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Short communication

Molecular evidence and phylogenetic affiliations of *Wolbachia* in cockroaches ☆

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1. Introduction

Wolbachia is a genus of obligate intracellular bacteria in the Anaplasmataceae family of the α-Proteobacteria that are transmitted through the egg cytoplasm and manipulate reproduction in their hosts in various ways (Werren, 1997). *Wolbachia* are associated with cytoplasmic incompatibility, parthenogenesis, feminization and male killing in arthropods and these aspects have been adequately reviewed recently (Charlat et al., 2003). *Wolbachia* are considered as potent evolutionary force, especially since these also harbor active bacteriophages like WO-A and WO-B, leading even to speciation in arthropods through effects such as reproductive isolation caused by cytoplasmic incompatibility (Shoemaker et al., 1999; Hurst and Werren, 2001; Charlat et al., 2003; Bordenstein and Wernegreen, 2004; Jaenike et al., 2006). *Wolbachia* are presently known from a large variety of arthropods and a few species of nematode worms.

Among arthropods, *Wolbachia* are known to be widely distributed in insects; the other groups being mites, spiders and terrestrial Crustacea (or Isopoda). In a survey of *Wolbachia* in different groups of insects from Panama, Werren et al. (1995) showed that over 16% of the species are infected. In a subsequent survey of temperate North American insects, Werren and Windsor (2000) found over 19% of the insect species to be infected with this endosymbionts. A further report by Jeyaprakash and Hoy (2000), using 'long PCR' modification, demonstrated that *Wolbachia* is present in over 76% of the arthropods tested. A survey of Japanese Lepidoptera (9 families and 49 species) revealed that almost 45% of Lepidoptera are infected (Tagami and Miura, 2004), while a similar survey of Malaysian ants indicated that 25 out of 50 (i.e., 50%) ant species are harboring *Wolbachia* (Wenseleers et al., 1998).

Apart from the above two groups (orders) of insects, many other insect groups are known to carry *Wolbachia*: thus it is known in wingless insects like springtails (Vandekerckhove et al., 1999), leafhoppers, thrips and whiteflies (Nirgianaki et al., 2003), termites (Bandi et al., 1997; Lo et al., 2002; Bordenstein and Rosengaus, 2005), beetles (Werren and Windsor, 2000; Nirgianaki et al., 2003); odonates or dragon and damselflies (Thipaksorn et al., 2003), and crickets (Komoda et al., 2000). In dipteran insects, especially mosquitoes, *Wolbachia* infections are known for a long time (Hertig and Wolbach, 1924 as cited by Bordenstein (2003)) and much work on drosophilids and tephritid flies have revealed that the latter are sometimes infected with as many as five distinct strains of *Wolbachia* (Jamnongluk et al., 2002).

Since *Wolbachia* are so widely spread in arthropods, especially in insects, and since insects form a vast proportion of biodiversity, it is no wonder that *Wolbachia* are perhaps the most abundant and globally distributed bacterial endosymbionts, as has been recently pointed out (Bordenstein, 2003).

In spite of global distribution, there is only a single formally named species, namely *Wolbachia pipientis* from *Culex* mosquito. Due to different divergent lineages that were subsequently discovered, it is now customary to refer *Wolbachia* to one or the other "Supergroup" (see Lo et al., 2002). There are now eight different supergroups of *Wolbachia* labeled A–H: of these A and B supergroups are present in arthropods (Werren et al., 1995a), C and D are found only in filarial nematode worms so far (Bandi et al., 1998),

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E is known only from springtails (Vandekerckhove et al., 1999; Czarnetzki and Tebbe, 2004), F exists in termites, crickets (Panaram and Marshall, in press), bedbugs (Sakamoto et al., 2006), louse (Covacin and Barker, 2007) and filarial parasite, *Mansonella ozzardi* (Casiraghi et al., 2001; Lo et al., 2002). Subsequently a lineage of *Wolbachia* outside of A–F supergroups was discovered in Australian spiders by Rowley et al. (2004) and was placed in new supergroup G, while another lineage in termites of the genus *Zootermopsis* is placed in supergroup H (Bordenstein and Rosengaus, 2005). In spite of these, there are some *Wolbachia* which are not placed in any existing supergroups, for example those from cat flea *Ctenocephalides felis*, and filarial nematode *Dipetalonema gracile* (Casiraghi et al., 2005). The phylogeny of all these groups is being investigated by many and it is still incompletely understood; however all these *Wolbachia* form a monophyletic group.

