Presence of *Wolbachia* in Insect Eggs Containing Antimicrobially Active Anthraquinones

Abstract. *Wolbachia* are obligatory, cytoplasmatically inherited α-proteobacteria which are common in arthropods where they may cause reproductive abnormalities. Many insects are well-known to protect themselves from deleterious microorganisms by antibiotic components. In this study, we addressed the question whether *Wolbachia* are able to infect insects containing antimicrobially active anthraquinones and anthrones, and if so, whether these genotypes of *Wolbachia* comprise a monophyletic cluster within one of the known supergroups. Leaf beetles of the taxon Galerucini (Galerucinae) are known to contain 1,8-dihydroxylated anthraquinones and anthrones. Also the scale insect *Dactylopius* contains an anthraquinone glycoside, carminic acid. Our analyses revealed that a representative of the Galerucini, *Galeruca tanaceti*, and *Dactylopius* are indeed infected by endosymbiotic *Wolbachia* bacteria. Phylogenetic analysis of the *wsp* and *ftsZ* genes of these bacteria revealed that strains in *G. tanaceti* cluster in supergroup A, while those present in *Dactylopius* are distinctive from each other and from those of *G. tanaceti*. They are clustering in supergroups A and B. *Wolbachia* strains present in close, but anthraquinone-free relatives of *G. tanaceti* were shown to belong also to supergroup A. From these results we can conclude (1) a double infection in *Dactylopius*, (2) that the presence of antimicrobially active compounds such as anthraquinones does not necessarily protect insects from infection by *Wolbachia* and (3) that genotypes of *Wolbachia* infecting anthraquinone-containing insects most likely do not comprise a unique genotype. These results show that *Wolbachia* bacteria might be adapted to cope with conditions usually detrimental to other bacteria and that these adaptations are widespread among *Wolbachia* supergroups.

Keywords. *Wolbachia*; anthraquinones; antimicrobial activity; leaf beetle; insect egg; *Galeruca tanaceti*

Introduction

Wolbachia (Rickettsiales) are obligatory intracellular, Gram-negative α-proteobacteria maternally transferred with the cytoplasm of the egg. An infection with these bacteria can cause different reproductive abnormalities in the host, like induction of parthenogenesis, male killing, cytoplasmatic incompatibility and feminisation (for reviews see: O'Neill et al. 1997; Stouthamer et al. 1999). These reproductive abnormalities have intensively been discussed also with respect to their impact on speciation processes in insects (e.g., Werren 1998; Koukou et al. 2006). *Wolbachia* have been detected in a broad range of insects, filarial nematodes, crustaceans, mites (Stouthamer et al. 1999) and spiders (Oh et al. 2000). Recent molecular studies using PCR amplification of the *wsp* gene revealed that *Wolbachia* are present in up to 76% of all tested species (Jeyaprakash and Hoy 2000), indicating that *Wolbachia* are more widely distributed than previously expected. Thus, *Wolbachia* seem to be very successful in exploitation of hosts. However, to date, little knowledge is available on the ability of *Wolbachia* to infect arthropods with eggs containing antimicrobially active components.

A first aim of this study was to analyse whether *Wolbachia* are able to infect eggs of leaf beetles of the tribe Galerucini (subfamily Galerucinae) that contain antimicrobially active anthraquinones (chrysophanol, chrysazin) and their precursors, the anthrones chrysarobin and dithranol (Cudlin et al. 1976; Howard et al. 1982; Hilker and Schulz 1991; Manojlovic et al. 2000; Izhaki 2002). These components are not sequestered by the beetles from their host plants, but instead are obviously produced in the insects. Anthraquinones and anthrones do not occur just sporadically in some eggs, but are present in all eggs investigated so far of 9 Galerucini species (Hilker et al. 1992; Kunze et al. 1996; Pankewitz and Hilker 2006). In addition to their antimicrobial activity, these components deter predators like ants and birds from feeding (Howard et al. 1982; Eisner et al. 1994; Hilker and Köpf 1995; Blum and Hilker 2002). Effects of anthraquinones and anthrones on endosymbiotic bacteria in insects or on α-proteobacteria other than *Wolbachia* have not been studied so far.

