Molecular Characterization of Wolbachia and its phage WO in the laboratory populations of Drosophila

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ABSTRACT

Wolbachia are a group of maternally inherited intracellular rickettsial α-proteobacteria, infecting wide range of arthropods and filarial nematodes. They infect around 66% of arthropods and impose various fitness related effects in their host populations to enhance their transmission. In the current study, four out of eight laboratory populations of Drosophila has been found positive for Wolbachia and its phage WO through PCR diagnostics. Four populations of D. ananassae were infected with wfilv strain of Wolbachia and its phage, while other four D. melanogaster populations do not have either of them. Further, phylogenetic characterization of Wolbachia and phage WO from D. ananassae indicates close relationship across other Drosophila species, suggesting possible horizontal transmission.

Keywords: Wolbachia, phage WO, Drosophila, phylogeny, reproductive manipulation.

1. Introduction

Majority of animal species on earth harbour intracellular endosymbionts, which induce either positive or negative fitness related effects on their hosts and play vital role in development, ecology, and evolution [1]. Such interactions have proved as good model systems for investigating the relationships between the host and their symbionts. In arthropods, mutualistic or commensalistic associations are more common apart from the conventional reproductive parasitism [2-4]. ‘Reproductive parasitism’ is a successful and stable association witnessed in endosymbionts that primarily promote maternal transmission in their hosts [5]. Such endosymbionts affects life histories of host and promotes insect diversity and speciation [6]. A few symbionts viz., Buchnera, Cardinium, Spiroplasma, Blochmannia, Wigglesworthia, Wolbachia etc., have drawn scientific interests due to their ability to affect and manipulate reproduction in different host species [7].

Among insect symbionts, Wolbachia an intracellular rickettsial α-proteobacteria, vertically transmitted in arthropods and nematodes [8] which infects around 66% of all known insect species [8] that has garnished the study of host-symbiont interactions over the last three decades. The major Wolbachia-mediated reproductive manipulations are, primarily, cytoplasmic incompatibility (CI), male killing, induction of parthenogenesis and feminization, thus selfishly enhancing their own transmission maternally [8]. In addition, Wolbachia influences many fitness related traits such as fecundity, lifespan, survival, feeding rate and sensitivity to various environmental conditions and provides resistance to RNA viruses [9-10]. Further, near obligate association have been established between Wolbachia and wasp Asobara tabida, bed bug Cimex lectularis and filarial nematodes for their normal reproduction and survival [11-12]. Additionally, the presence of Wolbachia can obstruct pathogens in the arthropods and found to have an impact on the innate immunity of the arthropods, which plays an important role in blocking pathogen infections [13-14]. Wolbachia also contribute to evolution of host genome, thus providing novel sets of gens and functions to exhilarate harsh environments [15]. Therefore, Wolbachia have context dependent effects in their host systems.
Wolbachia cannot be a complete obligate symbiont in majority of insect hosts, where it could eliminate through antibiotics or temperature treatment. In Uzi fly Exorista sorbillans antibiotics oxytetracycline eliminates Wolbachia and induces cytoplasmic incompatibility in crossing between Wolbachia infected males and antibiotic cures females [14-18]. However, some of the host populations have Wolbachia unable to cure with antibiotics or temperature treatment [13].

Phylogenetically, Wolbachia have fourteen super groups or clades (alphabetically labeled, A to N) based on evolutionary history of candidate gene sequences (16SrRNA, ftsZ, gltA, groEL, dnaA, and wsp). Wolbachia surface protein gene (wsp), which has high rate of recombination and variable gene are extensively used for discriminating A and B group of Wolbachia. Based on 16SrRNA phylogeny, 2% divergence between A and B super clades have been found, which diverged around 60 million years ago [16]. These two super-clades comprise mostly parasitic Wolbachia, which infects insects, mites and crustaceans. Studies show that super-clades A and B separated from C and D around 100 MYA ago [16]. The super-clade E to N Wolbachia are not so common and associated with insects, filarial nematodes, acari’s, crustaceans and arachnid’s [18].

