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Prakash BM
Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit, Jawarlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore – 560 064.
Email:prakashbm@gmail.com

Prathima A
Department of Biological Sciences, Bangalore University, Bangalore-560056. India
Email: prathima2805@gmail.com

Ravikumar H
Department of Biological Sciences, Bangalore University, Bangalore-560056. India
Email: ravikumarh79@gmail.com

Chandrashaker KT
Institution of Excellence, Vijnana Bhavana, University of Mysore, Manasagangotri, Mysore - 570 006. India
Email: ktseri@gmail.com

Puttaraju HP
Department of Biological Sciences, Bangalore University, Bangalore-560056. India
Email: puttarajuhp@hotmail.com

Correspondence:
Prakash BM
Evolutionary Biology Laboratory, Evolutionary and Organismal Biology Unit, Jawarlal Nehru Centre for Advanced Scientific Research, Jakkur, Bangalore – 560 064.
Email: prakashbm@gmail.com

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

Prakash BM, Prathima A, Ravikumar H, Chandrashaker KT, Puttaraju HP

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 *wRiv* 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 ricketsial α-proteobacteria, vertically transmitted in arthropods and nematodes [5] 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 [5]. 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 [2-4]. 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 [11-12]. *Wolbachia* also contribute to evolution of host genome, thus providing novel sets of gens and functions to exhilarate harsh environments [13]. 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 antibiotically cures females [14-15]. 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 (16S*rRNA, ftsZ*, *gltA*, *gro*EL, *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 [17]. 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 intracellular life style and lacks several genes involved in recombination pathways. Indeed, several genomes of primary endosymbionts lack such phage particles [19]. Screening for *Wolbachia* phage (*WO*) using PCR with minor capsid protein gene *orf*7 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 *WO* 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 ($JB_{1-4,}$ Joshi Base line) were maintained on banana-jaggery food since 40 years, and other four *D. ananassae* populations ($AB_{1-4,}$ 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 ºC 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 in 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: isoamyl alcohol, chloroform and finally two volumes of double distilled ice cold ethanol to precipitate DNA in the presence of 3M sodium acetate [23]. The precipitated DNA washed in 70% ethanol, dissolved in 200 µl TE (Tris-EDTA) buffer. The DNA was subjected to RNAse-A treatments followed by further re-extraction with above steps and finally the pellet was dissolved in 200 µl TE buffer. Quantified the DNA in spectrophotometer and stored at −20 ºC for further use.

Table1: Primers used for amplification of *Wolbachia*, phage *WO* and insect DNA.

Agent	Gene/ strain	Primer code	Primer sequence	Product size (bp)/ annealing temperature ºC
Wolbachia	*wsp*	*wsp81F* *wsp691R*	5'-TGGTCCAATAAGTGATGAAGAAAC-3' 5'-AAAAATTAAACGCTACTCCA-3'	632/55
	wspA[*]	*wspA136F*	5'-TGAAATTTTACCTCTTTTC-3'	556/50
	wspB	*wsp81F* *wsp522R*	5'-TGGTCCAATAAGTGATGAAGAAAC-3' 5'-ACCAGCTTTTGCTTGATA-3'	522/50
	wsp-Mel[*]	*wsp308F*	5'-TTAAAGATGTAACATTTG-3'	405/50
	wsp-Alb[*]	*wsp328F*	5'-CCAGCAGATACTATTGCG-3'	379/50
	wsp-Mors[*]	*wsp173F*	5'-CCTATAAGAAAGACAATG-3'	516/50
	wsp-Riv[*]	*wsp169* F	5'-ATTGAATATAAAAAGGCCACAGACA-3'	523/50
	wsp-Uni[*]	*wsp207F*	5'-AGTGATTACAGTCCATTG-3'	493/50
	wsp-Haw[*]	*wsp178F*	5'-AAAGAAGACTGCGGATAC-3'	581/50
Phage *WO*	*orf7*	*WOF* *WOR*	5'-CCCACATGAGCCAATGACGTCTG-3' 5'CGTTCGCTCTGCAAGTAACTCCATTAAAAC-3'	365/57
Insect	*rDNA*	*18SF1* *18SR1*	5'-TTGGAGGGCAAGTCTGGTGC-3' 5'-ACTTCGGCGGATCGCTAGCT-3'	555/63

[*]Reverse Primer was *wsp691R*

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 18SrDNA 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 $MgCl_2$ and 0.2µl (0.5 unit) *Taq* 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 ºC for 5 minutes followed by 36 cycles with denaturation at 94 ºC for 1min, primer annealing for 1min at specific melting temperature (Table 1), primer extension at 72 ºC for 2 min and final extension at 72 ºC 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 ºC, 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 at 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 *wsp* 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 *wsp* 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 *wsp* and 19 nucleotide sequences in *orf7* gene. Codon positions included were1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated. There were a total of 505 and 237 positions in *wsp* 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 *wsp* 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* (AB_{1-4} 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 before1970, 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 (JB_{1-4}) and a population from *Drosophila* stock center, Mysore, infected with *Wolbachia* [27] were crossed, CI was not observed (results not shown).

