Brief communication

Frequency of infection with A and B supergroup *Wolbachia* in insects and pests associated with mulberry and silkworm

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Wolbachia is a ubiquitous, Gram-negative, vertically transmitted, alpha-proteobacterium that causes an array of reproductive abnormalities including cytoplasmic incompatibility, feminization of genetic males, parthenogenesis in a number of insect species, among others. *Wolbachia* is now being exploited as an agent for pest and vector control. Previous surveys indicated that it is commonly seen in 16–76% of arthropods. In this paper, using polymerase chain reaction assay based on specific amplification of the *ftsZ*-A and -B supergroup *Wolbachia* gene fragments, we found that 30% of insects and pests screened were positive for *Wolbachia*. Among them 66.7% harbour double *Wolbachia* infection, while 33.3% harbour single *Wolbachia* infection. These results indicate widespread infection with both double and single *Wolbachia,* and provide a wealth of information to exploit this endobacterium for the management of pests and vectors.

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1. Introduction

Microorganisms are ubiquitous in most inhospitable domains across the various ecosystems and are considered as the masters of the biosphere (Bhatnagar and Bhatnagar 2005). Among them, members of the genuses *Rickettsia, Cowdria, Anaplasma* play an important role in causing disease in mammals (Weisburg *et al* 1989), whereas *Wolbachia,* a vertically transmitted alpha-proteobacterium of arthropods, induces an array of reproductive isolation mechanisms. Cytoplasmic incompatibility, parthenogenesis and feminization of genetic males (Stouthamer *et al* 1999; Werren 1997) are the most common phenotypes the *Wolbachia* imposes on its hosts. It is reported that *Wolbachia* is found in 16–76% of all known insect species (Werren *et al* 1995; Werren and Windsor 2000; Jeyaprakash and Hoy 2000; Prakash and Puttaraju 2006a) and comprises supergroups A–H based on the sequence of bacterial 16S rRNA, wsp and *ftsZ*, a cell cycle gene. The A and B supergroups of *Wolbachia* infect insects, mites and crustaceans (Werren *et al* 1995); the C, D, G and H supergroups infect filarial nematodes (Bandi *et al* 1998; Lo *et al* 2004), whereas supergroups E and F infect the springtail *Folsomia candida* of the order Collembola (Vendekerchove *et al* 1999), and termite *Kalotermes flavicollis* and *Microcerotermes* spp. of Isoptera (Lo *et al* 2002), respectively.

Holden *et al* (1993) have sequenced a protein-coding gene, *ftsZ* from *Wolbachia* of *Drosophila melanogaster.* The *ftsZ* is a bacterial cell cycle gene involved in the regulation of cell division (Lukenhaus 1990). It contains conserved and highly divergent regions, making it suitable for finer scale phylogenetic analysis within a bacterial gene. Using *ftsZ* sequence information from *Wolbachia* and three other bacterial species (*Escherichia coli, Bacillus subtilis* and *Rhizobium meliloti*), Werren *et al* (1995) designed *Wolbachia* A and B supergroup primers for PCR amplification of the gene from infected arthropods. Further, the *ftsZ* primer of the B supergroup of *Wolbachia* specifically has one AciI restriction site, producing two fragments, and A supergroup *Wolbachia* has two AciI

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restriction sites producing three fragments (Werren *et al* 1995).

The Indian ecosystem has a wide ecoclimatic variation that has diverse fauna and flora, of which insects and pests comprise a major part. They are economically very important and cause economic loss of agro-produce including sericulture. The cost incurred for the prevention and control of these insects and pests may run into millions of rupees. Though there are a number of curative measures, the loss of agro-products continues to be 25–30%. Our earlier work showed that some insects and pests of sericulture harbour *Wolbachia* (Madhu and Puttaraju 2001; Puttaraju and Madhu 2002; Prakash and Puttaraju 2006a) and suggested a novel method for the management of uzifly, a parasitoid of silkworn (Puttaraju and Prakash 2005a; Puttaraju and Prakash 2005b; Puttaraju and Prakash 2005c; Prakash and Puttaraju 2006b). In the present paper we demonstrate the frequency of double versus single infection of *Wolbachia* using *ftsZ* A- and B-specific primers (Prakash 2006) in some insects and pests associated with mulberry and silkworn. The present study explored different bacterial strains that infect insects and pests, and provides information that can be exploited for pest management in sericulture in particular and in agriculture in general.