Earlier studies hypothesized that obligate intracellular bacteria get protection from phage particles as they are protected intracellularly. Indeed, several genomes of primary endosymbionts lack such phage particles [19]. Screening for Wolbachia phage (WO) using PCR with minor capsid protein gene orf7 indicated that the phage infects 89% of Wolbachia of arthropods [20], out of these, 25 different WO phage particles have been identified [21]. Whereas, C and D super-group Wolbachia from nematode does not have phage WO [21]. These bacteriophage WQ particles undergo lateral transfer between Wolbachia and thus widespread source of genomic flux in Wolbachia and potentially the arthropod hosts [13, 20, 22]. Here we screened eight laboratory populations of Drosophila for the Wolbachia and phage WO infection and generated the molecular systematic relationships and discussed the results with the emphasis of Wolbachia acquisition and life history manipulation in Drosophila.

### 2. Materials and Methods

#### 2.1 Experimental populations

In this study, we used eight large laboratory populations, four D. melanogaster populations (JB1-4, Joshi Base line) were maintained on banana-jaggery food since 40 years, and other four D. ananassae populations (AB1-4, Ananassae Base line) were maintained on cornmeal food medium since 13 years. These populations have been maintained in the laboratory on a 21-day discreet generation cycle at 25 °C with relative humidity of about 90% and constant light. Sixty -80 eggs were collected in a vial (90 mm height and 24 mm diameter) containing approximately 6 ml of food, forty such vials were collected per population to maintain the appropriate population sizes of about 1800 breeding adults. After twelve days of egg collection, AB’s were dumped into Plexiglas cages (25 cm x 20 cm x 15 cm) provided with abundant food with moist cotton ball to provide humidity. For JB’s, on the 12th, 14th, and 16th day after egg collection, the flies were transferred to new food vial having approximately 4-ml of food. On the 18th day, these flies were collected in to the Plexiglas cages. Both AB and JB cages were provided food plate over-layered with yeast-acetic acid past on 18th day for two and half days than cult food plates on third day for 18 hrs followed by collection of eggs to initiate the next generation.

#### 2.2 DNA extraction

About 20 Drosophila flies were subjected for DNA extraction in each population following the usual stepwise methods of extraction with phenol, phenol: chloroform: isomyl alcohol, chloroform and finally two volumes of double distilled ice cold ethanol to precipitate DNA in the presence of 3M sodium acetate in the DNA in spectrophotometer and stored at −20 °C for further use.

| Table1: Primers used for amplification of Wolbachia, phage WO and insect DNA. |
|-----------------------------|----------------|----------------|
| Agent | Gene/ strain | Primer code | Primer sequence |
|-----------------------------|----------------|----------------|
| Wolbachia | wsp | wsp81F | 5'-TGGTCCAAATAAGTGAAGAAAC-3' |
| | | wsp91R | 5'-'AGTTAACTTCTTCACTCCAA-3' |
| | wspA' | wsp4136F | 5'-TGGATTACGATTCATTG-3' |
| | | wspA' | 5'-TGGTCCAAATAAGTGAAGAAAC-3' |
| | wspB | wsp81F | 5'-ACCACGTTTCTGATGTAAC-3' |
| | wsp22R | wsp22R | 5'-ACCACGTTTCTGATGTAAC-3' |
| | wsp-Mel* | wsp308F | 5'-TAAAGGATGTAACATTG-3' |
| | wsp-Alb* | wsp328F | 5'-CCACAGATCATTTGACG-3' |
| | wsp-Mors* | wsp317F | 5'-CTTAAAGGATGTAACATTG-3' |
| | wsp-Riv* | wsp169F | 5'-ATGGATTACGATTCATTG-3' |
| | wsp-Unit* | wsp207F | 5'-AGTGAAGACTGCCTGCTG-3' |
| | wsp-Haw* | wsp178F | 5'-AAAGAGACTGCCTGCTG-3' |
| Phage WO | orf7 | WOF | 5'-CCCATATGAGCCTGCTG-3' |
| | | WOR | 5'-CGTTCGCTGCTGCTGCTG-3' |
| Insect | rDNA | 18SF1 | 5'-TTGGAGGAGCAAGTCTGTTG-3' |
| | | 18SR1 | 5'-ACTTCCGGGATCGCTAGC-3' |