The Phage *WO* might have influenced genomic influx between

Wolbachia and their hosts [20]. 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 [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 [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.

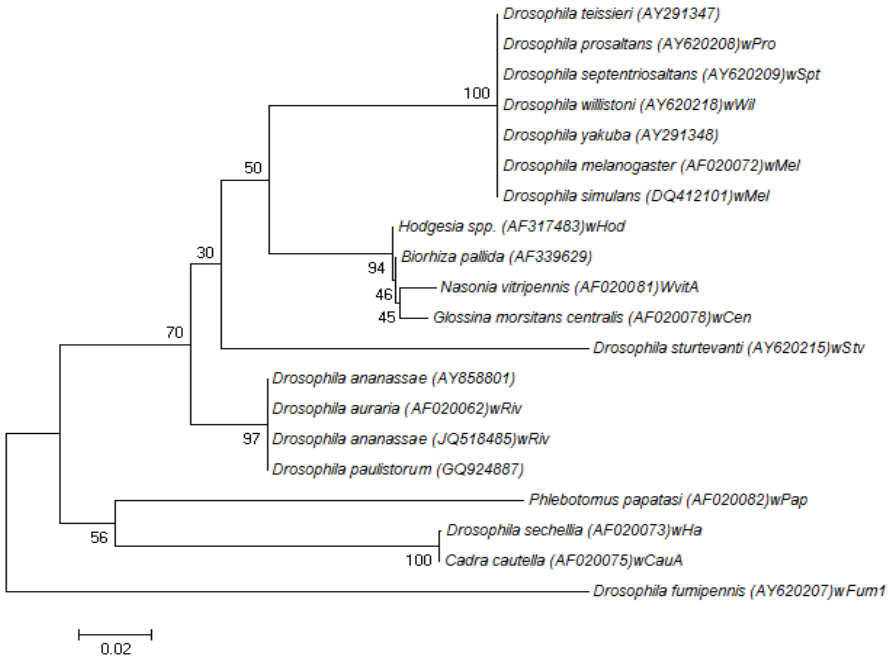

Fig 1 (a)

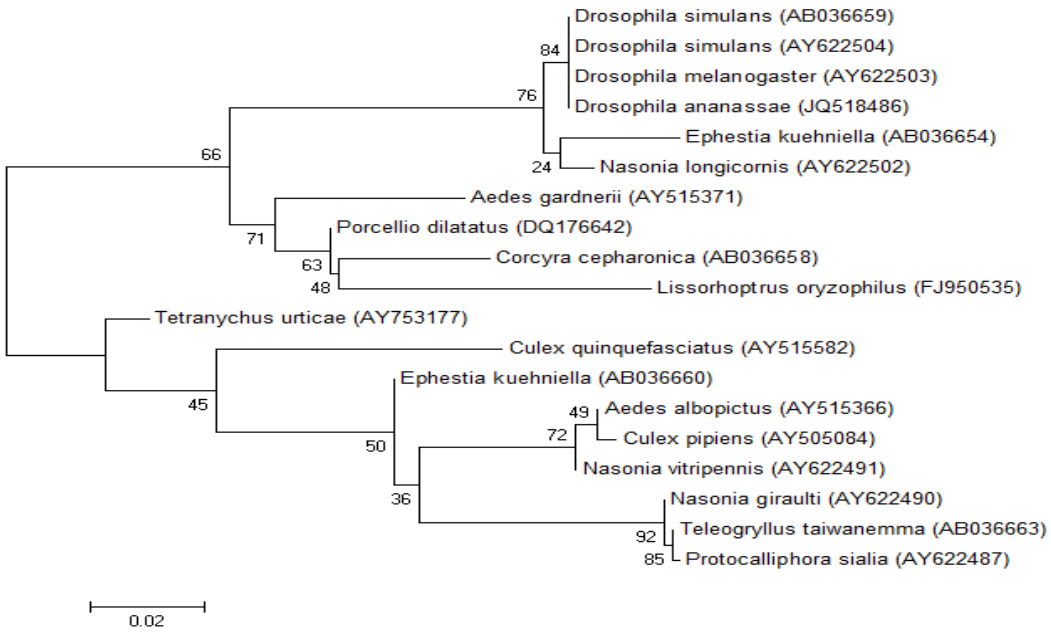

Fig 1 (b)

Fig 1: Phylogenetic trees of *Wolbachia* based on *wspA* (a) and *Wolbachia* phage *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 *wspA* and 19 in *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 able to 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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