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## Short Research Communications

# Molecular phylogenetic affiliation of *Wolbachia* and phage WO among *Mansonia* mosquitoes from Kerala, India

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Mosquitoes of the genus *Mansonia* (Diptera: Culicidae) are the main vectors of Brugian filariasis in India and other Southeast Asian countries. Cherthala in Alappuzha district, Kerala, India is highly endemic to *Brugia malayi* infection for several decades[1]. Application of insecticides as primary strategy for controlling mosquitoes over the years has led to resistance and environmental problems[2]. As a consequence, several alternative vector control strategies have attracted the attention in recent times**.** The ability of *Wolbachia* strains to affect population biology of their insect hosts ranging from their reproductive biology, ecology and evolution has provided an option to explore the use of *Wolbachia* in biocontrol of insect pests and biomedical applications[3].

*Wolbachia* are maternally inherited obligatory intracellular symbionts, which infect wide range of arthropods and filarial nematodes[4]. About 66% of all insect species, including mosquitoes are estimated to be infected with *Wolbachia*[5]. *Wolbachia* are known to have both parasitic and mutualistic association with their host's biology. They manipulate the reproductive fitness of insect hosts to enhance their transmission. Cytoplasmic incompatibility (CI) is one of the most widespread reproductive manipulation found in mosquitoes[6]. Thus, *Wolbachia* can be used as an important system to drive suitable gene into disease vector populations and disable them from transmitting the disease agents, induce life-shortening or strongly inhibit the development of pathogens within the mosquito[7-8].

Arthropod infecting *Wolbachia* commonly harbours a bacteriophage named WO. However, the presence of mobile elements have been detected, sometimes at high frequency, in the intracellular bacteria genome. Phage WO can be either lysogenic and integrated into the *Wolbachia* chromosome or lytic and free in the cytoplasm; and effect of *Wolbachia* may depend at least in part on the phage infection status[9]. Recently, phage WO has been exploited for its role to counter-attack the *Wolbachia* biology. The lytic phage is being targeted to destroy *Wolbachia* and aide filarial treatment[10].

Exploitation of *Wolbachia* in biological control of mosquitoes in any area requires knowledge of field parameters such as the vector species, the parasites or related micro-organisms, the rate of natural infection and the interaction of vector with the environment. In the present study, field collected *Mansonia* mosquitoes were examined for *Wolbachia* and phage WO using *wsp* and *orf7* genes. In addition, the phylogeny analysis was examined in relation to *Wolbachia* and phage WO infection.

Wild female mosquitoes, *Ma. uniformis* and *Ma. annulifera* were collected in stagnated water samples by root nodules of *Pistia stratiotes* planted from Kadakkarappally village, Cherthala taluk, Alappuzha district, Kerala state (9°42' N–76°19' E), India, during June-July 2012. The genomic DNA of mosquitoes were extracted using ZR Insect/Tissue DNA Kit-5™ (Zymo Research, USA). Individual mosquito was grounded in ZR Bashing Bead™ Lysis tube (Zymo Research Corporation, Irvine, USA) and homogenized in 600 µl lysis solution and DNA was extracted according to the manufacturer's protocol. DNA was stored at –20°C until it was further used for polymerase chain reaction (PCR).

PCR amplification was done using 25 µl reaction mixture volumes to check for the presence of *Wolbachia* and phage WO using *wsp* primers: A supergroup (136F 5'-TGAAATTTTACCTCTTTTC-3' and 691R 5'-AAAAATTAAACGCTACTCCA-3'), B supergroup (81F 5'-TGG TCCAATAAGTGATGAAGAAAC-3' and 522R 5'-ACCAGCTTTTGCTTGATA-3') and WO *orf7* primers: (*WO orf7*F-5'-CCCACATGA GCCAATGACGT CTG-3' and *WO orf7*R-5'-CGTTCGCTCTGCAAG TAACTC CATTAAAAC-3'). The PCR thermal profile was used as described by Ravikumar *et al*[6]. A negative (*Aedes aegypti*) and positive control (*Ae. albopictus*) were

