Indian J Microbiol (Apr–June 2016) 56(2):167–171
DOI 10.1007/s12088-016-0567-7

ORIGINAL ARTICLE

Detection and Localization of *Wolbachia* in *Thrips palmi* Karny (Thysanoptera: Thripidae)

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Received: 27 August 2015 / Accepted: 30 January 2016 / Published online: 13 February 2016

Abstract *Thrips palmi* Karny is a globally distributed polyphagous agricultural pest. It causes huge economic loss by its biological behaviors like feeding, reproduction and transmission of tospoviruses. Since *T. palmi* shows close morphological similarities with other thrips species, we employed mitochondrial cytochrome oxidase 1 (*mtCO1*) gene as a molecular marker. BLAST analysis of this sequence helped us to identify the collected specimen as *T. palmi*. We observed the female to male ratio of about 3:1 from collected samples and suspected the presence of *Wolbachia*. The presence of *Wolbachia* was detected by PCR using genus specific primers of *16S rRNA* gene. Further confirmation of *Wolbachia* strain was achieved by conducting PCR amplification of three ubiquitous genes *ftsZ, gatB* and *groEL*. A phylogenetic tree was constructed with concatenated sequences of *ftsZ* and *gatB* gene to assign supergroup to *Wolbachia*. Finally, we localized *Wolbachia* in abdominal region of the insect using fluorescent in situ hybridization with the help of confocal microscope. Our result confirmed the presence of *Wolbachia* supergroup B strain for the first time in *T. palmi*.

Keywords *mtCO1* gene · Ubiquitous genes · *16S rRNA* gene · PCR · FISH

Electronic supplementary material The online version of this article (doi:10.1007/s12088-016-0567-7) contains supplementary material, which is available to authorized users.

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Introduction

Many species of thrips cause huge economic loss in vegetable crops and ornamental plants worldwide [1]. They feed on plant sap by their piercing and sucking mouth parts resulting in curling and silvering of the leaves [2]. The reproductive behavior of thrips also results in fruit injury due to their oviposition [2]. Fourteen species of thrips belonging to the family Thripidae (order: Thysanoptera) are known to transmit 20 different tospoviruses (Tospovirus: Bunyaviridae) affecting around 1100 plant species worldwide [2–5]. *T. palmi* Karny is known to affect 50 different host plant species and has a global distribution [6]. *T. palmi* is considered as the one of most economically important thrips species in India [7]. So far, *T. palmi* is known to vector six tospoviruses; Calla lily chlorotic spot virus, Groundnut bud necrosis virus, Melon yellow necrotic virus, Tomato spotted wilt virus, Watermelon bud necrosis virus and Watermelon silver mottle virus [4, 5].

For an effective pest management strategy of thrips, accurate identification in the field and proper understanding of its biology are an essential pre-requisite. Adult thrips are very small in size (1–2 mm) and show many morphological similarities with other thrips species which make their identification difficult and require a trained manpower [2, 8]. Molecular markers assist in species level identification of such insects. Mitochondrial cytochrome oxidase 1 (*mtCO1*) gene has been used for identification of a number of organisms [9, 10] and is found to be a more reliable marker for distinguishing inter-specific variation [11]. *mtCO1* gene has been effectively used as molecular marker for identification of *T. palmi* and *Thrips tabaci* [12].

Wolbachia is a facultative bacterium which comes under the alpha-subdivision of proteobacteria [13]. It is known to infect large number of insects and is reported to be

transmitted maternally [13]. *Wolbachia* induces parthenogenesis, selective male killing and feminization of genetic males or cytoplasmic incompatibility by manipulating host reproduction [14]. *Wolbachia* has been reported in both thelytokous and arrhenotokous populations of thrips species such as *Hercinothrips femoralis, Franklinothrips vespiformis, Echinothrips americanus,* and *Suocerathrips linguis* [15, 16] and shown to induce thelytokous reproduction in *Franklinothrips vespiformis* [15].

