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Molecular strain typing of *Wolbachia* infection from Indian mosquitoes using *wsp* gene

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ABSTRACT

Objective: To determine the status of *Wolbachia* subgroup and phylogenetic relationships in Indian mosquitoes. **Methods:** Recently we reported *Wolbachia* infection in eight out of twenty field-caught mosquito species of India, using *wsp* specific primers. DNA extracted from these mosquito species were used for PCR amplification and sequencing. **Results:** *Wolbachia* A harboured in *Aedes albopictus* and *Culex gelidus* belongs to the subgroup *AlbA* whereas *Wolbachia* B harboured in *Aedes albopictus* and *Culex quinquefasciatus* belongs to the subgroup *Pip* and of *Culex vishnui* belongs to subgroup *Con*. However, *Wolbachia* harboured in *Armigeres subalbatus*, *Armigeres kesseli*, *Culex sitiens* and *Toxorhynchites splendens* could not be placed into any known subgroup and may represent other unknown strains of *Wolbachia*. Our phylogenetic analysis revealed eight novel *Wolbachia* strains, four in the A group and four in the B group. Most of the *Wolbachia* strains present in Indian mosquitoes belong to the *Albo*, *Pip* and *Con* groups. **Conclusions:** The similarities and differences between *Wolbachia* strains infecting different mosquito species are fundamental for estimating how easily mosquitoes acquire new infections.

1. Introduction

Wolbachia are maternally inherited bacteria infecting wide range of arthropods and nematodes[1,2]. The bacterium was first described in the ovaries of *Culex pipiens* and later, it was named as *Wolbachia pipientis*[3]. The bacteria mainly infect the reproductive tissues of arthropods and are vertically transmitted through the egg cytoplasm and alter their hosts' reproduction in various ways[4] such as cytoplasmic incompatibility, feminization of genetic males and induction of parthenogenesis[5,6]. The most common effect of *Wolbachia* infection in mosquitoes is cytoplasmic incompatibility, which was first described in *Culex pipiens*, when infected male mosquitoes mated with uninfected female mosquitoes of the same species, did not result in fertilization[7].

Molecular phylogeny represents a great source of information for better understanding the evolutionary relationships among these bacteria[8] and it is indicated that extensive horizontal transmission of *Wolbachia* has occurred between insect taxa[9]. Phylogenetic analysis of different strains of *Wolbachia* infecting arthropods using 16s rRNA gene did not show high level of divergences[10] and rapidly evolving bacterial cell-cycle gene ftsZ, has shown that there are two major groups of *Wolbachia* A and B, that were diverged 58-67 million years ago[11]. Further two additional groups C and D have also been reported in filarial nematode[2]. However, 16s rRNA gene and ftsZ gene have provided sufficient information to adequately resolve the relationships between individual *Wolbachia* strains that display different reproductive phenotypes[11]. Phylogenetic analysis of the highly variable *wsp* gene, a single copy gene coding for a surface protein of *Wolbachia*[12], has been most commonly used for resolving phylogenetic relationships among *Wolbachia* strains. It has been proposed that the *Wolbachia* A and B groups have been divided into 12 subgroups which was described by Zhou *et al*[13] and further subgroups have subsequently been added[14].

The importance of *Wolbachia* strains ranges from their effects on the reproductive biology, ecology and evolution of their hosts to their potential use in biocontrol of insect pests and biomedical applications[15,16]. Studies investigating similarities and differences in *Wolbachia* strains are important to understand how often mosquitoes encounter *Wolbachia* horizontal transmission events in nature over evolutionary time[17]. A recent PCR survey using *wsp* specific primers detected *Wolbachia* infection in eight out

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of twenty field-caught mosquito species of India[18] and in continuation of this an attempt has been made in the present study to describe the status of subgroups and phylogenetic relationships of *Wolbachia* strains present in Indian mosquitoes.

