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Wolbachia endobacteria in a natural population of *Culex quinquefasciatus* from filariasis endemic villages of south India and its phylogenetic implication

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Abstract. Understanding *Wolbachia* mosquito interactions have been recognized as an important concept to develop novel vector control strategies. The prevalence of *Wolbachia* endobacteria in a natural population of the filariasis vector *Culex quinquefasciatus* was determined by the polymerase chain reaction method. Earlier workers had estimated the infection rates of *Wolbachia* with only one or very few individuals per species. In our study large number of specimens were assayed, and a total of 750 adult *Culex quinquefasciatus* mosquitoes were collected from three south Indian villages of Tirukoilur and Mugaiyur blocks, monthly for a period of five months (December 2006 to April 2007) and screened for the presence of *Wolbachia*. The percentage prevalence in adult males ranged from 88% to 96%; while in females from 84% to 100%. An overall prevalence of 91.2% was observed. There was no significant difference observed in the proportion of mosquitoes positive for *Wolbachia* between males and females, and also between different months of the survey; except during the month of February '07. The *wsp* gene sequence of the *Wolbachia* strain of *Cx. quinquefasciatus* detected was BLAST analysed and showed 99% sequence similarity with *Wolbachia* sp. of *Culex pipiens* isolated from different geographical regions. Phylogenetic analysis based on *wsp* gene fragments showed that the present *Wolbachia* isolate was closely related with *Wolbachia* from *Culex pipens pipiens*, *Niphotettix virescens* (Order: Hemiptera) and *Cnaphalocrosis medinalis* (Order: Lepidoptera).

INTRODUCTION

Wolbachia are intracellular gram-negative bacteria that are found in association with a variety of invertebrate species, including insects, mites, spiders, terrestrial crustaceans, and nematodes. *Wolbachia* are transovarialy transmitted from females to their offspring, having been found to infect 20%–75% of invertebrate species sampled (Jeyaprakash & Hoy, 2000; Werren & Windsor, 2000). *Wolbachia* are members of the Rickettsiales order of the α-subdivision of the Proteobacteria phyla and belong to the Anaplasmataceae family, with members of the genera *Anaplasma*, *Ehrlichia*, *Cowdria*, and *Neorickettsia* (Dumler *et al.*, 2001). Six major clades (A–F) of *Wolbachia* have been identified to date (Lo *et al.*, 2002): A, B, E, and F have been reported from insects, arachnids, and crustaceans; C and D from filarial nematodes. *Wolbachia*–host interactions are complex and range from mutualistic to pathogenic, depending on the combination of host and *Wolbachia* involved. Most striking are the various forms of "reproductive parasitism" that serve to alter host reproduction in order to enhance the transmission of this maternally inherited agent. These include parthenogenesis (infected females reproducing in the absence of mating to produce infected female offspring), feminization (infected males being converted into functional phenotypic

females), male-killing (infected male embryos being selectively killed), and cytoplasmic incompatibility (the developmental arrest of offspring of uninfected females when mated to infected males) (O'Neill *et al*., 1997).

Wolbachia have been hypothesized to play a role in host speciation through the reproductive isolation they generate in infected hosts (Werren, 1998). They also provide an intriguing array of evolutionary solutions to the genetic conflict that arises from their uniparental inheritance. These solutions represent alternatives to classical mutualism and are often of more benefit to the symbiont than the host that is infected (Werren & O'Neill, 1997). From an applied perspective, it has been proposed that *Wolbachia* could be utilized to either suppress pest insect populations or sweep desirable traits into pest populations (e.g., the inability to transmit disease-causing pathogens) (Sinkins & O'Neill, 2000). In arthropods, in most cases, tetracycline treatment yields *Wolbachia* cured, healthy hosts, and related parasitic nematodes that do not harbour *Wolbachia* are unaffected by tetracycline treatment (Hoeruaf *et al.*, 1999).

In the present study, the prevalence of *Wolbachia* endobacteria in the natural population of the mosquito vector *Culex quinquefasciatus* where filariasis is endemic was determined by polymerase chain reaction, and its phylogenetic relationship with other known species was determined using the partial genomic nucleotide sequence of *Wolbachia* surface protein (*wsp*) gene.