used to confirm the PCR amplification[8]. The positive PCR products were purified using the Chromous PCR clean-up kit (Chromous Biotech™, Bengaluru, India) and directly sequenced with respective primers using an automated sequencer (3130 Genetic Analyzer, ABI, Foster City, California, USA). The sequence obtained has been deposited in GenBank with the accession number JF703674 and JN622145. The *Wolbachia* surface protein gene (*wsp*) and phage WO (*orf7*) sequences were compared for similarity with sequences in GenBank using BLAST-X programme at NCBI.

Multiple sequence alignment was done with closely related sequences using the ClustalW programme. Maximum-likelihood (ML) method was used to infer phylogenetic relationships. The appropriate model of evolution was estimated with jModelTest 0.1.1 and the best likelihood score was evaluated with a corrected version of the Akaike information criterion for small samples. Under the selected model, the Bayesian analysis was performed as implemented in MrBayes v.3.1.2[11].

In total, 153 samples were collected, of which 84 samples were identified as *Ma. uniformis* and 69 as *Ma. annulifera*. There was no *Ma. indiana* in the collected samples. Among these mosquito species, all the *Ma. uniformis* were infected with B supergroup *Wolbachia*. None of the *Ma. annulifera* samples were positive for *Wolbachia* infection based on PCR amplification of the *wsp* gene. There are no earlier reports about *Wolbachia* infections in the natural population of the mosquito vector *Ma. uniformis,* where filariasis is endemic due to *Brugia malayi* for several decades in India. These positive and negative results were similar to the study of *Wolbachia* incidence in Southeast Asia *Mansonia* species in Thailand[12-13].

The incidence of phage WO infections among *Wolbachia* in natural populations of *Ma. uniformis* was determined based on PCR detection of WO putative minor capsid protein (*orf7*). The present study detected 100% infection of phage WO in *Wolbachia* positive *Ma. uniformis*. The observations suggest that phage WO might have transmitted vertically among the infected mosquito species. Recent studies have recorded high prevalence of bacteriophage WO infection, ranging between 70 and 90% of the *Wolbachia* strains[9]. The widespread occurrence found in *Ma. uniformis* could be due to the integration of the phage into the chromosome of the associated *Wolbachia* strains. Phage WO is inversely associated with the density of *Wolbachia* as phage WO frequently toggles between the lytic and lysogenic phage. Variation in the *Wolbachia* density has a direct bearing on expression levels of CI[14].

The evolutionary history of *Wolbachia* and phage WO lineages in the *Ma. uniformis* were investigated by phylogenetic analysis performed by ML method, a GTR+G model for *wsp* and HKY+G model for *orf* gene. The direct sequencing of the PCR products gave only one sequence without double peaks, indicating the presence of only one strain of *Wolbachia* and phage WO. The construction of phylogenetic tree based upon *wsp* with closely related sequences revealed *Ma. uniformis* clustered with B supergroup as shown in the Fig. 1. The *wsp* gene sequence of *Wolbachia* from *Ma. uniformis* showed homology with various strains of *Wolbachia* from different hosts of mosquitoes such as *Culex pipiens*, *Cx. quinquefasciatus* and *Cx. fuscocephala*. The result revealed that in India *Mn. uniformis* were infected with a Pip strain similar to *Wolbachia* strains infecting *Cx. pipiens* and formed a separate lineage from *Ma. uniformis* (AF317492) and *Ma. indiana* (AF317493)[15] populations of Thailand.


*Fig. 1:* Maximum-likelihood (ML) phylogenetic tree of *Wolbachia* B supergroups (*wsp*) based upon sequences of the *wsp* gene. The tree is midpoint rooted. Name of the host species is followed by accession numbers. The nodes were supported by Bayesian inferences obtained by ML estimations.

