg hatchability. Phylogenetic analysis has revealed that a total of 8-11 distant *Wolbachia* supergroups designated as A-K has been proposed so far (Lo et al., 2002; Casiraghi et al., 2005). Among these supergroups, A and B are high presumably distributed in arthropods while supergroup C and D belong to terrestrial nematodes with the exception of endosymbionts from *Mansonella* spp., which are recognized in supergroup F. Anyhow, new supergroups with the range of novel *Wolbachia*-mediated hosts has not yet been completely investigated, although new phylogenetic lineages along with novel host are progressively discovered (Casiraghi et al., 2005).

Moreover, breeding behavior, (Gazla and Carracedo, 2009; Miller et al., 2010), sex determination, (Rigaud, 1997), eusociality (Stouthamer et al., 1999) and speciation (Bordenstein, 2003) of the host may also be profoundly affected or altered by *Wolbachia* sp. It is also known to play roles both protective or antagonistic in some RNA viral infections in *Drosophila* (Osborne et al., 2012) and establishes obligate associations as observed in all infected nematodes (Fenn and Blaxter, 2004; Fenn et al., 2006) and rarely in some arthropods (Dedeine et al., 2005; Hosokawa et al., 2010). Remarkable biology, and diverse phenotypic effects of *Wolbachia* and its potential application for the control of vector-borne diseases such as dengue fever, malaria or filariasis (Kambris et al., 2009) offers promising tools to control various economically important invasive species of agriculture and medical importance.

The beetle superfamily Curculionoidea (Insecta: Coleoptera) is one of the most plenteous, diverse, dominant and successful animal group containing more than 60,000 described and likely to contain further 220,000 undescribed species (Oberprieler et al., 2007). Among the Curculionoidea family, red palm weevil (RPW), *Rynchophorus ferrugineus* (Olivier) is one of most invasive pest of almost 26 palm species (Malumphy and Moran, 2009) including date palm, *Phoenix dactylifera* L, oil palm, *Elaeis guineensis* (Murphy and Briscoe, 1999), coconut palm, *Cocos Nucifera*, and Canary Island date palm, *Phoenix canariensis*. The RPW is a concealed tissue borer which undergoes complete metamorphosis (life cycle going through egg, larvae, pupae and adult stages as shown in Fig. 1) and the greater part of its life stages are found inside the palm tree.

Damage manifestations are showed by the presence of tunnels in the trunk, oozing of thick yellow to brown fluid from the tree, the presence of chewed up plant tissue in and around openings in the trunk, the occurrence of a fermented smell from the fluid inside infested tunnels in the trunk, and/or breaking of the trunk or toppling of the palm crown (Kaakeh et al., 2001). In China, damages of RPW initially reported in Guangdong during 1997 and spread rapidly to other parts (Chongqing, Fujian, Hainan Island, Hong Kong, Guangdong, Guangxi, Guizhou, Jiangsu, Jiangxi, Shanghai, Sichuan, Taiwan, Tibet, Yunnan and Zhejiang) (Li et al., 2009). Nowadays, this pest is widely distributed, found in Oceania, Asia, Africa, Europe (several countries around the mediterranean basin) and the Middle East (Howard et al., 2001).

Symbiotic association between beetles and microbial consortia have been broadly investigated. From last few decades, more than thirty weevils have been investigated to harbor *Wolbachia* (Lachowska et al., 2010), with number increasing gradually (41 beetles as shown in Table 1). Many of which belong to supergroup A or B, except the one discovered in *Rhinocyllus conicus* (Froehlich) that belongs supergroup F (Lo et al.,

2002). In view of the widespread distribution as well as the manipulation of host mating and reproduction, *Wolbachia* has attracted the interest of biologists because they maybe used as a novel environmentally friendly tool for insect pest control. Bearing in mind the importance of *Wolbachia*, first, we investigate the presence of *Wolbachia* infection in RPW with the aim to explore the potential use of this endosymbiont as biological control agent against it. This is the first novel study to interrogate the existence of *Wolbachia* in RPW using PCR-based approach. Secondly, according to our best knowledge we compiled all weevils which have been proven to *Wolbachia* positive from previous literatures (Table 1) and finally, we constructed a phylogenetic relationship between previously identified *Wolbachia* strains in closely related coleopterans species with RPW for further understanding the evolutionary consequences of *Wolbachia* infection in weevils.

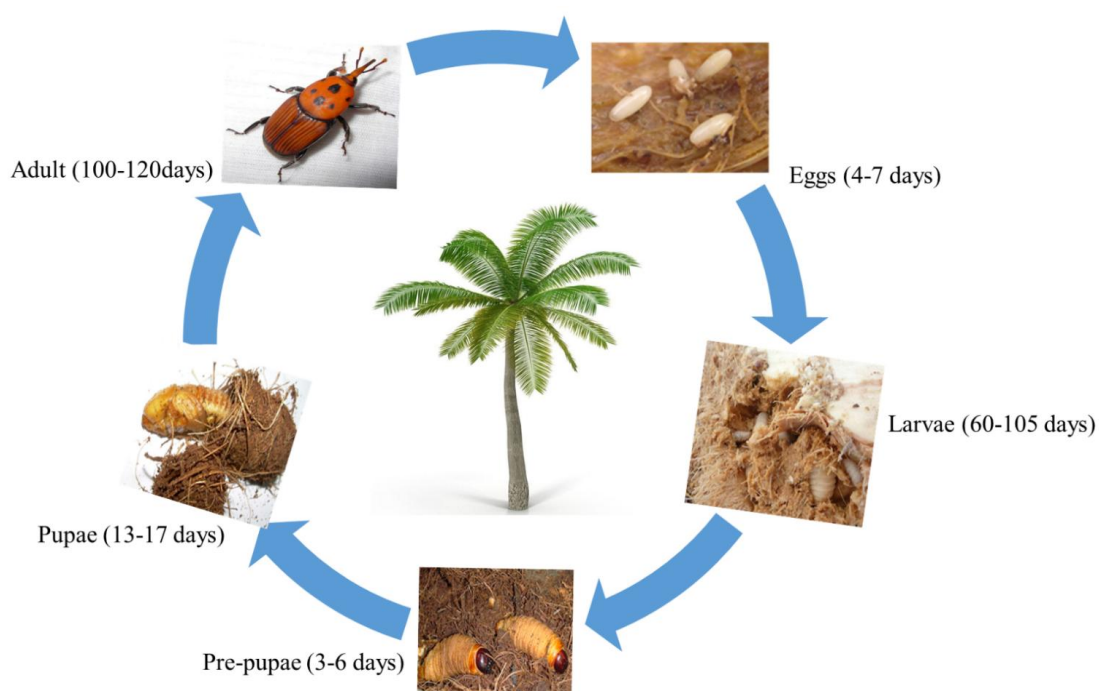


Figure 1. Life cycle (egg to adult stage) of red palm weevil (RPW), *Rynchophorus ferrugineus* (Curculionidae). Number of days may vary according to environmental conditions

Material and methods

Insect collections and rearing

The present study was conducted at the Key Laboratory of Insect Ecology, Department of Plant Protection, Fujian Agriculture and Forestry University, Fuzhou-China. Specimens of RPW were collected from different geographical locations (Table 2 and Fig. 2) during 2014-2017 from infested palm trees. From every location at least 10 specimens were captured. Collected adults and larvae were placed in perforated plastic boxes with host plant tissues and transported to the laboratory for further rearing and DNA extraction. Laboratory population was maintained under control conditions (Temp 25-28 °C, RH 65-70%, 12:12 light-dark cycle) (Hou and Weng, 2010) and provided with sugarcane (*Saccharum officinarum*) for feeding and oviposition.