*Reverse Primer was wsp91R*
2.3 PCR assay

*Wolbachia* specific surface protein (wsp) coding gene was amplified through general, A and B group specific wsp primers and strain characterization was carried out through wMel, wAlb, wMors, wRiv, wUni and wHaw primers [24]. The presence of Phage WO was confirmed through amplification of phage specific minor capsid protein gene *orf7* [25] and finally the insect specific 18S rDNA primer used to check the quality of extracted DNA [26] (Table 2). Amplification was carried out in a Eppendorf thermocycler in 20 µl reaction mixture having 2µl of 1x PCR buffer, 2µl of 0.2mM dNTP's, 2µl of 2.5mM MgCl2; and 0.2µl (0.5 unit) *Tag* DNA polymerase (MBI-Fermentas), 0.2µl of 0.1µM of each forward and reverse primer, 2µl (20ng) of template DNA and final volume of sterile water to make up 20µl. The cyclic conditions were initial denaturation at 94 °C for 5 minutes followed by 36 cycles with denaturation at 94 °C for 1min, primer annealing for 1min at a specific melting temperature (Table 1), primer extension at 72 °C for 2 min and final extension at 72 °C for 10 minute. *D. melanogaster* and *D. nasuta* of Mysore stock center populations were used as positive and negative controls during the PCR diagnostics respectively [27]. The PCR products were separated through 1.2% agarose gel run in1x TBE buffer for a length of 5-6 cm at a constant of 65 volts. The gel stained with 0.5 µg/ml gel of ethidium bromide just prior to gel casting. A standard molecular weight marker was used to identify the expected band. The expected PCR products of wsp-A and orf7 bands were excised and sequenced at Chromous Biotech Bangalore.

2.4 Antibiotics treatment

The corn-food was fortified with antibiotics oxy-tetracycline hydrochlorid (0.5µg/ml) to cure *Wolbachia* in *D. ananassae*. After food was cooled to below 40 °C, the antibiotics was thoroughly mixed and the larvae were reared as above for five generations. In each generation, the PCR was run to confirm the presence/absence of *Wolbachia* for five generation.

2.5 Phylogeny of *Wolbachia* and its phage WO

Manual primary alignment was done for raw sequences, followed by homologous sequence search was done on NCBI-BLAST. Pairwise and multiple sequence alignment was done for both *Wolbachia* specific *wspa* and phage specific *orf7* gene primers in MEGA 5 [28]. The aligned sequences were manually checked for gaps. For both *wspa* and *orf7* genes, the evolutionary history was inferred using the Maximum Likelihood method (ML) based on the HKY model [29]. The tree with the highest log likelihood -1844.8681 and -1222.6067 were shown for *wspa* and *orf7* genes respectively. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distance estimated using the Maximum Composite Likelihood approach, and then selecting the topology with superior log likelihood values. A discreet Gamma distribution was used to model evolutionary rate difference among sites (5 categories (+G, parameter = 0.3744 for *wspa* and 0.6151 for *orf7* genes)). The trees were drawn to scale with branch length measured in the number of substitutions per site. The analysis involved 20 in *wspa* and 19 nucleotide sequences in *orf7* gene. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 505 and 237 positions in *wspa* and *orf7* gene sequence final data sets, respectively. The sequences obtained in this study were deposited in GenBank under accession numbers JQ518485 and JQ518486 for *wspa* and orf7 respectively.

3. Results and discussion

Among the eight *Drosophila* populations screened, only four populations of *Drosophila ananassae* were positive for A group *Wolbachia* and other four populations of *Drosophila melanogaster* were free from *Wolbachia*. Further strain specific characterization with six primers revealed the presence of only wRiv strain in *D. ananassae*. All four *D. ananassae* populations infected with *Wolbachia* were also positive for phage WO. The *Wolbachia* negatives DNA quality was confirmed through insect specific 18S rDNA amplification, which amplify around 555 bp [26] to rule out the possibility of ‘false negative’ due to inferior quality of DNA. However, curing of *Wolbachia* infection in *D. ananassae* even after five generations with antibiotics was not successful. The phylogenetic characterization of *Wolbachia* and Phage WO of *D. ananassae* (AB1-4 populations) shows 100% congruence with other three *Drosophila Wolbachia* which infects A super clade Wolbachia viz., *D. auraria*, *D. paulistorum* and *D. ananassae*. The Phage WO showed homology with other phage WO sequences of *D. simulans* and *D. melanogaster*, suggesting possible horizontal transmission of both *Wolbachia* and phage WO particles. The failure of curing *Wolbachia* infections over five generations in *D. ananassae* could be due to the possible integration of *Wolbachia* genome into the host nuclear genome [13, 22]. Further, it could be possible that the concentration of tetracycline is not enough to cure *Wolbachia* or we treated less number of generations. Reports suggest [13] that the possible integration of *Wolbachia* in the *D. ananassae* could yielded increased genome size and thus has possible new sets of genes and functions that have increased fitness in the flies. Therefore, failure in curing *Wolbachia* in *D. ananassae* might be due to integration of *Wolbachia* genome in their nuclear genome. These integrations have occurred not only in *D. ananassae*, but also in many other organism including nematodes [13] and there might be in several other organisms, which remain unexplored. These interactions hamper the study of independently identifying and assorting the host symbiont interactions.