MATERIALS AND METHODS

Study Area

The study was carried out in three filarial endemic villages of Tirukoilur (latitude: 11°57'00"; longitude: 79°12'00") of Villupuram district, Tamil Nadu State, south India, viz; Kuladeepamangalam, Sorayapattu and Chozhavandipuram and lies roughly 80 km inland from the seacoast. The climate of the area is dry and hot with moderate rainfall. Most of the annual rainfall (mean = 1125 mm) is from the northeast monsoon months of October – December.

Mosquito collection

Culex quinquefasciatus mosquitoes (adults) were collected from 3 villages of Tirukoilur and Mugaiyur blocks monthly for a period of five months (December 2006 to April 2007). Each month, 50 adult mosquitoes (25 males + 25 females) were collected in human dwellings from each of the three villages by hand catch using oral aspirator. The male and female mosquitoes after confirmation of the species status as *Cx. quinquefasciatus* were stored at –80°C for DNA extraction. If females collected were blood fed, they were allowed to lay egg rafts in confined oviposition bowls. After egg laying, the parent females were stored for further PCR assaying. A total of 750 specimens were tested for the presence of endobacteria *Wolbachia*. The proportion of mosquitoes positive for *Wolbachia* were analysed by Chi square method, by taking into account the actual number of males and females positive, to determine any significant differences between the sexes and between the villages. The 95% confidence interval for each proportion value was also estimated.

DNA extraction and PCR assay

DNA extraction was carried out using 'DNA Extraction Solution' kit (Genie, Bangalore, India), as described by Dhananjeyan *et al.* (2010), following manufacturer's instructions with minor variations *viz;* each sample was homogenized in 100 µl of DNA extraction solution and incubated for 20 minutes at room temperature. The homogenate was then centrifuged at 10,000 rpm for 10 minutes. The supernatant was transferred to a fresh micro-centrifuge tube and centrifuged at 10,000 rpm for 5 minutes. The DNA pellet was washed twice with same volume of 95% ethanol with spin at 5000 rpm for 5 minutes. A final wash was given with same volume of 70% ethanol. The DNA pellet was air-dried and dissolved in 10 ml of DNAse free deionized water.

Two molecular markers *viz*; 16s rRNA gene (O'Neill *et al*., 1992) and a major cell surface coat protein gene (*wsp*) (Braig *et al*., 1998) has been used to screen *Wolbachia* in the mosquito, *Cx. quinquefasciatus*. PCR

reaction was carried out in a volume of 25 µl containing 2.5 µl of 10x Taq polymerase buffer with 1.5 mM MgCl2, 1.5 µl of dNTP mix (100 mMeach), 1.5 units of Taq polymerase enzyme, 2.4 nM of each primer, and 3 µl of the template DNA. The PCR products were visualized in 1.2% agarose gel stained with ethidium bromide on a UV transilluminator (Vilbert Lormet, France).

Sequencing of *Wolbachia* endobacterium

As a fast evolving gene (Baldo *et al.*, 2005), *wsp* was selected for further nucleotide sequencing and phylogenetic analysis. Nucleotide sequencing of the *wsp* gene was carried out using custom sequencing services (MWG. Bangalore). The nucleotide sequences thus obtained was BLAST analysed at NCBI.

Mutilple sequence alignment was carried out using Clustal-W programme. The evolutionary distances in the phylogenetic tree was constructed using Kimura – 2 distances and Neighbour-Joining algorithms with 1000 bootstrap replicates in MEGA 4.0 software (Tamura *et al.*, 2007).

RESULTS

Prevalence of *Wolbachia* in wild caught *Culex quinquefasciatus*

A total of 750 mosquitoes from 3 villages were screened for the presence of *Wolbachia* endobacteria by PCR. In all the villages together, the percentage positivity for *Wolbachia* in *Cx. quinquefasciatus* was 91.2% (Table 1). In males the percentage positive ranged from 88% to 93%; while in females it ranged from 89% to 100%. The results indicated that there was no significant difference observed in the proportion positive for *Wolbachia* between males and females, and also between different months surveyed, except during the month of February during which there was significant difference (Table 1).