Table 1. *Wolbachia* infection on the basis of *Wolbachia* outer surface protein (*wsp*) and filamenting temperature sensitive mutant Z protein (*FtsZ*) in all known Coleopteran species reported so far

Coleopteran hosts		Accession no. of <i>Wolbachia</i>		References
Scientific name	Family	<i>wsp</i> gene	<i>ftsZ</i> gene	
<i>Sitophilus zeamais</i>	Curculionidae	AB469362	-	Kageyama et al., 2010
<i>Otiorynchus sulcatus</i>	Curculionidae	-	-	Son et al., 2008
<i>Adalia bipunctata</i>	Curculionidae	AJ130714	-	Hurst et al., 1999
<i>Naupactus cervinus</i>	Curculionidae	GQ402145	-	Rodriguero et al., 2010
<i>Xylosandrus germanus</i>	Curculionidae	AB359039	-	Kawasaki et al., 2010
<i>Ceutorhynchus obstructus</i>	Curculionidae	-	HM012590	Floate et al., 2011
<i>Hypera postica</i>	Curculionidae	-	-	Iwase et al., 2015
<i>Sitophilus oryzae</i>	Curculionidae	-	KP762388	Li et al., 2015
<i>Lissorhoptrus oryzophilus</i>	Curculionidae	-	GU478334	Chen et al., 2012
<i>Rhinocyllus conicus</i>	Curculionidae	-	-	Campbell et al., 1992
<i>Conotrachelus nenuphar</i>	Curculionidae	GU013552	-	Zhang et al., 2010
<i>Tribolium madens</i>	Tenebrionidae	AF275546	-	Fialho and Stevens, 2000
<i>Tribolium confusum</i>	Tenebrionidae	AF020083	-	Ming et al., 2015
<i>Aulacophora nigripennis</i>	Chrysomelidae	GU236978	-	Jeong et al., 2009
<i>Chelymorpha alternans</i>	Chrysomelidae	DQ842458	-	Baldo et al., 2006
<i>Altica lythri</i>	Chrysomelidae	KF163375	-	Jackel et al., 2013
<i>Aphthona nigricutis</i>	Chrysomelidae	-	AY136550	Roehrdanz et al., 2006
<i>Hermaphysa mercurialis</i>	Chrysomelidae	-	KF163372	Jackel et al., 2013
<i>Callosobruchus latealbus</i>	Chrysomelidae	AB545610	-	Kondo et al., 2011
<i>Diabrotica virgifera zea</i>	Chrysomelidae	DQ091305	-	Giordano et al., 1997
<i>Oreina liturata</i>	Chrysomelidae	HG970634	-	Montagna et al., 2014
<i>Oreina cacaliae</i>	Chrysomelidae	HG325863	-	Montagna et al., 2014
<i>Diabrotica virgifera virgifera</i>	Chrysomelidae	-	AY136551	Roehrdanz and Levine, 2007
<i>Diabrotica cristata</i>	Chrysomelidae	-	AY007556	Clark et al., 2001
<i>Callosobruchus chinensis</i>	Chrysomelidae	AB038339	-	Kondo et al., 2011
<i>Diabrotica barberi</i>	Chrysomelidae	-	AY136552	Roehrdanz and Levine, 2007
<i>Brontispa longissima</i>	Chrysomelidae	MG345108	-	Ali et al., 2018
<i>Octodonta nipae</i>	Chrysomelidae	MG551861	-	Unpublished data
<i>Harmonia axyridis</i>	Coccinellidae	-	KM288833	Goryacheva et al., 2015
<i>Pityogenes chalcographus</i>	Scolytinae	DQ993183	-	Avtzis et al., 2008
<i>Hypothenemus hampei</i>	Scolytidae	AF389084	-	Vega et al., 2002
<i>Byturus tomentosus</i>	Byturidae	AJ585376	-	Malloch et al., 2005
<i>Megabruchidius sophorae</i>	Bruchidae	AB545607	-	Kondo et al., 2011
<i>Callosobruchus analis</i>	Bruchidae	AB469357	-	Kageyama et al., 2010
<i>Oryzaephilus mercator</i>	Silvanidae	KJ152808	-	Li et al., 2015
<i>Oryzaephilus surinamensis</i>	Silvanidae	AB469190	-	Kageyama et al., 2010
<i>Luciola unmunsana</i>	Lampyridae	FJ156729	-	Jeong et al., 2009
<i>Lasioderma serricorne</i>	Anobiidae	AB469359	-	Kageyama et al., 2010
<i>Stegobium paniceum</i>	Anobiidae	AB469917	-	Kageyama et al., 2010
<i>Byctiscus venustus</i>	Rhynchitidae	GU236986	-	Jeong et al., 2009
<i>Anthrenus verbasci</i>	Dermestidae	AB469915	-	Kageyama et al., 2010

Note: Scientific names, families and their accession numbers are given. Blank boxes indicates either not available or not known accession numbers from NCBI database

Table 2. Tested specimens of red palm weevil (RPW), *Rynchophorus ferrugineus* (Curculionidae), their collection year and place of collection