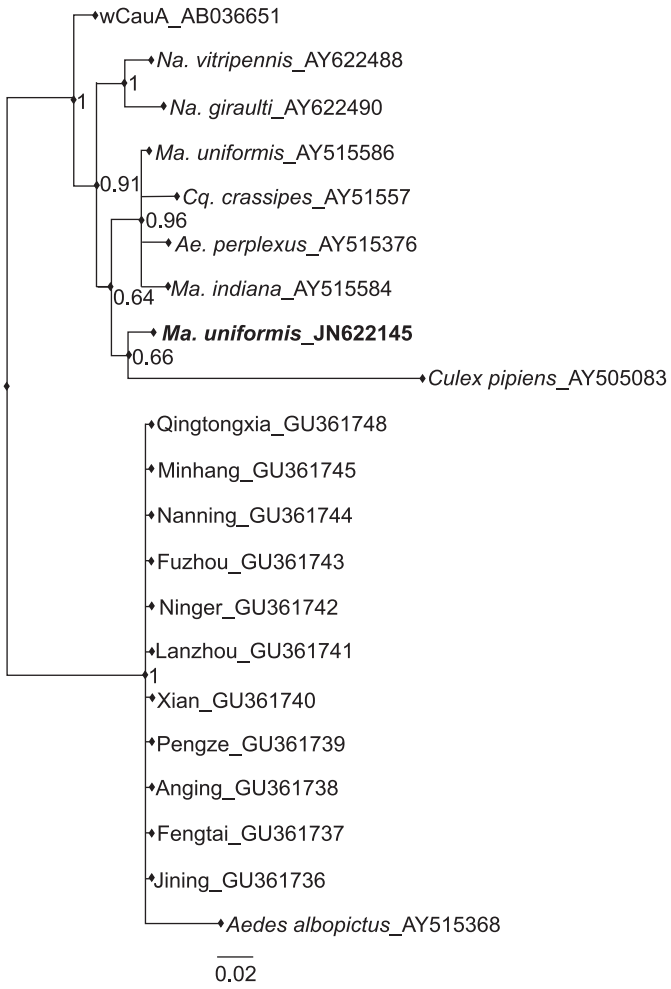


*Fig. 2:* Maximum-likelihood phylogenetic tree of phage WO (*orf7*) based upon sequences of the *orf7* gene. The tree is midpoint rooted. Name of the host species is followed by accession numbers. The nodes were supported by Bayesian inferences obtained by ML estimations.

The *orf7* gene sequences of phage WO from *Ma. uniformis* have formed a separate lineage in phylogeny tree, closer to *Cx. pipiens* in comparison to the previous reports of *Ma. uniformis* (AY515586)[16] as shown in the Fig. 2. The results revealed that both *Wolbachia* and phages WO, found in Indian *Ma. uniformis* matched Pip strains of *Wolbachia* and phage WO infecting *Culex quinquefasciatus*. The phage is known to have direct bearing on the phenotye induced by *Wolbachia*. Strains of distantly related A and B supergroups *Wolbachia,* which coinhabit single host share identical *orf7* sequences, indicating extensive lateral transmission, and therefore, the active phage in *Wolbachia* can act as a genetic tool to engineer *Wolbachia* for biocontrol[17].

It is now widely recognized that symbiotic microorganisms play a crucial role in the ecology and evolution of their hosts. The presence of *Wolbachia* in *Ma. uniformis* mosquitoes will help in understanding the *Wolbachia* and phage WO diversity, which can at later stages be exploited in developing suitable control measures. Recently, researchers are aiming on *Wolbachia* and its phage WO to devise anti-*Wolbachia* therapies to control filarial nematodes[18]. This study will provide basic descriptive information to devise experimental strategies by exploiting *Wolbachia*-WO cytoplasmic incompatibilities in the control of mosquitoes and mosquito-borne diseases. Once the tripartite associations among *Wolbachia*–phage WO–*Ma. uniformis* are established, it would pave the way for exploiting this association for controlling vector mosquitoes.