2. Materials and methods

2.1. Mosquito specimens

The mosquitoes were collected in India between June and December 2009 through dip sampling at appropriate habitats and adults were collected by aspiration from resting boxes in their natural habitats. The morphological keys were used to identify mosquito species[18]. DNA extraction from *Wolbachia*-infected mosquito specimens originally collected and reported by Ravikumar *et al*[19] were stored at −80 ℃. DNA extracted from a single *Wolbachia*-infected mosquito of each species was used for PCR amplification and sequencing.

2.2. PCR amplification and sequencing

PCR amplifications were performed using the primers *wsp* 136 F/691 R for A group, *wsp* 81 F/522 R for B group and subgroups, including *AlbA*, *Aus*, *Haw*, *Mel*, *Mors*, *Pap*, *Riv* and *Uni* for group A and *CauB*, *Con*, *Dei*, and *Pip* for group B[13]. Amplification was done with PCR thermocycler (Eppendorf AG, Hamburg, Germany) using Hot Start *Taq* polymerase (5 Prime Eppendorf, Hamburg, Germany) and 2 μL of DNA sample in a reaction volume of 50 μL. Each reaction consists of 20 μL 10X buffer (5 Prime Eppendorf), 6 μL 25 mM $MgCl_2$, 2.5 μL dNTPs (10 mM each), 2 μL 10 pmoles of both forward and reverse primers and 1 unit of *Taq* DNA polymerase (5 Prime Eppendorf). The PCR cycling conditions were 3 min at 95 ℃ for the initial denaturation step followed by 30 cycles of 1 min at 95 ℃, 1 min at 50 ℃, 1 min at 72 ℃ and 10 min at 72 ℃ for the final extension[18]. Analysis of the PCR products was conducted with gel electrophoresis. 10 μL of the PCR product was loaded onto 1.2% agarose gel containing ethidium bromide (0.5 μg/mL, GeNeiTM, Bangalore, India) and the amplicons were documented by gel documentation unit (Alpha Imager(R) EP, Canada). The size of the PCR product was determined using 3-kb ladder (GeNei™, Bangalore, India). All PCR products were purified using chromous PCR clean-up kit (Chromous Biotech™, Bangalore, 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 from HM007825 to HM007834.

2.3. Phylogenetic analysis

The *wsp* sequences were aligned to previously determined *Wolbachia wsp* sequences using Clustal W multiple sequence alignment program[20]. Gaps and regions with ambiguous alignments were excluded from the analysis[13,21] resulting in a data set of 449 and 360 nucleotide sites. The phylogenetic trees were constructed using Jukes-Cantor and Neighbor-Joining algorithm was computed using MEGA4 program[22].

3. Results

Subgroups typing of *Wolbachia* in Indian mosquito species were *AlbA*, *Pip* and *Con* as shown in Table 1 and Figure 1. *Wolbachia* A harboured in *Aedes albopictus* (*Ae. albopictus*) and *Culex gelidus* (*Cx. gelidus*) belongs to the subgroup *AlbA*; *Wolbachia* B harboured in *Ae. albopictus* and *Culex quinquefasciatus* (*Cx. quinquefasciatus*) belongs to the subgroup *Pip* whereas that of *Culex vishnui* (*Cx. vishnui*) belongs to subgroup *Con*. However, *Wolbachia* harboured in *Armigeres subalbatus* (*Ar. subalbatus*) (A group), *Armigeres kesseli* (*Ar. kesseli*) (B group), *Culex sitiens* (*Cx. sitiens*) (B group) and *Toxorhynchites splendens* (*Tx. splendens*) (AB group) could not be further classified to any known subgroup. DNA sequencing of these species is needed for confirmation of a new strain.

The evolutionary history was inferred using Neighbor-Joining method. The optimal tree with the sum of branch length 0.68540917 for A group and 0.53908534 for B group. The evolutionary distances were computed using the Jukes Cantor method and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 449 and 360 positions in the final dataset of the *wsp* gene sequences of A and B group. Whereas *Ae. albopictus*

Table 1
Subgroups of *Wolbachia* based on the *wsp* gene among mosquitoes of India.