S. No	Date of collections	Collected samples	Strain name	Host plant	Original place of collections
1	16/10/2015	Egg (lab collected)	LE	<i>S. officinarum</i>	Fuqing, Fujian, China
2	16/10/2015	Lab larvae	LL	<i>S. officinarum</i>	Fuqing, Fujian, China
3	16/10/2015	Lab pupa	LP	<i>S. officinarum</i>	Fuqing, Fujian, China
4	16/10/2015	Lab adult male	LM	<i>S. officinarum</i>	Fuqing, Fujian, China
5	16/10/2015	Lab adult female	LF	<i>S. officinarum</i>	Fuqing, Fujian, China
6	12/5/2014	Hainan adult male	HM	<i>P. canariensis</i>	Hainan province, China
7	12/5/2014	Hainan adult female	HF	<i>P. canariensis</i>	Hainan province, China
8	10/7/2014	Guanxi adult male	GM	<i>P. canariensis</i>	Guanxi province, China
9	10/7/2014	Guanxi adult female	GF	<i>P. canariensis</i>	Guanxi province, China
10	2/3/2015	Shangai adult male	SM	<i>P. canariensis</i>	Shangai, China
11	2/3/2015	Shangai adult female	SF	<i>P. canariensis</i>	Shangai, China
12	2/4/2015	Longyan adult male	LogM	<i>P. canariensis</i>	Longyan, Fujian, China
13	2/4/2015	Longyan adult female	Logf	<i>P. canariensis</i>	Longyan, Fujian, China
14	11/7/2015	Xiamen adult male	XM	<i>P. canariensis</i>	Xiamen, Fujian, China
15	11/7/2015	Xiamen adult female	XF	<i>P. canariensis</i>	Xiamen, Fujian, China
16	15/8/2015	Fuqing adult male	FM	<i>P. canariensis</i>	Fuqing, Fujian, China
17	15/08/2015	Fuqing adult female	FF	<i>P. canariensis</i>	Fuqing, Fujian, China
18	25/8/2016	Zhangzhou adult male	ZM	<i>P. canariensis</i>	Zhangzhou, Fujian, China
19	25/8/2016	Zhangzhou adult female	ZF	<i>P. canariensis</i>	Zhangzhou, Fujian, China
20	3/9/2017	Ningde adult male	NM	<i>P. canariensis</i>	Ningde, Fujian, China
21	3/9/2017	Ningde adult female	NF	<i>P. canariensis</i>	Ningde, Fujian, China

DNA extraction

For DNA extraction all samples (Table 2 and Fig. 2) were sterilized with 75% alcohol and rinsed three times with autoclaved double distilled water. Total genomic DNA extraction was performed from various life stages (egg, larvae, pupa, male and female) of lab-reared specimens, while only field caught adult stage (male or female) from different geographical locations using DNeasy Blood and Tissue Kit (Qiagen, Valencia, USA) in accordance with the manufacturer's protocol with final elution step repeated twice in 100 µl of buffer AE. The concentration and purity of the extracted DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Wilmington, DE).

PCR amplification for *Wolbachia* detection

The presence of *Wolbachia* in extracted DNA of RPW specimens was screened using PCR amplification with four *Wolbachia* SPs and a universal bacterial primer (Pu et al., 2016) (Table 3). PCR reactions contained a total volume of 25 µl comprised of 2 µl of template DNA, 12.5 µl of 2X Taq PCR Master-mix (Tiangen Biotechnology Beijing, China), 1 µl of each primer, and 8.5 µl double distilled water. Each reaction run with a positive control (*Wolbachia* DNA from *Sogatella furcifera* (Horvath) and negative control (without DNA). Amplified PCR products were run on 1% agarose gel (0.5 ug/ml ethidium bromide, TRIS-EDTA-Buffer; Fisher, Waltham, MA) and visualized using UV illuminator.

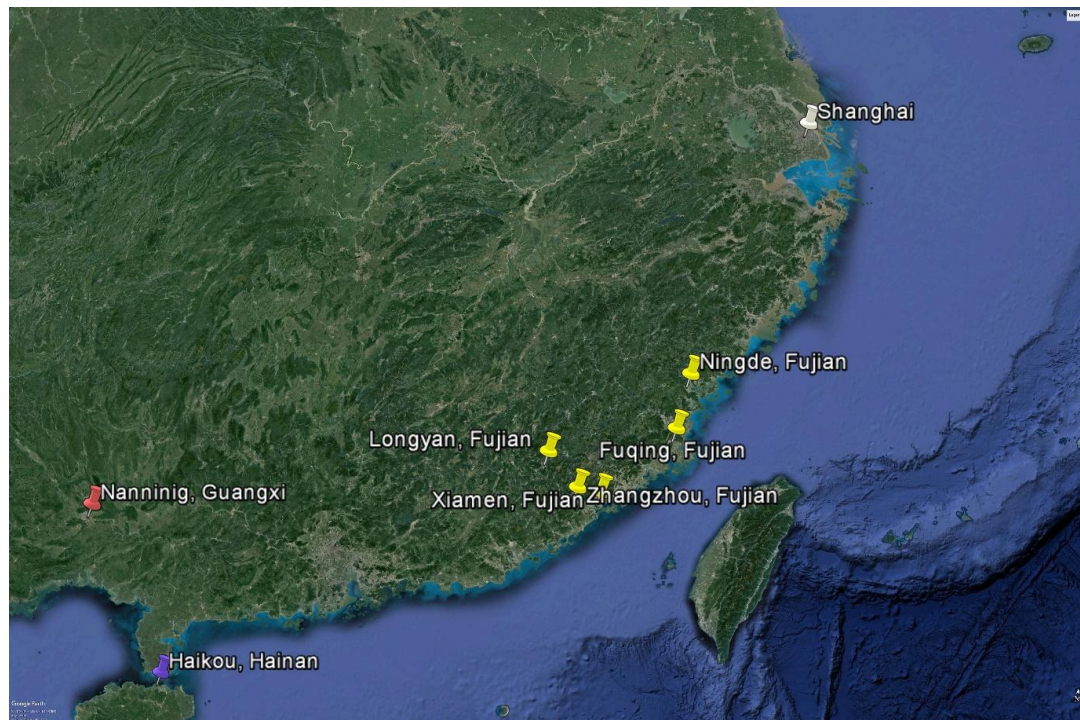


Figure 2. Map of sampling sites from different geographic locations (Fujian, Guangxi, Shanghai and Hainan province) of china. Red palm weevil (RPW), *Rynchophorus ferrugineus* (Curculionidae) specimens collected from same locations represent same color, while different colors represent variable provinces of china. The map was obtained from google earth (<https://www.google.com/earth/desktop/>)

Table 3. Four sets of *Wolbachia* specific primers (SPs) along with universal bacterial primer (BP) with PCR thermal cycling conditions used in this study

