RESEARCH NOTE

Molecular subgrouping of *Wolbachia* and bacteriophage WO infection among some Indian *Drosophila* species

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Introduction

Wolbachia are a group of obligate intracellular maternally inherited bacteria that infect arthropods and filarial nematodes worldwide (Werren *et al.* 2008). *Wolbachia* cause a variety of reproductive alterations in their arthropod hosts, including cytoplasmic incompatibility (CI), parthenogenesis, feminization and male killing (Werren *et al.* 2008). *Wolbachia* are not only speculated to have been associated with speciation in certain species of insects (Werren *et al.* 2008) but are also found to provide fitness benefits to their hosts (Teixeira *et al.* 2008). Therefore, characterization of the *Wolbachia* genotype, and determining its phenotypic effect in different hosts, is of significant importance in understanding their ecology and evolution. *Wolbachia* have been categorized into 11 different supergroups (alphabetically labelled A–K) on the basis of clades formed in gene phylogenies (Ros *et al.* 2009). Two of these supergroups, A and B, are found to infect only insects and comprise many subgroups that can be discriminated using the *Wolbachia* surface protein (*wsp*) gene. This gene, which has a very high rate of recombination, is extensively used for characterizing *Wolbachia* strains (Zhou *et al.* 1998).

Bacteriophages are widespread viruses infecting bacteria and constitute one of the most effective vectors of foreign DNA. Bacteriophages often encode proteins involved in important functions such as antibiotic resistance or virulence. A particularity of *Wolbachia* is the high number of mobile elements present in its genome despite the intracellular lifestyle of this bacterium (Moran *et al.* 2008). The phage WO is widespread in *Wolbachia* genomes: 90% of *Wolbachia* strains are infected by it including strains that are able to induce all the phenotypic effects known for *Wolbachia*, from parasitism of the host reproduction to mutualism. This phage can be either lysogenic and integrated into the *Wolbachia* chromosome or lytic and free in the cytoplasm (Guruprasad *et al.* 2011). The phage WO is an important source of genomic flux in *Wolbachia* and, thus, analysing the evolutionary dynamics and diversity of WO-*Wolbachia* association is a crucial factor for further research (Bordenstein *et al.* 2006). A recent discovery of phage WO of *Wolbachia* containing ankyrin-encoding genes and virulence factors has led to intensifying debate about whether the bacteriophage induces CI. Moreover, there are currently no tools available for the genetic manipulation of *Wolbachia* and the phage WO might be used as a vector for transgenes to recipient *Wolbachia* cells, providing an opportunity to overcome the current barriers to *Wolbachia* transformation (Kent and Bordenstein 2010; Ahantarig *et al.* 2011).

Although there have been many studies of *Wolbachia* and bacteriophage WO in different insect taxa (Gavotte *et al.* 2007), very few such studies have been carried out in India, in insect pests of rice, insect pest of sericulture, *Wuchereria bancrofti* a microfilariae (Vaishampayan *et al.* 2007) and some mosquito species (Ravikumar *et al.* 2010). Although the diversity of *Wolbachia* infection in *Drosophila* species have been well documented in respect of Bloomington Stock Centre (Clark *et al.* 2005), Tucson Stock Center (Mateos *et al.* 2006) and 16 Neotropical *Drosophila* species (Miller and Riegler 2006), there is a lack of such documentation for the National facility at the *Drosophila* Stock Centre, University of Mysore, Mysore, India. To fill this gap, an attempt has been made here for the first time to screen Indian *Drosophila* species for *Wolbachia* and bacteriophage WO infection.

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Table 1. Infection status of *Wolbachia*, phage WO, and subgroups based on the *wsp* and *orf7* gene in Indian *Drosophila* species.

	Species	No. tested	*Wolbachia* infection status	Subgroup[a] infection status	WO phage infection status
1	*D. melanogaster*	20	A	*Mel*	+
2	*D. bipectinata*	21	A	*Mel*	+
3	*D. nasuta*	28	–	–	–
4	*D. albomicans*	29	–	–	–
5	*D. pseudoobscura*	22	A	NS	+
6	*D. simulans*	24	A	*Mel, Haw*	+
7	*D. takahashi*	27	–	–	–
8	*D. yakuba*	20	A	NS	+
9	*D. virilis*	28	–	–	–
10	*D. annanassae*	23	A	NS	+
11	*D. malerkotliana*	25	A	*Mel*	+

[a]Gene fragments for identification of *Wolbachia* A included *Mel, AlbA, Mors, Riv, Uni, Haw, Pap* and *Aus* subgroups. NS, species not subgrouped.