2. Materials and methods

2.1 *Insect collection and preservation*

The insects listed in the table 1 were collected from mulberry gardens in Devanahalli and Mandya; the mulberry garden of the Silkworm Seed Technology Laboratory, Kodathi, Bangalore; Mulberry Germplasm Bank of the Department of Sericulture, Bangalore University, Bangalore and Silkworm and Mulberry Germplasm Bank, Hosur. The collected insects were classified by experts of the Entomology Department, GKVK and Zoology Department, Bangalore University. They were frozen at –80°C or preserved in 70% ethanol until further use for DNA isolation and subsequent screening for the presence of *Wolbachia.*

2.2 *DNA isolation*

DNA from a single insect was extracted by the method of Sambrook *et al* (1989). Single insects were ground separately in liquid nitrogen using a mortar and pestle. Extraction buffer (100 mM Tris-HCl, pH 8.0, 50 mM EDTA and 1% SDS) and proteinase K (100 μg/ml) were added to the ground tissue and incubated overnight at 37°C with occasional swirling. The DNA was extracted twice with phenol–chloroform–isoamyl alcohol (24:24:1) and once with chloroform. The supernatant DNA was ethanol-precipitated in the presence of 3 M sodium acetate. The pellet was washed in 70% ethanol, dried and resuspended in TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) buffer and incubated at 37°C for 1 h after addition of RNase A (100 μg/ml); DNA was then re-extracted with phenol–chloroform and ethanol-precipitated as described above. The genomic DNA was quantified on 0.8% agarose gels and diluted to a uniform concentration (10 ng/μl) for PCR amplification.

2.3 *PCR assay*

A PCR assay based on the amplification of the published *Wolbachia*-specific sequence primers, such as A and B supergroup *Wolbachia*-specific primers, were used to detect A and B supergroup *Wolbachia* in individual insect pests and silk moths. The *ftsZ* sequence of the A supergroup is *ftsZ Adf* 5′CTC AAG CAC TAG AAA AGT CG-3′; *ftsZ Adr* 5′-TTA GCT CCT TCG CTT ACC TG-3′; and *ftsZ* sequence of the B supergroup is *ftsZ Bf* 5′- CCG ATG CTC AAG CGT TAG AG-3′; *ftsZ Br* 5′-CCA CTT AAC TCT TTC GTT TG 3′. These primers were designed for the detection of *Wolbachia,* which amplifies the gene from 955 to 957 bp (Werren *et al* 1995). This was carried out with PTC 200 of an MJ Research Thermocycler, in a 20 μl reaction mixture containing 1x PCR buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 0.15 mM $MgCl_2$), 0.2 mM dNTPs (each of dATP, dGTP, dCTP and dTTP), 2.5 mM $MgCl_2$, and 0.5 U *Taq* DNA Polymerase (MBI Fermentas, USA), 0.1μM of each forward and reverse primer (Bangalore Genei), 20 ng template DNA and final volume of millipore water to make up 20 μl. The PCR was carried out with a cyclic condition of an initial denaturation step at 94°C for 2 min followed by 40 cycles with a denaturation step at 94°C for 1 min, primer annealing at 55°C for 1 min, primer extension in the presence of *Taq* DNA polymerase at 72°C for 2 min and final extension at 72°C for 5 min for both the primers. The amplified PCR products were separated through 1.3% agarose gel electrophoresis run in 1x TBE (89.2 mM Tris-HCl, 88.9 mM boric acid and 2 mM disodium ethylenediaminetetra acetic acid) buffer for a length of 5–6 cm at a constant of 65 Volts from a power supply unit EP S200V/400 mA (Amersham Pharmacia Biotech USA). The gel was stained with 0.5 μg/ml of ethidium bromide gel just prior to casting. Documentation was done with the gel documentation system.