In spite of enormous work and large number of insect species sampled, the only cockroach (*Percoblatta* sp.) that was tested turned out to be without *Wolbachia* infection (Werren and Windsor, 2000). There is no report of *Wolbachia* infection in any species of cockroach so far. As a part of work to sample relatively different tropical insect taxa to detect *Wolbachia*, we have started screening various insects from India. Earlier, in India *Wolbachia* has been shown in some insect pests of rice (Behera et al., 2001), in *Wuchereria bancrofti* microfilariae (Hoti et al., 2003) and insect pests of sericulture (Prakash and Puttaraju, 2006).

In this short communication, we intend to show that 2/5 species of cockroach species tested are positive for *Wolbachia*. Secondly, these *Wolbachia* harbor WO phage. Thirdly, we also show that in 16S, *ftsZ* based phylogeny, these cockroach *Wolbachia* are closer to F supergroup while in *wsp* based phylogeny, the placement is unresolved. Further, we also show that these cockroaches also co-harbor *Blattabacterium*. We used multigene approach by PCR amplifying three genes because 16S rRNA gene does not adequately resolve fine scale phylogeny in *Wolbachia* strains and a faster gene like cell cycle gene *ftsZ* and even faster evolving surface-protein gene *wsp*, are being used to improve phylogeny (Zhou et al., 1998) and this approach has also been advocated by others (see Bordenstein and Rosengaus, 2005). The trees drawn are unrooted as Casiraghi et al. (2005) and Bordenstein and Rosengaus (2005) have categorically stated that most outgroups like *Anaplasma marginale* and *Ehrlichia ruminantium* are extremely divergent from *Wolbachia* and hence those have not been useful in resolving basal relationship among supergroups (see also Lo et al., 2002 for additional discussion).

2. Materials and methods

2.1. Insects

Cockroaches were collected during 2005 from Pune, India. Five species of cockroaches (namely *Pycnoscelus surinamensis*, *Periplaneta americana*, *Blattella* sp., *Blattella nipponica*, and *Supella longipalpa*), males and females (minimum five each, except for *P. surinamensis* in which only females are known) from each species, were collected for this study. Small cockroaches were subjected to whole-body extraction (excluding wings), whereas large cockroaches were dissected to obtain gonads (some amount of fat was always associated) in PBS (137 mM NaCl, 7.8 mM Na_2HPO_4, 2.7 mM KCl, and 1.47 mM KH2PO4, pH 7.4). DNA was extracted from tissue using the QIAamp®DNA Mini Kit (QIAGEN®) following the manufacturer's instructions.

All PCR products were purified using PEG–NaCl method (Sambrook et al., 1989) and were sequenced with respective primers using an automated sequencer (3730 DNA analyser, ABI, Hitachi).

2.2. Detection of Wolbachia

PCR was performed in a 25 μL reaction mixture using three primer sets separately for amplification of *Wolbachia ftsZ* gene, *ftsZ* F (5′-GTT GTC GCA AAT ACC GAT GC-3′) and *ftsZ* R (5′-CTT AAG TAA GCT GGT ATA TC-3′) (Werren et al., 1995a), *Wolbachia* outer surface protein (*wsp*) wsp81 (5′-TGG TCC AAT AAG TGA TGA AGA AAC-3′) and wsp691R (5′-AAA AAT TAA ACG CTA CTC CA-3′) (Braig et al., 1998), 16S rRNA gene of *Wolbachia* 16wol F (5′-TTG TAG CCT GCT ATG GTA TAA CT-3′) and 16wol R (5′-GAA TAG GTA TGA TTT TCA TGT-3′) (O'Neill et al., 1992). Reactions contained 2 μL of the template DNA lysate, 10 pmol of each primer, 1.5 mM dNTP, and 0.5 μL of Taq (NEB) with a final $MgCl_2$ concentration of 1.5 mM in a total volume of 25 μL. PCR cycling conditions for all reactions were 5 min at 94 °C, 35 cycles (30 s at 94 °C, 30 s at 55 °C, 1 min at 72 °C) and 5 min at 72 °C.