Genus	Sub genus	Species	*Wolbachia* classification		
			Group	Subgroup	Strain
Aedes	*Stegomyia*	*Ae. albopictus*	A, B	*AlbA*	wAlbAI[a]
				Pip	wAlbB
Culex	*Culex*	*Cx. gelidus*	A	*AlbA*	wCgelI[a]
		Cx. vishnui	B	*Con*	wCVisI[a]
		Cx. sitiens	B	*	wCstinI[a]
		Cx. quinquefasciatus	B	*Pip*	wPip
Armigeres	*Armigeres*	*Ar. subalbatus*	A	*	wAsubI[a]
		Ar. kesseli	B	*	wAkesI[a]
Toxorhynchites	*Toxorhynchites*	*Tx. splendens*	A, B	*	wTspAI[a]
					wTspBI[a]

* Negative PCR amplifications for all *wsp* subgroup specific primers; a: New strains that were found in this study are indicated in bold.

(wAlbAI), *Cx. gelidus* (wCgelI), *Ar. subalbatus* (wAsubI) and *Tx.splendens* (wAspI) which were grouped in A group with its close member formed a separate lineage in the *Wolbachia* group A than the previously reported wAlbA (AF39411) and wsub (AF317488), indicating the presence of novel *Wolbachia* strain in *Ae. albopictus*, *Cx. gelidus*, *Ar. subalbatus* and *Tx. splendens* (A group) of Indian mosquitoes as shown in Figure 2(a).

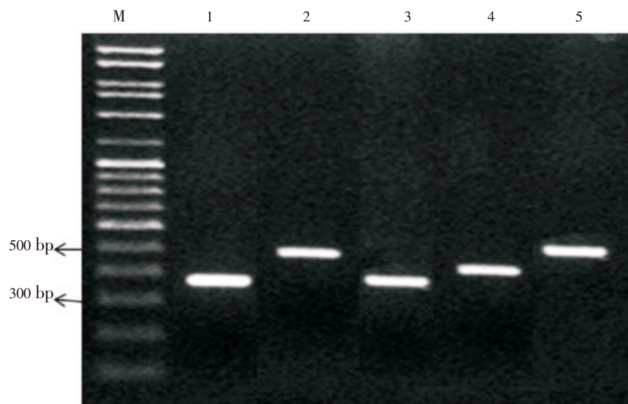

Figure 1. Subgroup of *Wolbachia* using specific *wsp* primers that amplifies 378-501 bp.
M: molecular weight marker; 1: *Ae. albopictus* (*AlbA*); 2: *Ae. albopictus* (*Pip*); 3: *Cx. gelidus* (*AlbA*); 4: *Cx. vishnui* (*Con*); 5: *Cx. quinquefasciatus* (*Pip*).

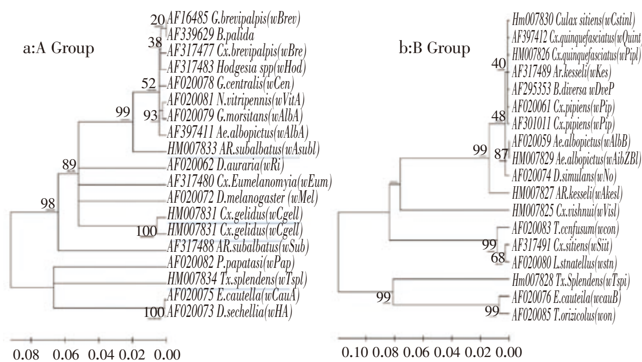

Figure 2. Phylogenetic tree based on *wsp* sequence of *Wolbachia*, constructed from Jukes-Cantor and Neighbour-Joining algorithm. Accession numbers with underlines indicate *Wolbachia* strains of the Indian mosquitoes examined in this study. *Wolbachia* strains are indicated by the host name. Numbers on the nodes indicates bootstrap percentage of 1000 replicates. The tree, restricted to (a) A group and (b) B group of *Wolbachia*, was midpoint rooted.