3. Results and discussion

A total of 40 arthropod species belonging to 10 different orders comprising 20 families were screened for the presence of *Wolbachia* infection, of which 30% were positive for *Wolbachia* (figures 1 and 2, and table 1). Earlier, Werren *et al* (1995) found that 16% of neotropical insect species were positive for *Wolbachia* and West *et al* (1998)

Table 1. *Wolbachia* infection status of insects

Sl. no.	Name of the insect	Race/population/location	Order/family	*Wolbachia* infection status
1	*Exorista* sp.	Laboratory population	Diptera/Tachinidae	AB
2	*Exorista* sp.	Bangalore population	Diptera/Tachinidae	AB
3	*Exorista* sp.	Tumkur population	Diptera/Tachinidae	AB
4	Exorista sp.	Mandya treated	Diptera/Tachinidae	AB
5	Exorista sp.	Kolar population	Diptera/Tachinidae	AB
6	*Exorista* sp.	Kollegal populations	Diptera/Tachinidae	AB
7	*Exorista* sp.	Tamil Nadu population	Diptera/Tachinidae	AB
8	*Exorista* sp.	Andhra Pradesh population	Diptera/Tachinidae	AB
9	*Bombyx mori*	Nistari	Lepidoptera/Bombycidae	–
10	*Bombyx mori*	Kollegal Jawan	Lepidoptera/Bombycidae	–
11	*Bombyx mori*	Mysore Princes	Lepidoptera/Bombycidae	–
12	*Bombyx mori*	Tamil Nadu White	Lepidoptera/Bombycidae	–
13	*Bombyx mori*	Pure Mysore	Lepidoptera/Bombycidae	–
14	*Philosomia recini*	Golden brown race	Lepidoptera/Saturnidae	–
15	*Antheraea mylitta*	Tropical tasar	Lepidoptera/Saturnidae	–
16	*Antheraea proyeli*	Temperate tasar	Lepidoptera/Saturnidae	–
17	*Antheraea assama*	Muga	Lepidoptera/Saturnidae	–
18	*Blepharipa zabina*	Madhya Pradesh	Diptera/Tachinidae	B
19	*Spilosoma oblique*	Bangalore University	Lepidoptera: Arctidae	–
20	*Margaronia pyloalis*	Bangalore University	Lepidoptera: Pyralidae	–
21	*Baris deplanata*	Kodathi	Coleoptera: Curculionidae	–
22	*Leptopterna dolabrata*	Kodathi	Hemiptera: Capsidae	–
23	*Neorthacris acuticeps nilgriensis*	Bangalore University	Coleoptera: Acrididae	–
24	*Hierodulla bipapilla*	Bangalore University	Dictyoptera: Mantidae	–
25	*Sehirus bicolor*	Bangalore University	Hemiptera: Pentotomidae	–
26	*Canthecona* sp.	Mandya	Hemiptera: Pentotomidae	–
27	*Apriona* sp.	Bangalore University	Coleoptera: Cerambycidae	–
28	*Neoperla edmundri*	Devanahalli	Plecoptera: Perlidae	B
29	*Tetranychus* sp.	Devanahalli	Acarina: Tetranychidae	B
30	*Udonga montane*	Devanahalli	Hemiptera: Pentatomidae	B
31	*Osmia lignaria propinqua*	Bangalore University	Hymenoptera: Megachilidae	–
32	*Tetigonia viridissima*	Bangalore University	Orthoptera: Tettigoniidae	–
33	*Adalia decempunctata*	Kodathi	Hemiptera: Coccinellidae	–
34	*Empoasca* sp.	Bangalore University	Hemiptera: Cicadellidae	–
35	*Maconellicoccus* sp.	Kodathi	Hemiptera: Psedococcidae	–
36	*Nesolynx thymus*	Ramanagara	Hymenoptera: Eulophide	–
37	*Saissetia niger*	Kodathi	Hemiptera: Coccidae	–
38	*Aleyrodicus dispersus*	Bangalore University	Homoptera: Aleyrodidae	–
39	*Crtacanthactis ranacea*	Kodathi	Orthoptera:Acrididae	–
40	*Elasmucha grisea*	Kodathi	Hemiptera: Pentotomidae	–