Materials and methods

Drosophila collection

The *Drosophila* species that are listed in table 1 were obtained from the *Drosophila* Stock Centre at University of Mysore, Mysore, India. The flies were reared in insectaria at 25°C ± 1°C and 70–80% relative humidity, under L : D 12 : 12 h, on a banana–jaggery food medium (Sheeba *et al.* 1998).

DNA isolation

The genomic DNA of *Drosophila* was extracted using ZR Insect/Tissue DNA kit-5™ (Zymo Research, Irvine, USA, http://www.zymoresearch.com). Individual whole insects were ground in ZR BashingBead™ lysis tube and homogenized in 600 μL lysis solution and DNA was extracted according to the manufacturer's protocol. DNA was quantified by spectrophotometer and stored at −20°C until further use for polymerase chain reaction (PCR) assay.

Polymerase chain reaction assay

A PCR assay based on the amplification of *Wolbachia* specific *wsp* gene primers, such as A and B supergroup, were used to detect *Wolbachia* in individual *Drosophila* species. The *wsp* sequence of the A supergroup primer is 136F: 5′-TGAAATTTTACCTCTTTTC-3′ and 691R: 5-′AAAAATTAAACGCTACTCCA-3′ and for B group 81F: 5′-TGGTCCAATAAGTGATGAAGAAAC-3′ and 522R: 5′-ACCAGCTTTTGCTTGATA-3′. Amplification was done with PCR thermocycler (Eppendorf AG, Hamburg, Germany) using Hot Start *Taq* polymerase (5 Prime Eppendorf) and 1 or 2 μL of DNA sample in a reaction volume of 25 μL. The PCR condition followed for each step include 3 min at 95°C for the initial denaturation step followed by 30 cycles of 1 min at 95°C (denaturation), 1 min at 50°C (annealing), 1 min at 72°C (primer extension) and 10 min at 72°C for the final extension. The PCR conditions for phage *WO* using *WO orf7* gene: (*WO orf7*F: 5′-CCC ACA TGA GCC AAT GAC GTC TG-3′ and *WO orf7*R: 5′-CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC-3′) (Ravikumar *et al.* 2010) were 1 min at 95°C for the initial denaturation step followed by 35 cycles of 30 s at 94°C (denaturation), 40 s at 57°C (annealing), 1 min 15 s at 72°C (primer extension) and 10 min at 72°C for the final extension. The reaction mixture contained 10 μL 10× PCR buffer (5 Prime Eppendorf), 3 μL 25 mM $MgCl_2$, 1.25 μL dNTPs (10 mM each), 1 μL 10 pmoles of both forward and reverse primers and 1 U of *Taq* DNA polymerase (5 Prime Eppendorf). A negative control for the PCR assay (sterile distilled water instead of DNA in the reaction mixture) and positive specimens from a colony of *Aedes albopictus* (Ravikumar *et al.* 2010) were used to confirm the PCR amplification. Analysis of the PCR products was conducted by gel electrophoresis. Ten μL of the PCR product were mixed with Green View™ DNA loading buffer (Chromous Biotech™, Bangalore, India) were loaded in to 2% agarose gel and the amplicons were documented by gel documentation unit (Alpha Imager EP, San Leandro, USA). The size of the PCR product was determined using 3-kb and 1-kb ladder (GeNei, Bangalore, India).

Subgrouping of Wolbachia

Samples that were positive for super group A were typed with *wsp* gene primers specific for the *Mel, AlbA, Mors, Riv, Uni, Haw, Pap* and *Aus* subgroups (Zhou *et al.* 1998). All samples that were positive for a particular subgroup were tested at least twice for confirmation of results.