Samples were also subjected to PCR using primers specific for insect mitochondrial 16S rDNA, 16s insF (5′-TTA CGC TGT TAT CCC TTA-3′) and 16s insR (5′-CGC CTG TTT ATC AAA AAC AT-3′) (Kambhampati, 1995). The PCR conditions were an initial denaturation step of 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 1 min, and 72 °C for 1.5 min. Homologous sequences from GenBank were searched using BLAST at NCBI.

2.3. Bacteroidetes symbionts

Primer pair which includes ChR (5′-GTG GAT CAC TTA ACG CTT TCG-3′) (Zchori-Fein and Perlman, 2004.) to target *Bacteroidetes* symbionts including *Blattabacterium* and 16F27 (5′-CCA GAG TTT GAT CMT GGC TCA G-3′) (Weisburg et al., 1991) to amplify 16S rRNA gene from all known bacteria was used to obtain a larger segment of the 16S rRNA gene using the parameters described as below. PCR parameters were: denaturation for 2 min at 95 °C, followed by 30 cycles of 30 s at 92 °C, 30 s at 57 °C, 30 s at 72 °C and a 5-min final extension at 72 °C.

2.4. Detection of Wolbachia phage (WO)

The putative phage capsid protein gene (*orf7*) encoded on the prophage WO was PCR amplified with the primers phgWOF (5′-CCC ACA TGA GCC AAT GAC GTC TG-3′) and phgWOR (5′-CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC-3′) as listed by Masui et al. (2000). The PCR conditions were 94 °C for 3 min followed by 35 amplification cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min, and finally 72 °C for 5 min.

2.5. Phylogenetic analysis

Wolbachia 16S rRNA, *ftsZ, wsp*, WO phage *orf7* gene and *Blattabacterium* 16S rRNA gene sequences generated in this study were aligned with homologous sequences deposited in GenBank comprising of supergroups A–H. All sequences were aligned unambiguously and manually edited using ClustalW (Higgins et al., 1994) and DAMBE (Xia and Xie, 2001). All uninformative sites were removed from further analysis. Phylogenetic analyses were performed using Bayesian inference, maximum likelihood (ML), and maximum parsimony (MP) methods for each dataset.

For Bayesian inference of phylogeny, the program MrBayes 3.0 (Huelsenbeck and Ronquist, 2001) was used. The analysis for each gene consisted of 3,000,000 generations. An appropriate model of sequence evolution for each data set were chosen via Akaike Information Criterion (AIC) using program MrModeltest 2.2 (Nylander, 2002). The selected models were as follows: (GTR+I+G) for *Wolbachia* 16S rRNA, *ftsZ*, *wsp* and *Blattabacterium* 16s rRNA gene fragments; (HKY+G) model for WO phage *orf7* gene. Trees were sampled for every 100 generations. First 3000 trees (10%) were discarded as burn in. Bayesian posterior probabilities were calculated using 50% majority rule consensus. Three independent runs were performed for each dataset.

ML and MP analysis was performed using PAUP* 4.0b10 (Swofford, 1998). For ML analysis appropriate model was selected using AIC in Modeltest 3.7 (Posada and Crandall, 1998). The models used for each dataset were as follows: (GTR+I+G) for *Wolbachia* 16S rRNA and WO phage *orf7* gene fragment; (TIM+I+G) for *Wolbachia ftsZ* gene fragment; (TVM+I+G) for *Wolbachia wsp* and *Blattabacterium* 16s rRNA gene fragment. ML heuristic search was performed with 10 random taxon addition replicates, tree-bisection and reconnection (TBR) branch swapping algorithm and 100 bootstrap replicates. MP heuristic search was performed using branch and bound search, 10 random taxon additions per replicate, TBR branch swapping algorithm and 100 bootstrap replicates with all characters weighted equally.

3. Results and discussion

All the sequences generated during this study have been deposited in the GenBank database and Accession numbers are as follows: for *wsp* gene, *Blattella* sp. (DQ354917, EF193197) and *S. longipalpa* (DQ354918, EF193198), for *ftsZ* gene, *Blattella* sp. (DQ457400, DQ457401) and *S. longipalpa* (DQ457402, DQ457403) for 16S rRNA gene, *Blattella* sp. (DQ354919, EF193196) and *S. longipalpa* (DQ354920, EF193195) for *Blattabacterium* 16S rRNA (EF423763 to EF423768) and finally for WO phage *orf7*gene *Blattella* sp (EF193194) and *S. longipalpa* (DQ354921). Only two cockroaches from each species tested positive for *Wolbachia*. Thus, the prevalence was 2/10 for each species tested. For WO infection, only one individual/specimen of each species yielded sequences. We did not check for multiple infections of *Wolbachia*.