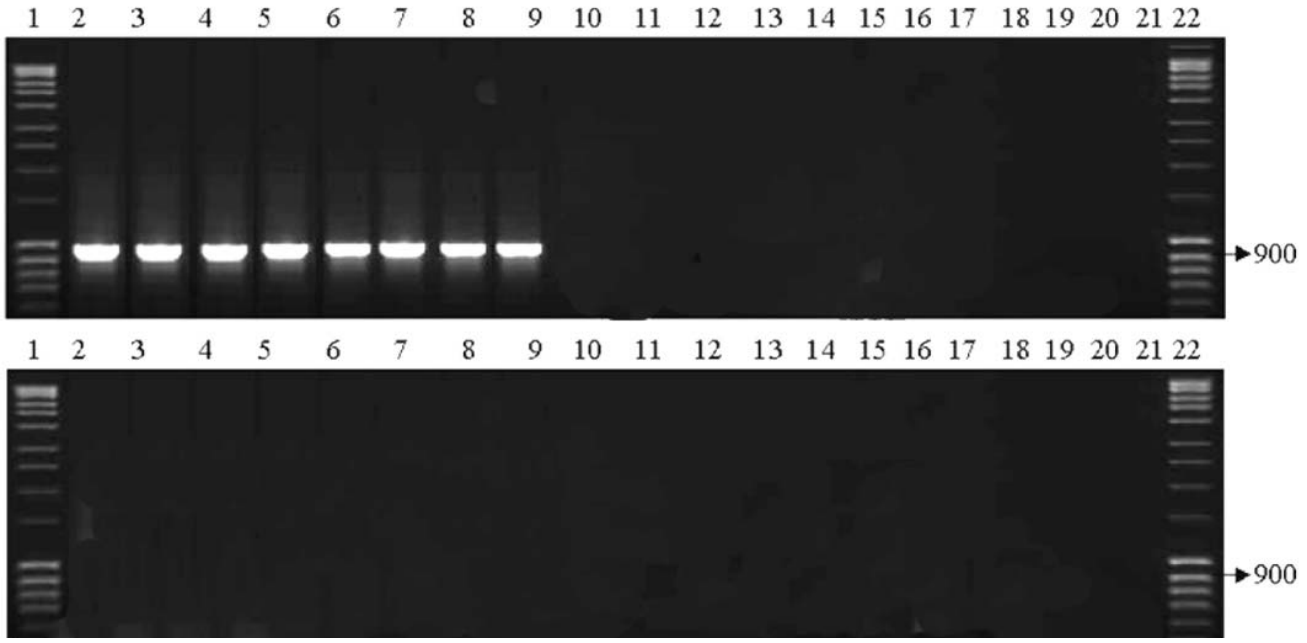

Figure 1. ftsZ A supergroup of *Wolbachia*-specific primer that amplifies at around 955–957 bp from insects in sericulture. Lanes 1 and 22, molecular weight marker; Lanes 2 to 21, insects as shown in table 1 from Sl. no. 1 to 20 (above) and 21 to 40 (below) (arrow in the marker indicates a 900 bp fragment).