In this study, we have focused on identification of *T. palmi* Karny in our collected sample. We achieved the same with conventional morphological study followed by molecular identification using *mtCO1* gene. Later, we looked for the presence of *Wolbachia* in three different populations of *T. palmi* using PCR with its genus specific *16S rRNA* gene primers. Three ubiquitous genes namely *ftsZ, gatB* [17] and *groEL* [18] were also analyzed to reconfirm presence of *Wolbachia. ftsZ* and *gatB* [17] genes were used for genotyping of *Wolbachia* into particular supergroup strain. To localize *Wolbachia* as a bacterial endosymbiont in *T. palmi,* fluorescent in situ hybridization (FISH) was performed using confocal microscopy. This study will provide a base for understanding the role of *Wolbachia* in reproductive behavior of *T. palmi.*

Materials and Methods

Sample Collection and DNA Isolation

Three different populations of thrips were collected from agricultural fields of Indian Agricultural Research Institute (IARI), Pusa, New Delhi. Later these specimens were identified by their morphological features with the help of compound microscope as *T. palmi* (Online Resource 1; Table S1). The identified specimens were stored at −20 °C in 70 % ethanol and absolute acetone for DNA isolation and FISH analysis respectively. For PCR amplification reactions, DNA isolation was done as previously performed by Rana et al. [19].

Molecular Identification of *T. palmi* Based on *mtCO1* Gene

PCR amplification of *mtCO1* gene was carried out with two sets of primers (Table 1). PCR reaction conditions and product visualization were same as previously described by Asokan et al. [12]. PCR products were eluted using HiPurA^{TM} Quick Gel Purification Kit and were subsequently sequenced commercially (Macrogen Inc., Korea). The sequences were subjected to BLAST analysis in NCBI server. Sequence alignment was done with Clustal X2.1 and maximum likelihood analysis with Jukes-Cantor correction was performed for phylogenetic tree construction using MEGA6 [20]. Resultant tree topology was evaluated by bootstrap analysis based on 1000 repeats.

Wolbachia Screening Using its Genus Specific Primer Based on *16S rRNA* Gene

Presence of *Wolbachia* was detected using its genus specific primers of *16S rRNA* gene (Table 1). PCR reaction of 25 µL consisted of 100 ng of DNA template isolated from a pool of 20 *T. palmi* individuals, 2.5 mM dNTP mixture, 10× Taq buffer, 10 pmol of each primer, 1 U Taq polymerase and volume was made up to 25 µL with autoclaved MQ water. PCR parameters were: 94 °C for 1 min, followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, 72 °C for 1 min and final extension of 10 min at 72 °C. For positive control, *16S rRNA* gene of *Wolabachia* cloned in vector pGEM-T® Easy (Promega) was taken as template and negative control was performed without DNA template. PCR product was eluted using HiPurA^{TM} Quick Gel Purification Kit and cloned directly into TA cloning vector pGEM-T® Easy (Promega, Madison, WI). *E. coli* DH5α strain was used for transformation of ligated product. Blue-white screening was performed for selection of positive colonies as per manufacturer's instructions (Promega, Madison, WI). Three white colonies were selected for plasmid isolation using mdi fastlyse pDNA miniprep kit. Such purified plasmids were sequenced commercially (Macrogen Inc., Korea) followed by BLAST analysis of the sequence on the NCBI for similarity searches.

PCR Amplification of *ftsZ*, *gatB* and *groEL* Genes of *Wolbachia*

ftsZ, *gatB* and *groEL* genes of *Wolbachia* were amplified using *Wolbachia* specific primers (Table 1). Chemicals in PCR reaction were same as previously performed experiment. PCR parameters were: 94 °C for 2 min, followed by 42 cycles of 94 °C for 30 s, optimal annealing temperature for 45 s (see below), 72 °C for 1 min and final extension of 10 min at 72 °C. The optimal annealing temperature was 52 °C for *ftsZ* and *gatB* and 53 °C for *groEL*. As a negative control, mock reaction was set up without DNA template. Elution, cloning, sequencing and BLAST analysis of PCR products were performed as described earlier. Clustal X2.1 was used for sequence alignment of a concatenated data set of *ftsZ* and *gatB* genes and its phylogenetic analysis was performed as discussed earlier.