Primer	Primer sequence	PCR cycling conditions	FL	Reference
Wolbachia SPs	1) wsp81F: 5'-TGG TCC AAT AAG TGA TGA AGA AAC-3' wsp691R: 5'-AAA AAT TAA ACG CTA CTC CA-3'	94 °C for 4 min, 40 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C, Extension step of 10 min at 72 °C	≈600bp	Zhou et al., 1998
	2) wsp1: 5'-GGATCCGGGTCCAATAAGTGATGAAGAAAC-3' wsp2: 5'-GGATCCTTAAACGCTACTCCAGCTTCTGC-3'	-do-	-do-	Baldo et al., 2006
	3) wspF: 5'-GGGTCCAATAAGTGATGAAGAAAC-3' wspR: 5'-TTAAACGCTACTCCAGCTTCTGC-3'	-do-	-do-	Ming et al., 2015
	4) FtsF: 5'-GTA TGC CGA TTG CAG AGC TTG-3' FtsR: 5'-GCC ATG AGT ATT CAC TTG GCT-3'	95 °C for 10 min, 1 min at 95 °C, 1 min at 55 °C, 2 min at 70 °C, Extension step of 5 min at 72 °C	≈750bp	Kondo et al., 2011
Universal BP	1) 27F: 5'-TCG ACATCGTTTACGGCGTG-3' 1492R: 5'-CTA CGGCTACCTTGTTACGA-3'	94 °C for 3 min, 40 s at 94 °C, 40 s at 55 °C, 1 min at 72 °C, Extension 5 min at 72 °C	≈1400bp	Pu and Hou, 2016

SPs: specific primers; BP: bacterial primer; FL: fragment length; PCR: polymerase chain reactions

***Wolbachia* positive strain**

Wolbachia strain used in this study as a positive control was originally isolated from a lab-reared adult of *S. furcifera* (Horvath) (Homoptera, Delphacidae) known to be infected by *Wolbachia* under the accession number of FJ713766.1 retrieved from National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/nucore/224797910>). PCRs were carried with total volume of 50 µl comprised of 4 µl of template DNA, 50 µl of 2X Taq PCR Master mix (Tiagen Biotechnology Beijing, China), 2 µl of each primer, and 17 µl double distilled water to amplify the targeted genes. The PCR products, after evaluation of positive amplification verified through gel electrophoresis, were subjected to cloning and transformations. A 2 µl of purified amplification product from 1% agarose gel extracted using MiniElute Gel Extraction Kit (Qiagen) was directly ligated into the pGEM T-Easy Cloning Vector (Promega, Madison, WI) in accordance with the protocol. Ligation products were transformed into T1 Competent Cells (Qiagen), which were plated on 0.5% ampicillin Luria-Bertani broth (LB) selection plates (S-Gal LB Agar Blend, Sigma-Aldrich, St Louis, MO) and incubated overnight at 37 °C. At least three positive clones were picked and labeled according to sample and incubated in 1000 µl LB broth (0.5% ampicillin) for 10 h at 37 °C and 300 rpm. After shaking, 2 µl finally confirmed sample via diagnostic PCR were send to sequencing company (life science Company).

Phylogenetic analysis

We retrieved the sequences of previously identified *Wolbachia* strains (41 species) from closely related weevils to RPW by running search on NCBI GeneBank database (<http://www.ncbi.nlm.nih.gov/BLAST>). All *Wolbachia* strains retrieved from the NCBI GeneBank were aligned using ClustalW and tree was constructed with MEGA5.05 software (Tamura et al., 2011). The aligned sequences were corrected manually if necessary and best fit model was chosen for cladogram analysis.

Results

Detection of *Wolbachia* in RPW

According to our PCR results, all four *Wolbachia* SPs (*wsp*81F-*wsp*691R, *wsp*F-*wsp*R, *wsp*1-*wsp*2 and *fts*F-*fts*R) yielded negative results except for the positive control as shown in Table 4. Based on these results, we comprehensively rejected the hypothesis of the presence of this endosymbiont in field-collected, or lab-reared RPW samples (egg, larvae, pupa, and adult) and concluded that RPW is not naturally infected by *Wolbachia* sp. (Table 4). On the other hand, bacterial universal primer (27F, 1492R) showed visible bands (≈1400 bp) on agarose gel across the all tested life stages (Table 4) except for the negative control which shows the purity of DNA isolated from all targeted specimens. Contrary with above assumptions, all four *Wolbachia* SPs including *Wolbachia* outer surface protein (*wsp*) and filamenting temperature sensitive mutant Z protein (*ftsZ*) genes were used against positive control (*S. furcifera*) yielded ≈600 bp and ≈750 bp respectively after running on 1% agarose gel electrophoresis (Table 4). To ensure the authenticity of targeted regions of *wsp* and *ftsZ* genes from positive control were blasted against NCBI database and the results demonstrated the same sequence fragments under the accession numbers of FJ713766 and JN560721 respectively. Visible fragments only in positive control indicates the integrity of DNA.

Table 4. *Wolbachia* infected (+) or uninfected (–) diagnostic PCR results against laboratory reared and field collected specimens of red palm weevil (RPW), *Rynchophorus ferrugineus* (Curculionidae) with four pairs of *Wolbachia* SPs and one pair of universal BP

	Sample	Universal BP	<i>Wolbachia</i> SPs			
		27F 1492R	wsp81 F wsp691R	wspF wspR	wsp1 wsp2	ftsF ftsR
Laboratory reared	LE	+	-	-	-	-
	LL	+	-	-	-	-
	LP	+	-	-	-	-
	LM	+	-	-	-	-
	LF	+	-	-	-	-
Field collected	HM	+	-	-	-	-
	HF	+	-	-	-	-
	GM	+	-	-	-	-
	GF	+	-	-	-	-
	SM	+	-	-	-	-
	SF	+	-	-	-	-
	LogM	+	-	-	-	-
	Logf	+	-	-	-	-
	XM	+	-	-	-	-
	XF	+	-	-	-	-
	FF	+	-	-	-	-
	ZM	+	-	-	-	-
	ZF	+	-	-	-	-
	NM	+	-	-	-	-
	NF	+	-	-	-	-
	NC	-	N/A	N/A	N/A	N/A
	PC	N/A	+	+	+	+

SPs: specific primers; BP: bacterial primer; N/A: not applied; NC: negative control; PC: positive control

Phylogenetic relationship of previously described Wolbachia strains from order Coleoptera

Wolbachia is widely distributed in Coleopteran insects and majority of weevil species have been reported *Wolbachia* infected which are taxonomically closely related to RPW. However, in our PCR assay, we were unable to detect the presence of *Wolbachia* sp. in RPW. Total length of alignments were ≈600 bp for 26 *wsp* and ≈750 bp for 10 *ftsZ* gene sequences were used to construct phylogenetic tree, while 3 weevils (*Otiorhynchus sulcatus*, *Hypera postica*, *Rhinocyllus conicus*) (Table 1), we were unable to find their *Wolbachia* sequence from NCBI database. We construct a phylogenetic relationship between previously identified *Wolbachia* strains in closely related coleopterans species with RPW in order to understand the future of *Wolbachia* transfection into RPW and exploit it in pest management. *Wolbachia* strains were named after the host insect species, and the GeneBank accession number are listed as well (Fig. 3).