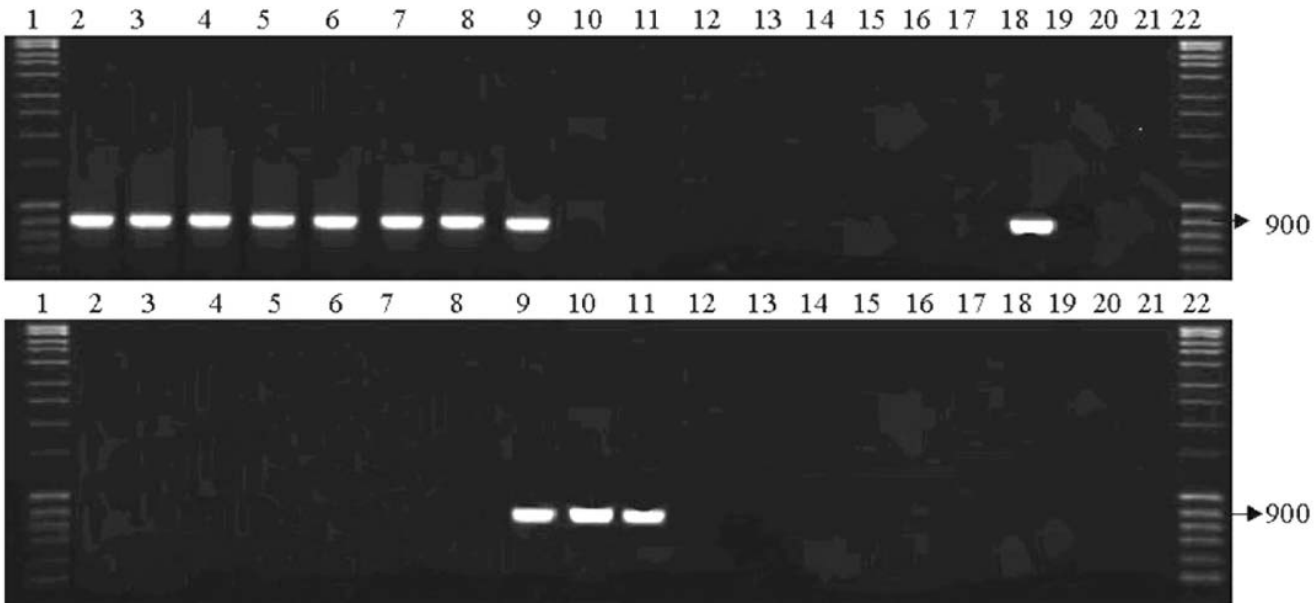

Figure 2. ftsZ B supergroup of *Wolbachia*-specific primer that amplifies at around 955–957 bp from insects in sericulture. Lanes 1 and 22, molecular weight marker; Lanes 2 to 21, insects as shown in table 1 from Sl. no. 1 to 20 (above) and 21 to 40 (below) (arrow in the marker indicates a 900 bp fragment).

found 22% of British insects infected with *Wolbachia,* whereas Jeyaprakash and Hoy (2000) found 76% of insects positive for *Wolbachia* infection. In addition to the earlier reports, in the present study *Wolbachia* were further found in members of the order Diptera, Plecoptera, Acarina and Hemiptera. Amplified *Wolbachia* gene fragments revealed

that 12 species of arthropods tested positive for *Wolbachia,* of which 66.66% were doubly infected with A and B supergroups of *Wolbachia* and another 33.33% were singly infected with B supergroup *Wolbachia,* but infection with A supergroup *Wolbachia* alone was not found.

The overall infection rate due to A plus B and B supergroups was 30% and due to B supergroup *Wolbachia* bacterium alone it was 10%. This indicates that the overall infection frequency with both bacterial types (A and B) was 20%. Werren and Windsor (2000) found 19.3% of insect species positive for *Wolbachia* infection of which 67.9% were singly infected with A supergroup *Wolbachia*; 25.0% were singly infected with B supergroup *Wolbachia* and 7.1% were doubly infected with A and B supergroup *Wolbachia.* All the nine tested Dipteran members in the present study were positive for *Wolbachia* infection. Among them, 88.89% were doubly infected with A and B supergroup *Wolbachia* and 11.11% were singly infected with B supergroup *Wolbachia*. The other orders that were positive for *Wolbachia* infection are Plecoptera (Family: Perlidae), Acarina (Family: Tetranychidae) and Hemiptera (Family: Pentatomidae) wherein only B supergroup *Wolbachia* was amplified. Werren and Windsor (2000) found *Wolbachia* infection in members from major insect orders such as Lepidoptera, Colioptera, Dictyoptera, Hymenoptera and Orthoptera. Further, they demonstrated that a high temperature (above 30°C) was lethal for *Wolbachia.* These findings demonstrate the high infection level with *Wolbachia* among various arthropod species, which can be exploited for pest management through the release of paratransgenic insects into the existing population with the aim of replacing natural populations. Future work on this fastidious microorganism will generate enormous potential for application in the management of agriculturally important insect pests and medically important vectors.

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