FISH Analysis

FISH technique was used as previously performed by Gottlieb et al. [21] with modifications. Insect specimens

Table 1 Primers used in this study

Gene	Product	Primer name	Primer sequence (5′–3′)	References
mtCO1	Mitochondrial cytochrome oxidase 1	mtD7.2F	ATTAGGAGCHCCHGAYATAGCATT	[12]
		mtD9.2R	GAGGCAAGATTAAAATATAAACTTCTTG	
		1 RA f	TTGACTTCTTCCACCCTCTTTAACTCTT	
		5 RA r	TAGATGTTGATAAAGTACAGGATCT	
16S rRNA	Ribosomal small subunit 16S	WF	CGGGGGAAAATTTATTGCT	[22]
		WR	AGCTGTAATACAGAAAGGAAA	
ftsZ	Cell division protein	ftsZ_F1	ATYATGGARCATATAAARGATAG	[17]
		ftsZ_R1	TCRAGYAATGGATTRGATAT	
gatB	Glutamyl-tRNA (Gln) amidotransferase, subunit B	gatB_F1	GAKTTAAAYCGYCCAGGBGTT	[17]
		gatB_R1	TGGYAAYTCRGGYAAAGATGA	
groEL	Chaperon protein	WgroEL_F	GGATCCATGGCTAACATAGT	This study
		WgroEL_R	GTCGACTTAGAATCCACCCA	

were incubated overnight at room temperature in Carnoy's fixative (chloroform: ethanol: glacial acetic acid, 6:3:1) and then decolorized in 6 % H_2O_2 (Merck) in absolute ethanol for 4 h. Later, the specimens were hybridized overnight at 30 °C in hybridization buffer (20 mM Tris–Cl pH 8.0, 0.9 M NaCl, 0.01 % Sodium dodecyl sulfate, 50 % Formamide) with 0.6 pmol/μL fluorescent labeled probe. For detection of *Wolbachia*, LNA probe from Exiqon (TEX615-5′-CTTCTGTGAGTACCGTCATTATC-3′) was used. Specimens were then washed with washing buffer (0.3 M NaCl, 0.03 M sodium citrate, 0.01 % SDS) twice for 10 min and then mounted on glass slide using Fluoroshield (Sigma). To check the specificity of FISH, RNase digested control and no probe control were performed. Mounted specimens were observed under Nikon A1 confocal microscope and images were analyzed using NIS-Element (V 3.21.02) software (Nikon).

Results and Discussion

Sex Ratio of *T. palmi* Populations

Three different populations of thrips were identified by analyzing their morphological features with the help of compound microscope (Online Resource 1; Table S1). We observed the female to male ratio, on an average as 3:1 in the three different populations of *T. palmi*.