In Kuladeepamangalam village, the overall percentage of *Cx. quinquefasciatus* with *Wolbachia* was found to be 90.8% (86%-94%). In the other 2 villages studied, the pattern of prevalence of *Wolbachia* showed a similar trend (Table 2). The overall percentage positive was 90.4% and 92.4% in Sorayapattu and Chozhavandipuram villages respectively. In these two villages, the prevalence of *Wolbachia* was slightly higher in the female *Cx. quinquefasciatus* than males. This difference was not significant ($P > 0.05$).

Sequencing of *Wolbachia* endobacterium

The *Wolbachia* partial nucleotide sequence obtained was used to analyze the phylogenetic relationships with other known sequences in the NCBI databases.

A total of 556 positions were present in the final dataset. The *Wolbachia* sequence in the present study showed close relationship with the known sequences obtained from

Table 1. Prevalence of *Wolbachia* endobacteria in males and female *Cx. quinquefasciatus*

Months	*% Cx. quinquefasciatus* positive for *Wolbachia*		*P* value
	Males (n=75)	Females (n=75)	
December '06	88.0 (79.1, 94.0)*	89.3 (80.8, 94.9)	1.000
January '07	93.3 (85.8, 97.5)	89.3 (80.8. 94.9)	0.560
February '07	90.7 (82.4, 95.8)	100.0 (96.1, 100.0)	0.013**
March '07	90.7 (82.4, 95.8)	89.3 (80.8, 94.9)	1.000
April '07	89.3 (80.8, 94.9)	92.0 (84.1, 96.7)	0.779
Total	90.4 (87.1, 93.1)	92.0 (88.9, 94.4)	0.519

*95% CI in parenthesis; **Significant by X^2 test

Table 2. Prevalence of *Wolbachia* endobacteria in *Cx. quinquefasciatus* in three villages

Months/ Villages	*% Cx. quinquefasciatus* positive for *Wolbachia* with 95% CI		
	Kuladeepamangalam (n=50)	Sorayapattu (n=50)	Chozhavandipuram (n=50)
December '06	90 (79.2, 96.2)*	88 (76.7, 95.0)	88 (76.7, 95.0)
January '07	94 (84.5, 98.5)	90 (79.2, 96.2)	90 (79.2, 96.2)
February '07	94 (84.5, 98.5)	94 (84.5, 98.5)	98 (90.5, 99.9)
March '07	90 (79.2, 96.2)	88 (76.7, 95.0)	92 (81.8, 97.4)
April '07	86 (74.3, 93.7)	92 (81.8, 97.4)	94 (84.5, 98.5)

*95% CI in parenthesis

the NCBI database (Figure 1). The *wsp* gene sequence from *Wolbachia* strain of *Cx. quinquefasciatus*, i.e., Tirukoilur (GenBank accession number EU194487) showed 99% nucleotide sequence homology with *Wolbachia* sp. in *Cx. pipiens* in different geographical regions. Phylogenetic analysis showed that the present *Wolbachia* isolate is closely related with *Wolbachia* from *Culex pipiens pipiens*, *Niphotettix virescens* (Order: Hemiptera) and *Cnaphalocrosis medinalis* (Order: Lepidoptera).

DISCUSSION

Wolbachia have attracted considerable recent interest for several reasons. First, given their widespread distribution and effects upon hosts, *Wolbachia* have implications for important evolutionary processes. The number of known infected species is increasing rapidly, and limits of distribution for this bacterial group are unknown. Systematic surveys of *Wolbachia* distribution and diversity are now possible using PCR-based methodologies (Werren, 1997). The 16S rRNA and *ftsZ* studies have provided a number of useful molecular tools for such surveys. These include general *Wolbachia* specific primers and A-*Wolbachia* and B-*Wolbachia* specific primers for both 16S rDNA and *ftsZ* genes (Werren *et al*., 1995). Infections were detected in each of the major insect orders, including Coleoptera, Diptera, Hemiptera/Homoptera, Hymenoptera, Lepidoptera, and Orthoptera. Surveys of neotemperate insects give similar percentages of infected species extrapolating from the percentage of infected species observed to the estimated number of species existent globally (10–30 million) yields 1.5–5.0 million infected insect species worldwide, making *Wolbachia* among the most abundant of parasitic bacteria.