The *wsp* gene sequences of *Ae. albopictus* (wAlbBI), *Cx. sitiens* (wstinBI), *Cx. quinquefasciatus* (wPipB), *Cx. vishnui* (wVisBI), *Ar. Kesseli* (wKesBI) and *Tx. splendens* (wTSpBI) formed a monophyletic group with representative members of arthropods *Wolbachia* comes under B group. The *wsp* gene phylogeny indicated that the *Ae. Albopictus* (wAlbBI) and *Cx. quinquefasciatus* (wPipI) were clustered together with previously reported *wsp* gene sequences of *Ae. Albopictus* (AF020059) and *Cx. quinquefasciatus* (AF397412), showed homology with same strains of *Wolbachia*, whereas *Cx. vishnui* (wVisBI), *Ar. Kesseli* (wKesBI) and *Tx. splendens* (wTSpBI) formed a separate lineage in a phylogenetic tree indicating the presence of novel *Wolbachia* strains in these mosquito species as shown in the Figure 2(b). Among these mosquito species, *Tx. splendens* is reported for the first time, and infected with both A and B group *Wolbachia*.

4. Discussion

Mosquitoes are medically important insects that transmit a variety of diseases like malaria, filarial, dengue, yellow fever and Japanese encephalitis. The endosymbiont *Wolbachia* could be genetically transformed to modify the disease transmitting abilities of these mosquitoes[23,24]. To make this approach successful it is important to understand the strain variation, if any, in *Wolbachia* infection in Indian mosquito populations. Subgroup typing of *Wolbachia* is of evolutionary significance as it provides information about the relationship between symbionts and host[25].

The *wsp* gene is known to possess 10 times the variability of the ftsZ gene[11,13] and thus more suitable for elucidating the evolutionary relationships among *Wolbachia* infection. Even so, some *wsp* positive *Wolbachia* could not be appropriately placed into any known subgroup, suggesting that some *Wolbachia* infection actually belonged to a subgroup not described previously by Zhou *et al*[13]. It would be interesting to determine whether coinfection of two subgroups of *Wolbachia* also could cause cytoplasmic incompatibility or other reproductive distortions in a host[26]. It is crucial to know which *Wolbachia* strains are present in populations before releasing infected individuals because pre-existing natural infections can interact with and alter the dynamics of introduced strains[27-30]. Classification of *Wolbachia* by major groups A and B may miss infection involving subgroup within the same group. Based on the *wsp* subgroup specific primers, we found that two species were infected with A strains and three species were infected with B strains.

The topology of the phylogenetic tree indicated that *Wolbachia* strains could be placed into both group A and B, similar to those described by Zhou, Van Meer and Ruang-Areeerate *et al*[13,14,21]. The placement of *Wolbachia* strains into these two groups corresponded with trees derived from sequences of the 16S rRNA and ftsZ genes[10,11]. The C and D groups were not included in the analysis because *Wolbachia* strains in C and D groups have been documented only in filarial nematodes and none of *Wolbachia* strains in mosquitoes assembled out of A and B groups. The phylogenetic position of *Wolbachia* groups in Indian mosquitoes has not previously been determined. In the present study we found that eight novel strains (wAlbAI, wCgelI, wAsubI and wTspAI in group A and wVisI, wCstinI, wAkesI and wTspBI in group B) were designated. 12 previously strains were determined by Zhou *et al*[13] unlike Van Meer, Ruang-Areeerate and Behbahani *et al*[14,21,31] who found new strains in the B and A group. Most of the novel strains found in our study were in both A and B groups. The similarities and differences between *Wolbachia* strains infecting different mosquito species are fundamental for estimating how mosquitoes acquire new infections. This kind of basic descriptive information helps to devise experimental strategies by exploiting a *Wolbachia*-cytoplasmic incompatibility based mechanism to control vectors.

Conflict of interest statement

We declare that we have no conflict of interest.

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