Molecular Identification of *T. palmi*

T. palmi shows similar morphological appearance to *T. flavus* Schrank and *T. alatus* Bhatti. *T. flavus* can be distinguished by the position of interocellar setae, which arise from inside the ocellar triangle posterior to the anterior ocellus, in contrast, it arise from outside the ocellar triangle in *T. palmi* [6]. While in *T. alatus* the metanotal sculpture is striate but not converged on the posterior margin as is usual in *T. palmi* [6]. To avoid any confusion, morphologically identified specimens were subjected to molecular identification based on *mtCO1* gene PCR amplification to make sure that they were *T. palmi* and not any other thrips species [2, 8]. Sequencing of PCR amplification products of *mtCO1* gene resulted in a sequence of 461 and 366 bp with the primer set 1 and set 2 respectively. These resultant sizes were close to the expected sizes of *mtCO1* amplified product of *T. palmi*. Further, the nucleotide sequences from four different *T. palmi* specimens showed no variation. Similarity search result using BLAST algorithm in NCBI showed that these sequences were 99 % identical to previously deposited sequences of *T. palmi* in the GenBank. The partial *mtCO1* gene sequence of *T. palmi* obtained in this study was submitted to NCBI GenBank under accession number KM393212. Phylogenetic tree analysis of our specimen and *T. palmi mtCO1* gene sequences (retrieved from NCBI database) showed that our specimen was closest to *T. palmi* of India and formed a separate cluster (Online Resource 1; Fig. S1).

Detection and Reconfirmation of *Wolbachia* in *T. palmi*

During our attempts to collect the three different populations of *T. palmi,* it was observed that the female to male ratio, on an average was 3:1 indicating a strong female bias population. Since, *Wolbachia* was already reported in a few species of thrips and in *Franklinothrips vespiformis*, it was reported to induce thelytoky reproduction [15, 16]. The occurrence of female biased population in *T. palmi* prompted us to investigate for the presence of *Wolbachia.*

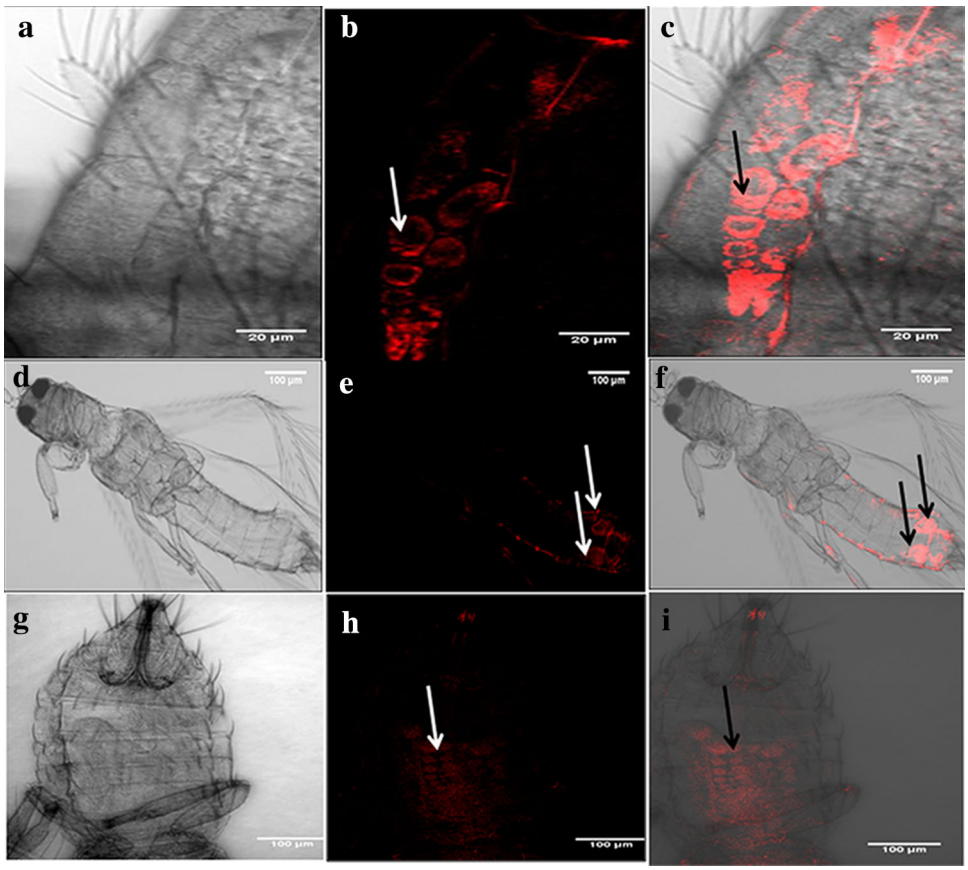

Fig. 1 FISH staining of *Wolbachia* in the whole mount of *T. palmi* (Female). **a**, **d** and **g** show bright field images. **b, e** and **h** show bacterial endosymbiont *Wolbachia* localized in abdomen region. **c**, **f** and **i** show merged images