PCR assay using primers for *wsp* and *ftsZ* gave expected amplification product of 650 bp and 1000 bp, respectively, from *Blattella* sp. and *S. longipalpa*. The other cockroach spp. such as *B. nipponica*, *Periplaneta americana, P. surinamensis* showed no PCR amplification products. These results suggested that only two of the five cockroach species are infected with *Wolbachia*. These results were further confirmed by PCR amplification using primers for *Wolbachia* specific 16S rRNA. PCR products of expected size of 900 bp were obtained from *Blattella* sp. and *S. longipalpa* but no amplification product was obtained from the other species of cockroaches.

PCR amplification product of 415 bp was obtained with primers specific for insect mitochondrial 16S rRNA from all DNA samples. The nucleotide sequences generated with these PCR products helped in further taxonomic confirmation of cockroach species involved. PCR amplification was observed in all the species of cockroaches tested using primers designed to target *Bacteroidetes* symbionts. All the sequences exhibited high sequence similarity with *Blattabacterium* as shown in Fig. 1. It is evident from the placement of cockroach endosymbionts under study that these are *Blattabacterium* as they are clustering with the known *Blattabacterium* sequences in the database.

In Fig. 2, we show the phylogenetic relationship of cockroach *Wolbachia* based on 16S, *ftsZ* and *wsp* genes. 16S rRNA gene sequence of *Wolbachia* from *Blattella* and *Supella* that we determined in our study showed 98% similarity to the *Wolbachia* endosymbiont of termite *Kalotermes flavicollis* (Y11377) (Lo et al., 2002).

In the phylogenetic analysis with the 16S rRNA genes, independent of the method for tree reconstruction, the two cockroach *Wolbachia* sequences clustered as a sister clade in supergroup F. Phylogenetic trees with the *ftsZ* genes, independent of the tree construction method, also led to similar tree topologies as found with the 16S rRNA genes. In all the trees, cockroach *Wolbachia* sequences are close to the supergroup F clade with high bootstrap and posterior probability support.

Phylogenetic trees inferred from the *wsp* gene however showed different topology with respect to placement of cockroach *Wolbachia*. Here, it is close to supergroup A. One possible explanation can be that because there is only