The absence of *Wolbachia* infections in four *D. melanogaster* populations might be due to 40 years of continues adaptation to laboratory condition. However, this might not be the only hypothesis for such observation, as Ravikumar et al. [27] showed the presence of natural *Wolbachia* infections in *D. melanogaster* populations from *Drosophila* stock center, Mysore where the *Drosophila* populations are being maintained for several hundred generations. Similar studies by Miller and Riegler [30] showed the absence of *Wolbachia* in *D. willistoni* collected before 1970, however recent samples of *D. willistoni* are shown to be naturally infected with wWil strains of *Wolbachia*. Several such observations prove beyond doubts that infection polymorphism exists in different species/populations and the “reproductive parasite” invade/abandon host insects. These interactions are of evolutionary significance as they describe and define the life history traits of arthropod hosts.

Through several studies, the baffling phenomenon of CI has been majorly attributed to density and diversity of *Wolbachia* strains; however, modulation of bacterial densities alters the expression levels of CI. Further, when the *D. melanogaster* populations from our laboratory (JB1-4) and a population from *Drosophila* stock center, Mysore, infected with *Wolbachia* [28] were crossed, CI was not observed (results not shown).

The Phage WO might have influenced genomic influx between...
Wolbachia and their hosts \cite{28}. Although Wolbachia has an endosymbiotic association within the host cell vacuoles, they are infected with phage WO. It is estimated that around 89% of all Wolbachia clades (except C & D) infects phage WO and have played a fundamental role in the evolution of the symbiont’s as well as host genome \cite{13}. The phage particles are inversely associated with the Wolbachia density and their phenotype in their respective hosts. The phage particles can be either being in lysogenic phase or lytic phase and thus modulates the bacterial densities \cite{31}. Drosophila are the model organism for the study of genetics and other life history traits such as reproductive fitness, ageing, genetic makeup, adaptability, etc., Since Wolbachia infection in the Drosophila greatly hamper the life history traits, it would be feasible to investigate Drosophila - Wolbachia interactions and characterize the role of Wolbachia and their phage WO before conducting life history studies.

\textbf{Fig 1 (a)}

\textbf{Fig 1 (b)}

\textbf{Fig 1:} Phylogenetic trees of \textit{Wolbachia} based on wspA (a) and \textit{Wolbachia} phage \textit{orf7} (b) gene sequences. The tree was generated by Maximum Likelihood method based on the HKY model. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The branch lengths measured in the number of substitution per sites. All positions containing gaps and missing data were eliminated. The analysis involved 20 in \textit{wspA} and 19 in \textit{orf7} gene nucleotide sequences. Name of the arthropod host followed by accession number and strain designation are shown wherever available.
4. Conclusion

Wolbachia infections in laboratory populations of D. ananassae has not been cured with antibiotics; this might be due to possible integration of Wolbachia genome with host nuclear genome [13]. Further, D. melanogaster do not have Wolbachia, and opens up a major debate on the factors responsible for the ‘entry’ and ‘exit’ of Wolbachia infection in the insect hosts. The comparable reproductive fitness between Wolbachia infected and free Drosophila spp. paves way to investigate the possible role of the endosymbionts. Future studies needed to quantify Wolbachia induced possible phenotypes in D. ananassae in laboratory.

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6. Reference