Results and discussion

Wolbachia infection was screened in 11 species of *Drosophila* through polymerase chain reaction assay using *Wolbachia* specific *wsp* gene primers as shown in figure 1a and table 1. Seven species were positive for *Wolbachia* infection, whereas *D. nasuta*, *D. albomicans*, *D. takahashi* and

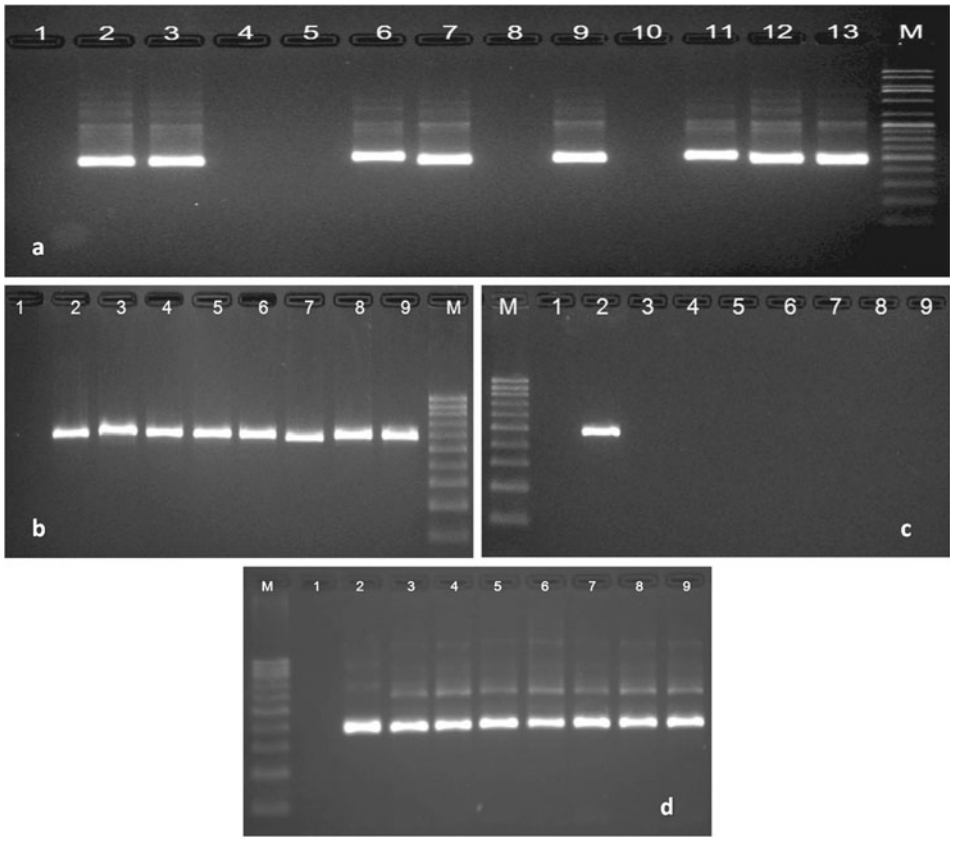

Figure 1. (a) *Wolbachia* specific *wsp* general primer that amplifies at around 600 bp; lane M, molecular weight marker (3-kb ladder GeNei^{TM}, Bangalore, India), lanes 1 & 13, negative and positive controls respectively, lanes 2 to 12, *Drosophila* species as shown in table 1. (b) *wsp* A supergroup of *Wolbachia* specific primer that amplifies around 550 bp; lane M, molecular weight marker (1-kb ladder GeNei^{TM}, Bangalore), lanes 1 & 2, negative and positive controls, lanes 3 to 9, *Drosophila* species. (c) *wsp* B supergroup of *Wolbachia* specific primer that amplifies around 450 bp; lanes 1 & 2, negative and positive controls; lanes 3 to 9, *Drosophila* species as shown in table 1. (d) WO phage of *orf7* gene that amplifies at around 400 bp; lane M, molecular weight marker (1-kb ladder GeNei^{TM}, Bangalore), lanes 1 & 2, negative and positive controls; lanes 3 to 9, *Drosophila* species as shown in table 1.