The infection rate of *Wolbachia* is generally estimated to be at least 20% (Werren *et al*., 1995; Werren & Windsor, 2000). The estimate of infection rate emerges as the result of several *Wolbachia* screenings. In majority of the cases, only one or few individuals per species are tested and qualitative estimate for the presence or absence of the endobacterium was estimated (Ravikumar *et al*., 2010). In our study conducted at Tirukoilur, 750 specimens of *Cx. quinquefasciatus* collected from the three villages were individually assayed for *Wolbachia*, and the overall percentage positivity was determined to be 91.2%. In Kuladeepamangalam village, the prevalence was 90.8% (86%-94%), while in the other two villages; the positivity was 90.4% and 92.4% in Sorayapattu and Chozhavandipuram villages respectively. A beta-binomial model describes the distribution of infection frequencies of *Wolbachia*, shedding light on the overall infection rate as well as on the infection frequency within species. Kirsten *et al.* (2008) observed (1) the proportion of *Wolbachia*-infected species to be 66%, and that (2) within species the infection frequency

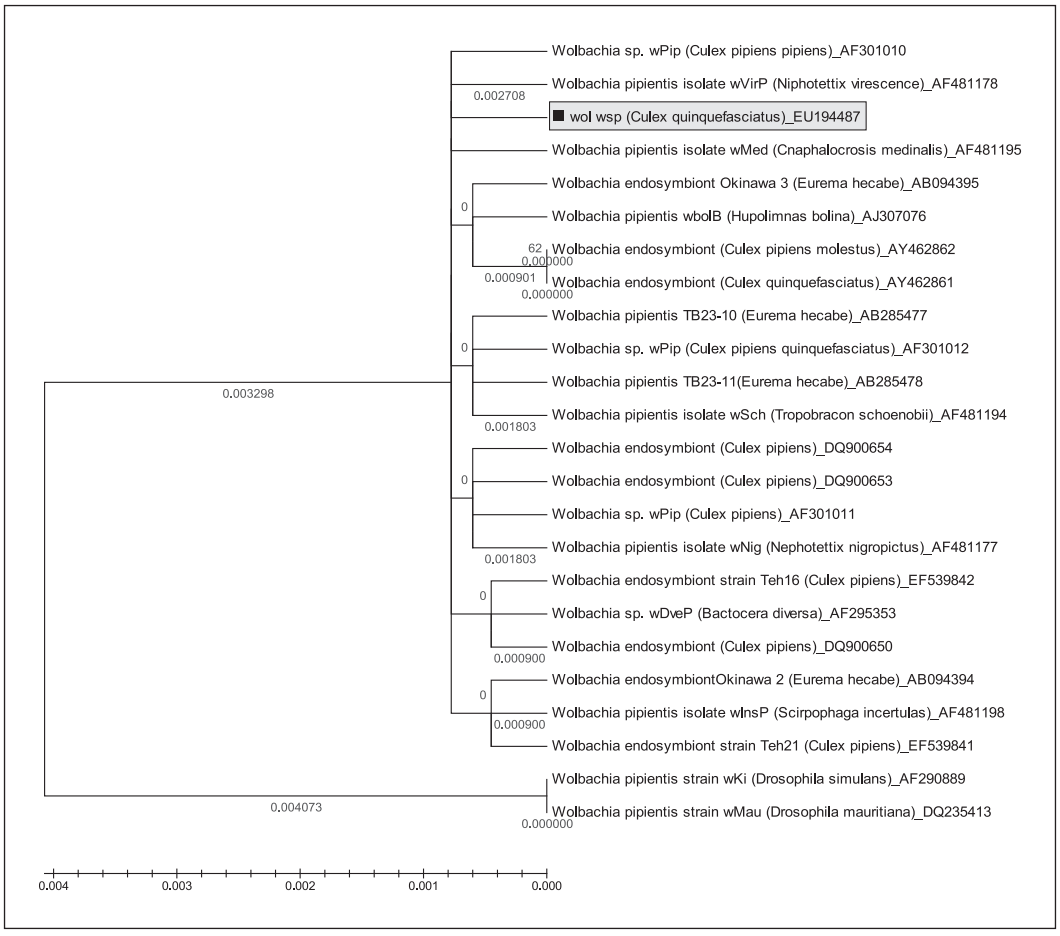

Figure 1. Phylogenetic tree of the *Wolbachia* strain, isolated from *Cx. quinquefasciatus* (Tirukoilur) based on partial sequences of *wsp* gene, constructed using the program MEGA 4.0 on the basis of the neighbor-joining method

follows a 'most-or few' infection pattern. It was observed by Tiawsirisup *et al.* (2008) that fifty-four percent (15/28 pools) of *Culex gelidus* and none (0/20 pools) of *Culex tritaeniorhynchus* was found positive for *Wolbachia* infection collected from the immigration bird-nested area, Pathumthani province of Thailand. *Wolbachia* infection was observed to be prevalent in 15 of the 29 (51.72%) field-caught mosquitoes in Taiwan (Tsai *et al.*, 2004). Three mosquito species were identified as having *Wolbachia* A infection, eight species as having *Wolbachia* B, and four other species were dually infected by both groups. Using a *Wolbachia*-specific polymerase chain reaction assay, Rasgon & Scott (2004) tested 14 North American mosquito species in five genera (*Aedes*, *Anopheles*, *Culiseta*, *Culex*, and *Ochlerotatus*) for *Wolbachia* infection, but the infections were only detected in members of the *Cx. pipiens* (L.) species complex. In Italy, Ricci *et al.