Genomic DNA samples of three populations of well identified *T. palmi* were subjected to PCR for detection of *Wolbachia* with genus specific *16S rRNA* gene primers. Conventionally, a *16S rRNA* gene library is made to study bacterial endosymbiont population, but this technique does not detect bacterial endosymbionts which are rare [22]. Genus specific primers have been reported to be more sensitive for the detection of less populated bacterium [21].

We observed an amplification product of 632 bp which was in accordance with the expected size of *16S rRNA* gene with genus specific primers of *Wolbachia* [22] in all three populations. Similarity search result using BLAST algorithm (search was performed against nucleotide sequence nr/nt, scoring parameter; match/mismatch scores were 1/−2 and gap cost set as linear) in NCBI showed that sequence of this product was 99 % identical to previously deposited sequences of *Wolbachia* from other insects in the GenBank. Thus sequence and BLAST analysis of this product confirmed the presence of *Wolbachia* in *T. palmi*. Partial *16S rRNA* gene sequence of *Wolbachia* was submitted to NCBI GenBank under accession number KP889218.

Furthermore, all three populations of *T. palmi* were subjected to PCR amplification of three ubiquitous gene *ftsZ, gatB* [17] and *groEL* [18] for reconfirmation of presence of *Wolbachia* in *T. palmi*. We got amplification of *ftsZ, gatB* and *groEL* genes in all the three populations of *T. palmi.* Sequencing and BLAST analysis of these products reconfirmed the presence of *Wolbachia* in *T. palmi*. Partial sequences of *ftsZ, gatB* and *groEL* genes were submitted under accession numbers KT250944, KT250945 and KP889217 respectively. Phylogenetic tree analysis of concatenated sequences of *ftsZ* and *gatB* genes of *Wolbachia* from *T. palmi* and other species helped us to identify strain of *Wolbachia* in *T. palmi* as belonging to supergroup B (Online Resource 1; Fig. S2).

Localization of *Wolbachia* in *T. palmi* by FISH

We performed FISH using confocal microscopy for localization of *Wolbachia* in *T. palmi*. A strong signal was detected which indicated the presence of *Wolbachia* in the abdominal region of *T. palmi* (Fig. 1). The presence of such precise *Wolbachia* signals were seen on an average in 20 % of specimens screened from all the three populations (100 individuals from each population). Besides this, we could observe some auto fluorescence in the legs and chitinous parts of the body which was also observed in no probe and RNase digested controls. Such structures have

earlier been observed to show auto fluorescence in RNase digested controls and in absence of probe during *Rickettsia sp.* detection in mealybug [22].

Thus, through this study, we were able to detect and localize *Wolbachia* supergroup B strain in *T. palmi,* a condition which has not been reported yet. *T. palmi* are economically important to agriculture because of their ability to transmit viruses and monitoring their population and understanding their biology are important aspects. Ascertaining the role of bacterial endosymbionts like *Wolbachia* in biology and population dynamics of *T. palmi* is a critical part which may give us better insight for understanding the life history parameters of this insect. Further study is required to understand the functional role of *Wolbachia* in *T. palmi*.

Acknowledgments ORP-SP and NFBSFARA, of the Indian Council for Agricultural Research (ICAR), Govt. of India and Faculty Research Grants of University of Delhi funded this work. SP was provided research fellowship by UGC/DSKPDF. GD was provided research fellowship by UGC. We also thank Mr. Ashok Pal for laboratory assistance.

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