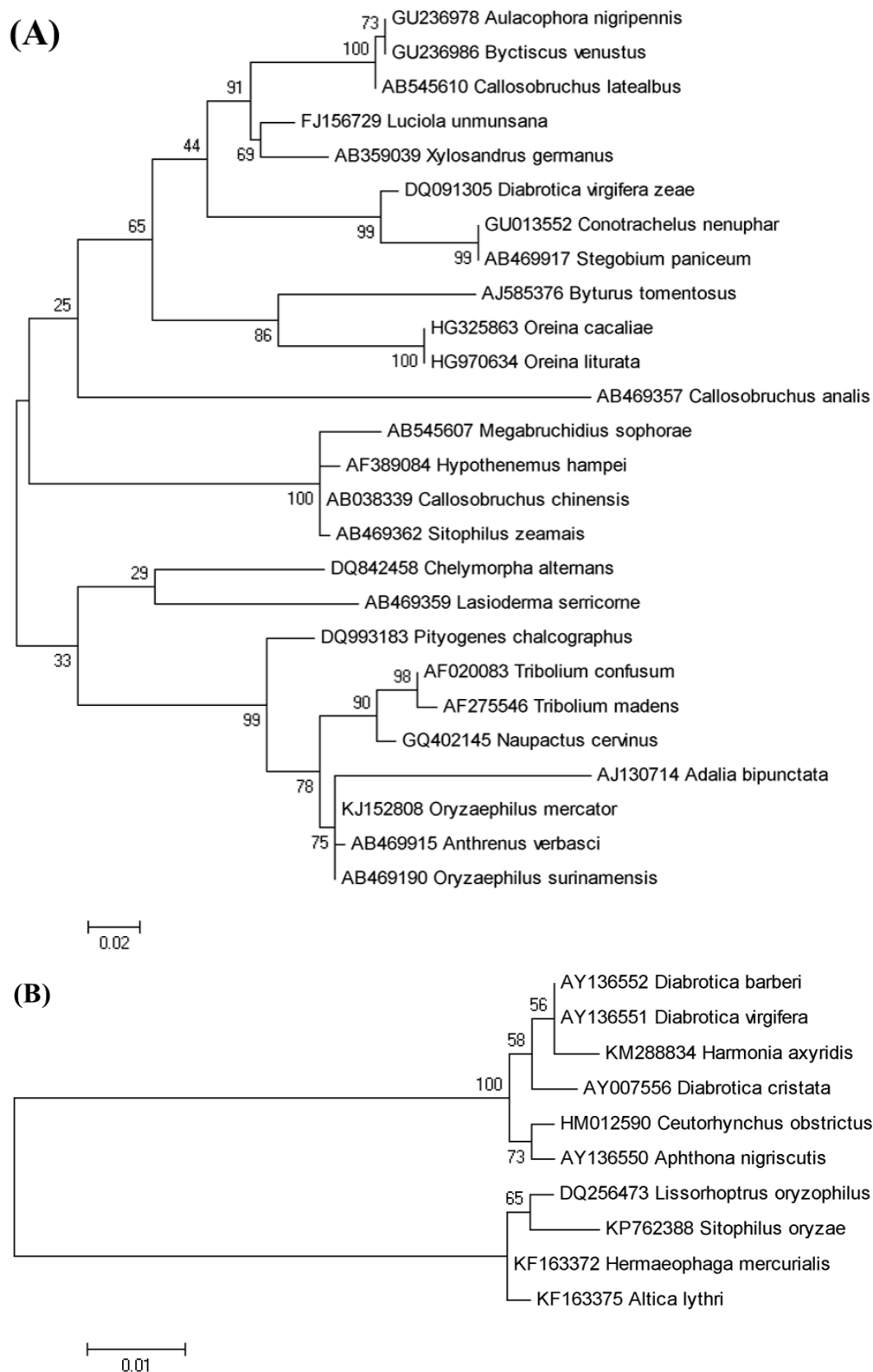


Figure 3. Maximum likelihood (ML) phylogenetic tree of previously reported *Wolbachia* positive individuals in coleopteran insects based on *wsp* (A) and *ftsZ* (B) gene sequences. Bootstrap values shown next to nodes are based on 1000 replicates

Discussion

Numerous invertebrates harbor endosymbiotic microorganisms, including terrestrial isopods (Rigaud et al., 2001), mites (Breeuwer and Jacobs, 1996), and all major orders of insects (Werren et al., 2000). Every insect species has its own set of endosymbiotic species that can vary greatly, even within the same insect species. The variation depends on factors such as sex, life stage, biotype, and geographic location (Pan et al., 2012). *Wolbachia* is one of the widespread ubiquitous intracellular bacterial symbionts that have evolved several phenotypic strategies (male killing, parthenogenesis, feminization and cytoplasmic incompatibility) to manipulate the reproductive system of the various insect, nematodes, crustacean etc.

In various weevils, these endosymbionts are broadly investigated and reported to have extremely interdependent associations like in *Nardonella* and many members of Dryophthoridae. For example, West Indian sweet potato weevil, *Euscepes postfasciatus* (Fairmaire), when endosymbionts were removed through the utilization of antibiotics, its structure, size, body weight, and developmental rate was significantly reduced (Kuriwada et al., 2010). *Wolbachia* were reported from ≈ 30 weevils (Lachowska et al., 2010) so far which is quite a large number as its abundance. However, as a whole, current knowledge of the bacterial communities and their associations with natural hosts are still limited. In the context of *Wolbachia* importance as a bio-control agent and broad existence in all arthropods especially in Coleopteran insects, we planned a study to unravel the question about the presence of *Wolbachia* sp. in RPW. Furthermore, to improve our previous knowledge about the existence of *Wolbachia* in weevils, we compiled up to date known *Wolbachia*-inherited weevils in this study. Based on our results, we confirmed that *Wolbachia* is not present in both laboratory reared and field collected specimens of RPW in any developmental stage. A PCR assay based study was conducted by Bordenstein et al. (2003) in a diverse set of nonfiliariid species and the results demonstrated the absence of *Wolbachia* in Nonfiliariid Nematodes which is quite similar to our study conclusion. Other studies such as Voronin et al. (2015), McNulty et al. (2012) are also in line with our results. There could be several possible reasons such as co-evolution, stochasticity (Jansen et al., 2008), fitness costs incurred by infection (Sarakatsanou et al., 2011; Suh et al., 2013; Dykstra et al., 2014), high temperature, (Clancy et al., 1998), imperfect maternal transmission and/or agricultural application of bactericide (McManus et al., 2002), high host specificity all are behind the absence of *Wolbachia* in RPW but without any authentic surveys all explanation remains possibilities which still need to be determined.