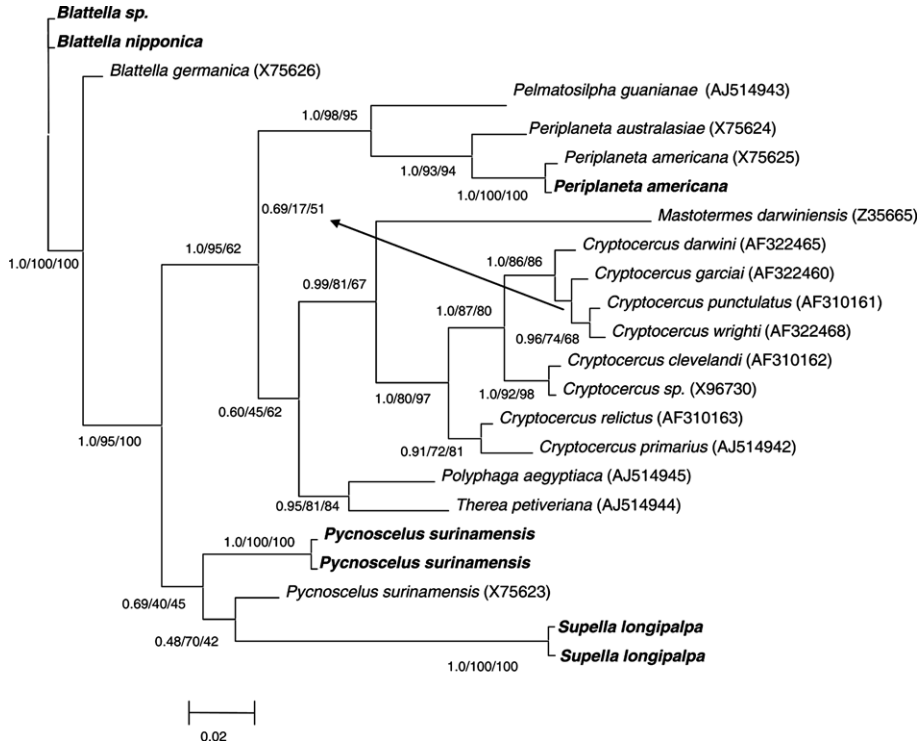

Fig. 1. Unrooted phylogenetic relationships between *Blattabacterium* from our cockroaches samples (bold) and those from other cockroaches in the GenBank, based on 16S rRNA. Names are those of the host species. The topology was inferred using the program Mr.Bayes, with the GTR+G+I model of sequence evolution for each gene. Levels of confidence for each node are shown in the form of posterior probabilities (PP; Bayesian analysis). Trees inferred from maximum likelihood and maximum parsimony using PAUPb10 program were similar though less resolved (data not shown). Bootstrap values obtained from maximum likelihood and maximum parsimony are shown under PP. Accession numbers are shown after each species name in parenthesis. Scale bar represents substitutions per site.

one *wsp* sequence from supergroup F (bedbug). Another possible reason is *wsp* has hyper-variable regions.

The expected 394 bp amplification product was observed from DNA samples of *S. longipalpa and Blattella sp*., indicating presence of WO phage in these samples. The species that did not have *Wolbachia* were also negative for WO phage. WO phage phylogeny based on *orf7* gene placed the cockroach *Wolbachia* in WO-B group clade (Fig. 2). Presence of bacteriophage in endosymbionts is an interesting fact recently discovered and this is the first ever report of WO phage in any supergroup F *Wolbachia*. It has been pointed out by Bordenstein and Wernegreen (2004) that mobile elements like WO can figure prominently in promoting recombination, just as it does in *Escherichia coli* bacteriophages. Strains of distantly related A and B group *Wolbachia* which co-inhabit single host share identical *orf7* sequences indicating extensive lateral transfer and, therefore, it has been stated that active phage in *Wolbachia* can be a genetic tool to engineer *Wolbachia* for biocontrol (Bordenstein et al., 2006). Role of mobile elements in evolution is also reviewed by Hurst and Werren (2001).

Wolbachia of supergroup F are known in divergent groups such as termites, bedbugs, crickets, louse and nematodes but there is no satisfactory explanation how nematodes and insects come to possess related *Wolbachia*. Recently, while discussing the phylogeny of *Wolbachia* in F group, Casiraghi et al. (2005) have stated that clustering of *Wolbachia* spp. From insect and nematode host suggest that an independent horizontal transfer of the bacteria between these host phyla might have occurred recently. Transfer of *Wolbachia* can happen between predators and prey, as indicated by closely related *Wolbachia* strains in parasitoids and their hosts (Werren et al., 1995a) but similar transfer cannot be expected in nematodes and insects. Is it likely that nematode *Wolbachia* were passed on to vector insects like mosquito that are known to transmit filariasis?

All the samples of cockroach species were found to harbour *Blattabacterium*. Thus two cases of double infection with *Wolbachia* and *Blattabacterium* were observed in roaches *Blattella* sp. and *S. longipalpa*.

It is now widely recognized that symbiotic microorganisms play a crucial role in the ecology and evolution of their hosts and the discovery *Wolbachia* in cockroaches will further help understanding the phylogeny of *Wolbachia*.

To summarize: the present investigation reports (1) presence of *Wolbachia* in cockroaches for the first time (2) first detection of WO phage in *Wolbachia* strains from two different cockroach species, viz *S. longipalpa and Blattella* sp. (3) coexistence of intracellular bacteria, *Wolbachia* and *Blattabacterium* in cockroaches. (4) Phylogenetic analysis revealing the fact that *Wolbachia* from cockroaches forms a sister clade with supergroup F.