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## REFERENCES

1. Kumar NP, Sabesan S, Panicker KN. Susceptibility status of *Mansonia annulifera* to *Brugia malayi* parasites in Cherthala, Alappuzha district, Kerala. *Indian J Exp Biol* 1998; *36*(8): 829–31.
2. Hemingway J, Ranson H. Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol* 2000; *45:* 371–91.
3. Zabalou S, Riegler M, Theodorakopoulou M, Stauffer C, Savakis C, Bourtzis K. *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proc Natl Acad Sci USA* 2004; *101*(42): 15042–5.
4. Werren JH, Baldo L, Clark ME. *Wolbachia*: Master manipulators of invertebrate biology. *Nat Rev Microbiol* 2008; *6:* 741–51.
5. Hilgenboecker K, Hammerstein P, Schlattmann P, Telschow A, Werren JH. How many species are infected with *Wolbachia*?: A statistical analysis of current data. *FEMS Microbiol Lett* 2008; *281:* 215–20.
6. Ravikumar H, Ramachandraswamy N, Sampathkumar S, Prakash BM, Huchesh HC, Uday J, *et al*. A preliminary survey for *Wolbachia* and bacteriophage WO infections in Indian mosquitoes (Diptera: Culicidae). *Trop Biomed* 2010; *27*(3): 384–93.
7. McMeniman CJ, Lane RV, Cass BN, Fong AW, Sidhu M, Wang YF, *et al*. Stable introduction of a life-shortening *Wolbachia* infection into the mosquito *Aedes aegypti*. *Science* 2009; *323:* 141–4.
8. Kambris Z, Blagborough AM, Pinto SB, Blagrove MS, Godfray HC, Sinden RE, *et al. Wolbachia* stimulates immune gene expression and inhibits plasmodium development in *Anopheles gambiae*. *PLoS Pathog* 2010; *6*(10): e1001143.
9. Guruprasad NM, Mouton L, Sumithra, Puttaraju HP. Survey of *Wolbachia* and its phage WO in the Uzifly *Exorista sorbillans* (Diptera: Tachinidae). *Curr Microbiol* 2011; *63*(3): 267–72.

10. Metcalf JA, Bordenstein SR. The complexity of virus systems: The case of endosymbionts. *Curr Opin Microbiol* 2012; *15:* 546–52.
11. Ronquist F, Huelsenbeck JP. MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* 2003; *19:* 1572–4.
12. Kittayapong P, Baisley KJ, Baimai V, O'Neill SL. The distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae). *J Med Entomol* 2000; *37*(3): 340–5.
13. Wiwatanaratanabutr I. Geographic distribution of *Wolbachial* infections in mosquitoes from Thailand. *J Invertebr Pathol* 2013; *114*(3): 337–40.
14. Kent BN, Bordenstein SR. Phage WO of *Wolbachia*: Lambda of the endosymbiont world. *Trends Microbiol* 2010; *18*(4): 173–81.
15. Ruang-Areerate T, Kittayapong P, Baimai V, O'Neill SL. Molecular phylogeny of *Wolbachia* endosymbionts in Southeast Asian mosquitoes (Diptera: Culicidae) based on *wsp* gene sequences. *J Med Entomol* 2003; *40:* 1–5.
16. Chauvatcharin N, Ahantarig A, Baimai V, Kittayapong P. Bacteriophage *WO*–B and *Wolbachia* in natural mosquito hosts: Infection incidence, transmission mode and relative density. *Mol Ecol* 2006; *15:* 2451–61.
17. Ravikumar H, Prakash BM, Sampathkumar S, Puttaraju HP. Molecular subgrouping of *Wolbachia* and bacteriophage WO infection among some Indian *Drosophila* species. *J Genet* 2011; *90*(3): 507–10.
18. LePage D, Bordenstein SR. *Wolbachia*: Can we save lives with a great pandemic?. *Trends Parasitol* 2013; *29*(8): 385–93.

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