D. virilis were negative. A broad classification of *Wolbachia* into major supergroups A and B showed that all these seven species of *Drosophila* were singly infected with A subgroup and negative for B or AB supergroup as shown in figure 1, b&c. Earlier, *Wolbachia* has been shown to infect at least 19 species of the genus *Drosophila* (Mateos *et al.* 2006), from Bloomington Stock Center, 30 per cent of *D. melanogaster* meigen stocks (Clark *et al.* 2005) and 16 neotropical *Drosophila* species (Miller and Riegler 2006). The present survey revealed that 63 per cent of *Drosophila* species from the *Drosophila* Stock Centre, Mysore, are infected with *Wolbachia* endosymbionts.

The bacteriophage WO in *Drosophila* species were amplified by polymerase chain reaction using WO putative minor phage caspid protein gene primer (*orf7*). The results revealed 100% infection of bacteriophage WO in *Wolbachia* positive *Drosophila* species as shown in figure 1d. Recent studies have recorded high prevalence of bacteriophage WO infection, between 70% and 90% of the *Wolbachia* strains (Guruprasad *et al.* 2011). The widespread occurrence found in *Drosophila* species could be due to the integration of the phage into the chromosome of the associated *Wolbachia* strains. However, our PCR survey does not allow determining whether phage are lytic and free or integrated in the *Wolbachia* genome. Further study is required to investigate the phage diversity by phylogenetic analysis. The tripartite arthropod, *Wolbachia*-phage WO system is an emerging model to study the role of mobile elements in *Wolbachia*. Phage WO is inversely associated with the density model of CI in which lytic phage repress *Wolbachia* densities and lead to reduction in CI (Bordenstein *et al.* 2006; Kent and Bordenstein 2010).

Indian *Drosophila* species were found to be infected with subgroups *Mel* and *Haw* strains of *Wolbachia*. *D. melanogaster*, *D. malerkotliana* and *D. bipectinata* were infected with *Mel* strain, whereas, *D. simulans* was infected with both *Mel* and *Haw* strains (table 1). However, *Wolbachia* harboured in *D. pseudoobscura*, *D. yakuba* and *D. annanassae* could not be further classified to any known subgroups and these might represent other strains of *Wolbachia*. Perhaps gene sequencing studies need to be carried out through multilocus sequence typing for *Wolbachia*

instead of relying on the single gene *wsp*. Recent studies have demonstrated that the *wsp* gene sequences experience extensive recombination and were subjected to strong diversifying selection (Baldo and Werren 2007). Hence, we used only *wsp* gene to confirm *Wolbachia* infection and to discriminate between *Wolbachia* strains. Earlier study has shown that *D. simulans* harbours at least five phylogenetically and phenotypically distinct strains such as *w*Riv, *w*Ha, *w*No, *w*Ma and *w*Au (Mercot and Charlat 2004). The present study found that in India *D. simulans* harbour two strains *w*Riv and *w*Haw. It would be interesting to determine whether coinfection of two subgroup of *Wolbachia* also could cause cytoplasmic incompatibility or other reproductive distortions in *Drosophila* species.

Although the relationship between *Wolbachia* and the host species is usually symbiotic, a virulent form of *Wolbachia* that shortens lifespan has been found in *D. melanogaster* (Hedges *et al.* 2008). Recently, *Wolbachia* infection in *D. melanogaster* has also been shown to increase resistance to some viral infections (Teixeira *et al.* 2008). *Wolbachia* strain *w*MelPop, which normally infects *D. melanogaster*, lacks normal replication control, shortening the lifespan of the flies (McMeniman *et al.* 2009). The present survey revealed that Indian *D. melanogaster*, *D. simulans*, *D. bipectinata*, *D. pseudoobscura*, *D. yakuba*, *D. annanassae* and *D. malerkotliana* were infected by both *Wolbachia* and bacteriophage WO, whereas *D. nasuta*, *D. albomicans*, *D. takahashi* and *D. virilis* were free from *Wolbachia.* The results are of importance to *Drosophila* researchers in India, as fitness components are typically affected by *Wolbachia* infection in laboratory stocks. The similarities and differences between *Wolbachia* strains and the phage WO play a major role in providing basic descriptive information in understanding *Drosophila* biology, its fitness and reproduction. The phenotypic analysis will also provide opportunities to study the genetic basis of host-symbiosis in these Indian *Drosophila* species.

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