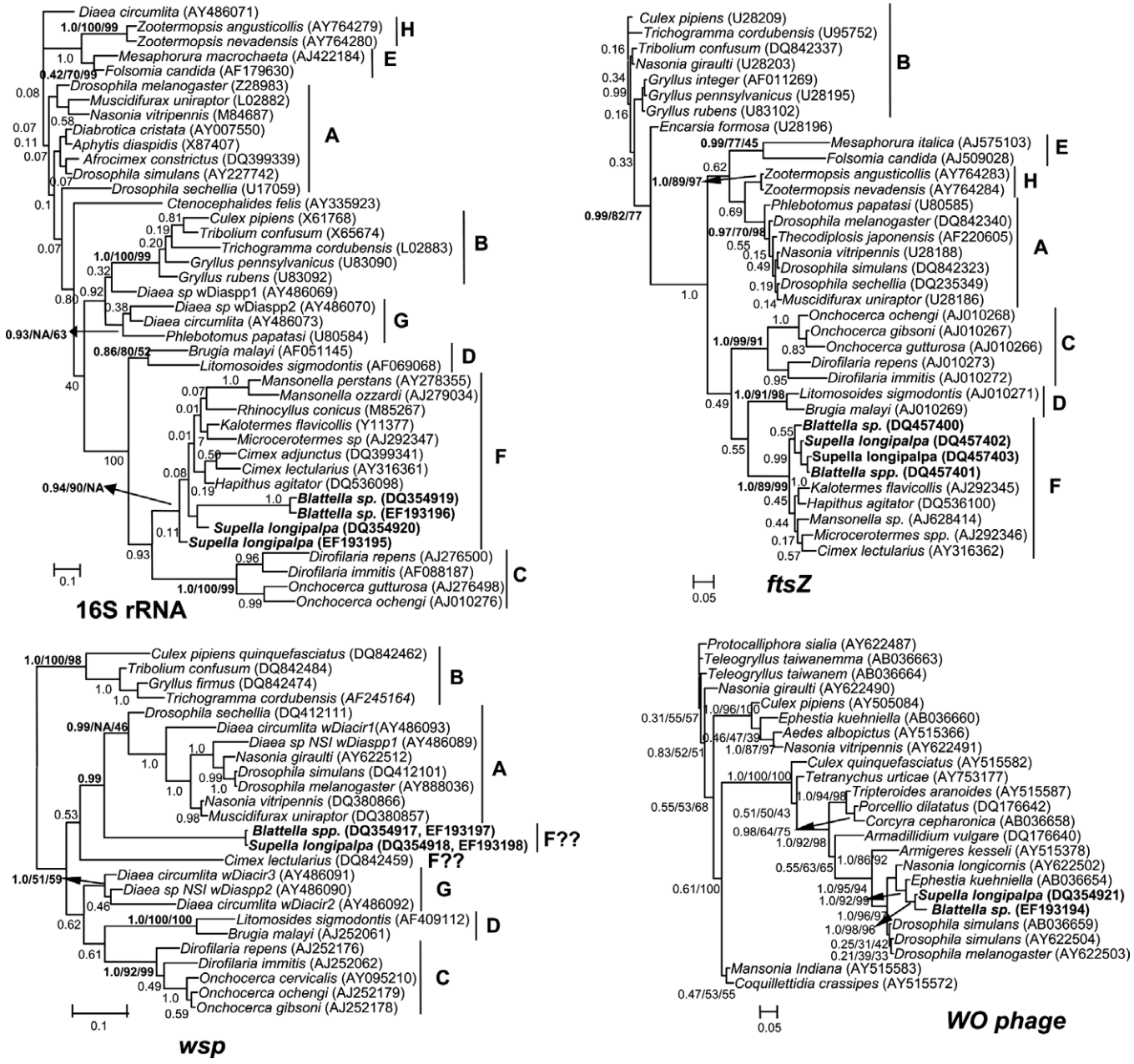

Fig. 2. Unrooted phylogenetic relationships between *Wolbachia* from cockroaches (bold) and those infecting other organisms, based on 16S rRNA, *ftsZ* and *wsp* genes. WO phage refers to *orf7* gene of WO phage from there specific host. Names are those of the host species. The topology was inferred using the program Mr.Bayes, with following nucleotide substitution models.(GTR+I+G) for *Wolbachia* 16S rRNA, *ftsZ*, *wsp* gene fragments; (HKY+G) for WO phage *orf7* gene fragment. Levels of confidence for each node are shown in the form of posterior probabilities (PP; Bayesian analysis). Trees inferred from maximum likelihood and maximum parsimony using PAUPb10 program were similar though less resolved (data not shown.) Bootstrap values obtained from maximum likelihood and maximum parsimony are shown after PP, respectively (only for supergroup clade support). Accession numbers are shown after each species name in parentheses. Supergroups are shown to the right side of the host species names (except for WO phage). Scale bar represents substitutions per site.

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