The congruence of *Wolbachia* sp. with filariid dated back to about 100 million years suggesting that *Wolbachia* sp. has coevolved with the host organism and became an integral organ of the host (Casiraghi et al., 2001). Lo et al. (2002) has suggested that *Wolbachia* sp. are highly host specific. However, recent studies have shown that this can be cultured in vitro as well as can be injected and manipulated in other non-host organisms which not only infects host soma but also infects somatic tissue and maintain this infection into various generation (stock populations of *Drosophila melanogaster*) after initial injections of *Wolbachia* sp. Transfection of *Wolbachia* sp. from naturally infected host to non-host organism such as RPW would open new avenues for developing an effective bio-control strategy against this devastating pest of palm trees.

Conclusion and future prospects

Wolbachia species are widely distributed in arthropods, mites, terrestrial isopods and all major insect orders particularly in Coleopteran insects. However, in this study, there is no evidence of *Wolbachia* presence in all tested developmental stages either laboratory reared or field collected specimens of RPW at any geographical locations of China. Therefore, on the basis of our results we concluded that *Wolbachia* sp. is not present in the population of RPW. Greater awareness, in combination with rapidly expanding knowledge base of *Wolbachia* (particularly in the areas of genomics, cell biology and molecular biology) and comparable endosymbionts, offers new directions for incorporating them in bio-control programs. The capacity of *Wolbachia* that cause CI in arthropod species has created interest in their use as a mechanism to drive desirable traits (for example resistance to disease) into insect vector populations. The use of *Wolbachia* infected males is also being developed as a mechanism to decrease pest populations by inducing elevated CI, similar to the use of sterile male programs to control pest insects. However, incorporation of *Wolbachia* in biocontrol research strategies may be restricted by technical challenges. For example, infections can be manipulated by elimination, transfection or genetic modification. The former has been achieved in many cases, transfection has been accounted less frequently, and genetic modification has yet to be achieved. However, given advances in recent years, we are optimistic that results of ongoing and future research will expand opportunities to use *Wolbachia* and similar endosymbiotic bacteria in bio-control programs.

Acknowledgements. We are very grateful to the grant from the National Key R & D Program of China (2017YFC1200605) and Fujian Science and Technology Special Project (2017NZ0003-1-6).

Conflict of interest. The authors declare to have no potential conflicts of interests.

REFERENCES

- [1] Ali, H., Hou, Y., Tang, B., Shi, Z., Huang, B., Muhammad, A., Sanda, N. B. (2016): A way of reproductive manipulation and biology of *Wolbachia pipientis*. – Journal of Experimental Biology and Agricultural Sciences 4: 156-168.
- [2] Ali, H., Muhammad, A., Hou, Y. (2018): Infection density dynamics and phylogeny of *Wolbachia* associated with coconut hispine beetle, *Brontispa longissima* (Gestro) (Coleoptera: Chrysomelidae) by multilocus sequence type (MLST) genotyping. – Journal of Microbiology and Biotechnology (In press).
- [3] Avtzis, D. N., Arthofer, W., Stauffer, C. (2008): Sympatric occurrence of diverged mtDNA lineages of *Pityogenes chalcographus* (Coleoptera, Scolytinae) in Europe. – Biological Journal of the Linnean Society 94: 331-340.
- [4] Baldo, L., Hotopp, J. C. D., Jolley, K. A., Bordenstein, S. R., Biber, S. A., Choudhury, R. R., Hayashi, C., Maiden, M. C., Tettelin, H., Werren, J. H. (2006): Multilocus sequence typing system for the endosymbiont *Wolbachia pipientis*. – Applied and Environmental Microbiology 72: 7098-7110.
- [5] Blagrove, M. S., Arias-Goeta, C., Failloux, A. B., Sinkins, S. P. (2012): *Wolbachia* strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. – Proceedings of the National Academy of Sciences 109: 255-260.
- [6] Bordenstein, S. R. (2003): Symbiosis and the origin of species. – Insect Symbiosis 1: 347.
- [7] Bordenstein, S. R., Fitch, D. H., Werren, J. H. (2003): Absence of *Wolbachia* in nonfilariid nematodes. – Journal of Nematology 35: 266.

- [8] Breeuwer, J., Jacobs, G. (1996): *Wolbachia*: intracellular manipulators of mite reproduction. – Experimental and Applied Acarology 20: 421-434.
- [9] Campbell, B. C., Bragg, T. S., Turner, C. E. (1992): Phylogeny of symbiotic bacteria of four weevil species (Coleoptera: Curculionidae) based on analysis of 16S ribosomal DNA. – Insect Biochemistry and Molecular Biology 22: 415-421.
- [10] Casiraghi, M., Favia, G., Cancrini, G., Bartoloni, A., Bandi, C. (2001): Molecular identification of *Wolbachia* from the filarial nematode *Mansonella ozzardi*. – Parasitology Research 87: 417-420.
- [11] Chen, S. J., Lu, F., Cheng, J. A., Jiang, M. X., Way, M. O. (2012): Identification and biological role of the endosymbionts *Wolbachia* in rice water weevil (Coleoptera: Curculionidae). – Environmental Entomology 41: 469-477.
- [12] Clancy, D. J., Hoffmann, A. A. (1998): Environmental effects on cytoplasmic incompatibility and bacterial load in *Wolbachia*-infected *Drosophila simulans*. – Entomologia Experimentalis et Applicata 86: 13-24.
- [13] Clark, T. L., Meinke, L. J., Skoda, S. R., Foster, J. E. (2001): Occurrence of *Wolbachia* in selected diabroticite (Coleoptera: Chrysomelidae) beetles. – Annual Entomological Society of America 94: 877-885.
- [14] Dedeine, F., Bouletreau, M., Vavre, F. (2005): *Wolbachia* requirement for oogenesis: occurrence within the genus *Asobara* (Hymenoptera, Braconidae) and evidence for intraspecific variation in *A. tabida*. – Heredity 95: 394-400.
- [15] Dykstra, H. R., Weldon, S. R., Martinez, A. J., White, J. A., Hopper, K. R., Heimpel, G. E., Asplen, M. K., Oliver, K. M. (2014): Factors limiting the spread of the protective symbiont *Hamiltonella defensa* in *Aphis craccivora* aphids. – Applied and Environmental Microbiology 80: 5818-5827.
- [16] Fenn, K., Blaxter, M. (2004): Are filarial nematode *Wolbachia* obligate mutualist symbionts?. – Trends in Ecology and Evolution 19: 163-166.
- [17] Fenn, K., Conlon, C., Jones, M., Quail, M. A., Holroyd, N. E., Parkhill, J., Blaxter, M. (2006): Phylogenetic relationships of the *Wolbachia* of nematodes and arthropods. – PLoS Pathogens 2: e94.
- [18] Fialho, R. F., Stevens, L. (2000): Male-killing *Wolbachia* in a flour beetle. – Proceedings of the Royal Society of London. Series B 267: 1469-1473.
- [19] Floate, K. D., Coghlin, P. C., Dosdall, L. (2011): A test using *Wolbachia* bacteria to identify Eurasian source populations of cabbage seedpod weevil, *Ceutorhynchus obstrictus* (Marsham), in North America. – Environmental Entomology 40: 818-823.
- [20] Gazla, I., Carracedo, M. (2009): Effect of intracellular *Wolbachia* on interspecific crosses between *Drosophila melanogaster* and *Drosophila simulans*. – Genetics and Molecular Research 8: 861-869.
- [21] Giordano, R., Jackson, J. J., Robertson, H. M. (1997): The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. – Proceedings of the National Academy of Sciences 94: 11439-11444.
- [22] Goryacheva, I., Blekhman, A., Andrianov, B., Gorelova, T., Zakharov, I. (2015): Genotypic diversity of *Wolbachia pipientis* in native and invasive *Harmonia axyridis* Pall., 1773 (Coleoptera, Coccinellidae) populations. – Russian Journal of Genetics 51: 731-736.
- [23] Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J. H. (2008): How many species are infected with *Wolbachia*? A statistical analysis of current data. – FEMS Microbiology Letters 281: 215-220.
- [24] 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 107: 769-774.
- [25] Howard, F. W., Giblin-Davis, R., Moore, D., Abad, R. G. (2001): Insects on Palms. – CABI, Wallingford, UK.