* (2002) observed *Wolbachia* infection in five out of 26 mosquito species tested and phylogenetic analysis positioned the five *Wolbachia* strains in the phyletic subdivision. Kittayapong *et al.* (2000) carried out a systematic survey of *Wolbachia* in mosquitoes, and classified *Wolbachia* infections by subgroup. *Wolbachia* were detected in 28.1% of 89 wild-caught mosquito species, based on a polymerase chain reaction assay using *ftsZ* and *wsp* gene primers. Infections were found in all major disease vector genera except *Anopheles*.

Wolbachia infection was detected in *Cx. pipiens* complex mosquitoes from the Upper Rhine Valley, Germany, and Cebu City, Philippines, with the use of polymerase chain reaction (PCR) amplification of the 16S rRNA of the bacteria and further confirmation by electron microscopy. All *Culex* populations assayed by PCR showed infection of *Wolbachia* at rates between 10 and 100%. Females from different populations exhibited higher infection rates than did the males. The ultrastructure of *Wolbachia* in the ovaries of *Cx. pipiens* complex exhibited typical morphology for *Wolbachia* with 3 enveloping membranes (Mahilum *et al.*, 2003). In our study, the *Wolbachia* infection in female *Cx. quinquefasciatus* was found slightly higher than in males but was not statistically significant ($P>0.05$); except during the month of February '07. Tirukoilur isolate of *Wolbachia* analyzed was found to be closely related to *wMed*, Okinawa 3, AJ 307076 isolates, but far related to Teh 21. The phylogeny of *Wolbachia* sp., based on fast evolving gene, *wsp* gene, has been accepted (Braig *et al.*, 1998). *Wolbachia* sp. that infects *Cx. pipiens* complex belongs to Pip group of B super group (Zhou *et al.*, 1998; Pidiyar *et al.*, 2003). Phylogenetic analysis of *Wolbachia* spp. in the present study based on the *wsp* gene fragments showed that the present *Wolbachia* isolate from Tirukoilur was closely related with *Wolbachia* from *Cx. pipiens pipiens*, *N. virescence* and *C. medinalis*. These four strains appear to have branched out earlier than the other isolates considered in the study. *Wolbachia* isolates from hosts in the phylogenetic analysis belonged to four insect orders viz; Hemiptera, Lepidoptera, Hymenoptera and Diptera. Except for the isolate from Drosophila, all other isolates from various hosts of the 4 orders were grouped together.

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