- [26] Hurst, G. D., Jiggins, F. M., von der Schulenburg, J. H. G., Bertrand, D., West, S. A., Goriacheva, I. I., Zakharov, I. A., Werren, J. H., Stouthamer, R., Majerus, M. E. (1999): Male-killing *Wolbachia* in two species of insect. – Proceedings of the Royal Society of London. Series B 266: 735-740.
- [27] Iwase, S., Tani, S., Saeki, Y., Tuda, M., Haran, J., Skuhrovec, J., Takagi, M. (2015): Dynamics of infection with *Wolbachia* in *Hypera postica* (Coleoptera: Curculionidae) during invasion and establishment. – Biological Invasions 17: 3639-3648.
- [28] Jackel, R., Mora, D., Dobler, S. (2013): Evidence for selective sweeps by *Wolbachia* infections: phylogeny of *Altica* leaf beetles and their reproductive parasites. – Molecular Ecology 22: 4241-55.
- [29] Jansen, V. A. A., Turelli, M., Godfray, H. C. J. (2008): Stochastic spread of *Wolbachia*. – Proceedings of the Royal Society of London. Series B 275: 2769-2776.
- [30] Jeong, G., Kang, T., Park, H., Choi, J., Hwang, S., Kim, W., Choi, Y., Lee, K., Park, I., Sim, H. (2009): *Wolbachia* infection in the Korean endemic firefly, *Luciola unimansana* (Coleoptera: Lampyridae). – Journal of Asia-Pacific Entomology 12: 33-36.
- [31] Jiggins, F. M., Hurst, G. D., Schulenburg, J. H. G., Majerus, M. E. (2001): Two male-killing *Wolbachia* strains coexist within a population of the butterfly *Acraea encedon*. – Heredity 86: 161-166.
- [32] Kaakeh, W., Khamis, A., Aboul-Nour, M. M. (2001): The Red Palm Weevil: the Most Dangerous Agricultural Pest, pp. 90-165. – United Arab Emirates University Press, Dubai.
- [33] Kageyama, D., Narita, S., Imamura, T., Miyanooshita, A. (2010): Detection and identification of *Wolbachia* endosymbionts from laboratory stocks of stored-product insect pests and their parasitoids. – Journal of Stored Products Research 46: 13-19.
- [34] Kambris, Z., Cook, P. E., Phuc, H. K., Sinkins, S. P. (2009): Immune activation by life-shortening *Wolbachia* and reduced filarial competence in mosquitoes. – Science 326: 134-136.
- [35] Kawasaki, Y., Ito, M., Miura, K., Kajimura, H. (2010): Superinfection of five *Wolbachia* in the alnus ambrosia beetle, *Xylosandrus germanus* (Blandford) (Coleoptera: Curculionidae). – Bulletin of Entomological Research 100: 231-239.
- [36] Kondo, N. I., Tuda, M., Toquenaga, Y., Lan, Y. C., Buranapanichpan, S., Horng, S. B., Shimada, M., Fukatsu, T. (2011): *Wolbachia* infections in world populations of bean beetles (Coleoptera: Chrysomelidae: Bruchinae) infesting cultivated and wild legumes. – Zoological Science 28: 501-508.
- [37] Kuriwada, T., Hosokawa, T., Kumano, N., Shiromoto, K., Haraguchi, D., Fukatsu, T. (2010): Biological role of *Nardonella* endosymbiont in its weevil host. – PloS One 5: e13101.
- [38] Lachowska, D., Kajtoch, L., Knutelski, S. (2010): Occurrence of *Wolbachia* in central European weevils: correlations with host systematics, ecology, and biology. – Entomologia Experimentalis et Applicata 135: 105-118.
- [39] Li, Y., Zeng-Rong, Z., Ruiting, J., Lian-Sheng, W. (2009): The red palm weevil, *Rynchophorus ferrugineus* (Coleoptera: Curculionidae), newly reported from Zhejiang, China and update of geographical distribution. – Florida Entomologist 92: 386-387.
- [40] Li, Y. Y., Fields, P., Pang, B. P., Coghlin, P., Floate, K. (2015): Prevalence and diversity of *Wolbachia* bacteria infecting insect pests of stored products. – Journal of Stored Products Research 62: 93-100.
- [41] Lo, N., Casiraghi, M., Salati, E., Bazzocchi, C., Bandi, C. (2002): How many *Wolbachia* supergroups exist? – Molecular Biology and Evolution 19: 341-346.
- [42] Malloch, G., Fenton, B. (2005): Super-infections of *Wolbachia* in byturid beetles and evidence for genetic transfer between A and B super-groups of *Wolbachia*. – Molecular Ecology 2: 627-637.

- [43] Malumphy, C., Moran, H. (2009): Red palm weevil, *Rhynchophorus ferrugineus*. Plant pest factsheet. – www.fera.defra.gov.uk/plants/publications/documents/factsheets/redPalmWeevil.pdf (accessed 7 June 2011).
- [44] McManus, P. S., Stockwell, V. O., Sundin, G. W., Jones, A. L. (2002): Antibiotic use in plant agriculture. – Annual Review Phytopathology 40: 443-465.
- [45] McNulty, S. N., Fischer, K., Mehus, J. O., Vaughan, J. A., Tkach, V. V., Weil, G. J., Fischer, P. U. (2012): Absence of *Wolbachia* endobacteria in *Chandlerella quiscali*, an avian filarial parasite. – Journal of Parasitology: 98(2): 382-387.
- [46] Miller, W. J., Ehrman, L., Schneider, D. (2010): Infectious speciation revisited: impact of symbiont-depletion on female fitness and mating behavior of *Drosophila paulistorum*. PLoS Pathogens. – 6: e1001214.
- [47] Ming, Q. L., Shen, J. F., Cheng, C., Liu, C. M., Feng, Z. J. (2015): *Wolbachia* infection dynamics in *Tribolium confusum* (Coleoptera: Tenebrionidae) and their effects on host mating behavior and reproduction. – Journal of Economic Entomology 108: 1408-1415.
- [48] Montagna, M., Chouaia, B., Sacchi, L., Porretta, D., Martin, E., Giorgi, A., Lozzia, G. C., Epis, S. (2014): A new strain of *Wolbachia* in an Alpine population of the viviparous *Oreina cacaliae* (Coleoptera: Chrysomelidae). – Environmental Entomology 43: 913-922.
- [49] Murphy, S., Briscoe, B. (1999): The red palm weevil as an alien invasive: biology and the prospects for biological control as a component of IPM. – Biocontrol News and Information 20: 35-46.
- [50] Negri, I., Pellecchia, M., Mazzoglio, P., Patetta, A., Alma, A. (2006): Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system. – Proceedings of the Royal Society of London. Series B 273: 2409-2416.
- [51] Oberprieler, R. G., Marvaldi, A. E., Anderson, R. S. (2007): Weevils, weevils, weevils everywhere. – Zootaxa 1668: 491-520.
- [52] Osborne, S. E., Iturbe-Ormaetxe, I., Brownlie, J. C., O'Neill, S. L., Johnson, K. N. (2012): Antiviral protection and the importance of *Wolbachia* density and tissue tropism in *Drosophila simulans*. – Applied and Environmental Microbiology 78: 6922-6929.
- [53] Pan, H., Li, X., Ge, D., Wang, S., Wu, Q., Xie, W., Jiao, X., Chu, D., Liu, B., Xu, B. (2012): Factors affecting population dynamics of maternally transmitted endosymbionts in *Bemisia tabaci*. – PloS One 7: e30760.
- [54] Poinot, D., Charlat, S., Mercot, H. (2003): On the mechanism of *Wolbachia*-induced cytoplasmic incompatibility: Confronting the models with the facts. – Bioessays 25: 259-265.
- [55] Hou, Y., Weng, Z. (2010): Temperature-dependent development and life table parameters of *Octodonta nipae* (Coleoptera: Chrysomelidae). – Environmental Entomology 39: 1676-1684.
- [56] Pu, Y. C., Hou, Y. M. (2016): Isolation and identification of bacterial strains with insecticidal activities from *Rhynchophorus ferrugineus* Oliver (Coleoptera: Curculionidae). – Journal of Applied Entomology 140: 617-626.
- [57] Rigaud, T. (1997): Inherited Microorganisms and Sex Determination of Arthropod Hosts. – In: O'Neill, S. L., Hoffmann, A. A., Werren, J. H. (eds.) Influential Passengers: Inherited Microorganisms and Arthropod Reproduction, pp. 81-102. – Oxford University Press, Oxford.
- [58] Rigaud, T., Pennings, P., Juchault, P. (2001): *Wolbachia* bacteria effects after experimental interspecific transfers in terrestrial isopods. – Journal of Invertebrate Pathology 77: 251-257.
- [59] Rodriguero, M., Confalonieri, V., Guedes, J., Lanteri, A. (2010): *Wolbachia* infection in the tribe Naupactini (Coleoptera, Curculionidae): association between thelytokous parthenogenesis and infection status. – Insect Molecular Biology 19: 631-640.

- [60] Roehrdanz, R., Levine, E. (2007): *Wolbachia* bacterial infections linked to mitochondrial DNA reproductive isolation among populations of northern corn rootworm (Coleoptera: Chrysomelidae). – Annual Entomological Society of America 100: 522-531.
- [61] Roehrdanz, R., Olson, D., Bouchier, R., Sears, S., Cortilet, A., Fauske, G. (2006): Mitochondrial DNA diversity and *Wolbachia* infection in the flea beetle *Aphthona nigriscutis* (Coleoptera: Chrysomelidae): an introduced biocontrol agent for leafy spurge. – Biological Control 37: 1-8.
- [62] Sarakatsanou, A., Diamantidis, A. D., Papanastasiou, S. A., Bourtzis, K., Papadopoulos, N. T. (2011): Effects of *Wolbachia* on fitness of the Mediterranean fruit fly (Diptera: Tephritidae). – Journal of Applied Entomology 135: 554-563.
- [63] Son, Y., Luckhart, S., Zhang, X., Lieber, M. J., Lewis, E. E. (2008): Effects and implications of antibiotic treatment on *Wolbachia*-infected vine weevil (Coleoptera: Curculionidae). – Agricultural and Forest Entomology 10: 147-155.
- [64] Stouthamer, R., Breeuwer, J. A., Hurst, G. D. (1999): *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. – Annual Reviews in Microbiology 53: 71-102.
- [65] Suh, E., Dobson, S. L. (2013): Reduced competitiveness of *Wolbachia* infected *Aedes aegypti* larvae in intra- and interspecific immature interactions. – Journal of Invertebrate Pathology 114: 173-177.
- [66] Tagami, Y., Miura, K. (2004): Distribution and prevalence of *Wolbachia* in Japanese populations of Lepidoptera. – Insect Molecular Biology 13: 359-364.
- [67] Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S. (2011): MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. – Molecular Biology and Evolution 28: 2731-2739.
- [68] Vega, F. E., Benavides, P., Stuart, J. A., O'Neill, S. L. (2002): *Wolbachia* infection in the coffee berry borer (Coleoptera: Scolytidae). – Annual Entomological Society of America 95: 374-378.
- [69] Voronin, D., Abeykoon, A. M., Gunawardene, Y. S., Dassanayake, R. S. (2015): Absence of *Wolbachia* endobacteria in Sri Lankan isolates of the nematode parasite of animals *Setaria digitata*. – Veterinary Parasitology 207(3-4): 350-354.
- [70] Werren, J. H., Windsor, D. M. (2000): *Wolbachia* infection frequencies in insects: evidence of a global equilibrium?. – Proceedings of the Royal Society of London. Series B 267: 1277-1285.
- [71] Zhang, X., Luckhart, S., Tu, Z., Pfeiffer, D. G. (2010): Analysis of *Wolbachia* strains associated with *Conotrachelus nenuphar* (Coleoptera: Curculionidae) in the eastern United States. – Environmental Microbiology 39: 396-405.
- [72] Zhou, W., Rousset, F., O'Neill, S. (1998): Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. – Proceedings of the Royal Society of London. Series B 265: 509-515.
- [73] Zug, R., Hammerstein, P. (2012): Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. – PloS one 7: e38544.