Chrysophanol and also structurally very similar anthraquinones like emodin and physcion have been proved to inhibit growth of free living Gram-negative bacteria (Le Van 1984; Manojlovic et al. 2000; Kambizi et al. 2004). For example, the anthraquinone emodin is

toxic against several bacteria in a very low concentration of 10-200 µg ml^{-1} (Le Van 1984). The concentrations of anthraquinones detected in the eggs of *G. tanaceti* (Pankewitz and Hilker 2006) are within the range of antibiotic activity (Le Van 1984; Kambizi et al. 2004). Anthraquinones, like many other antibiotics, do not show a general antibacterial activity. The anthraquinone chrysazin present in Galerucini has an antimicrobial effect on two Gram-positive bacteria (*Bacillus subtilis*, *B. cereus*), whereas no effect was found towards Gram-negative *E. coli* (Cudlin et al. 1976). The anthraquinone chrysophanol is present in about 30 fold higher concentrations than chrysazin in the eggs of the Galerucini species *G. tanaceti* (Pankewitz and Hilker 2006). Chrysophanol inhibits some Gram-positive (*B. subtilis*, *Staphylococcus epidermis*) and Gram-negative bacteria (*E. coli*), but has no antimicrobial effect against closely related species like *B. cereus*, *S. aureus* and the proteobacterium *Shigella sonnei* (Kambizi et al. 2004). Other anthraquinones like carminic acid are present in the scale insect *Dactylopius* within the range of such concentrations that display cytostatic activity (Eisner et al. 1980; Gálvez et al. 1996). Up to date it is unknown whether these compounds are effective against intracellular bacteria.

Given that *Wolbachia* are present in anthraquinone-containing insect eggs, a further aim was to study the hypothesis that these *Wolbachia* represent a specific, anthraquinone-resistant genotype within the phylogenetic tree of *Wolbachia*. Based on molecular analysis of 16S rDNA, the *ftsZ* and *wsp* genes, six so-called *Wolbachia* supergroups (A-F) can be differentiated (Lo et al. 2002; Czarnetzki and Tebbe 2004). While the product of the *ftsZ* gene is involved in the control of cell division (Holden et al. 1993), the latter gene is encoding a major cell surface protein (Braig et al. 1998; van Meer et al. 1999).

To test our hypothesis, first we searched for the presence of *Wolbachia* in the following insect species by targeting on the *ftsZ* and *wsp* genes: (1) *Galeruca tanaceti*, a leaf beetle species belonging to the tribe Galerucini and containing anthraquinones (chrysophanol, chrysazin) and anthrones (chrysarobin, dithranol), (2) *Xanthogaleruca luteola*, a close relative of *G. tanaceti* belonging to the same tribe and containing the same anthraquinones and anthrones, (3) *Agelastica alni*, a leaf beetle species of the tribe Sermylini that is closely related to Galerucini, but eggs of this species do not contain any anthraquinones (negative control species) and (4) a distantly related species to *G. tanaceti*, the scale insect *Dactylopius* that contains the anthraquinone glycoside carminic acid in all developmental stages (Eisner et al. 1980). Second, the 16S rDNA, the *ftsZ* and *wsp* gene sequences of

Wolbachia found in the species studied here were subjected to a phylogenetic analysis by comparison with *Wolbachia* strains in 46 (16S rDNA), 48 (*ftsZ*) and 69 (*wsp*) other arthropod species. We used sequences of the respective *Wolbachia* genes presented by Shoemaker et al. (2002) and Czarnetzki and Tebbe (2004).

Materials and Methods

Insects

Beetles of *Galeruca tanaceti* and *Agelastica alni* were collected near Berlin, Germany and kept in perforated plastic boxes in the laboratory at 20°C and 16 h light/ 8 h darkness until oviposition. *A. alni* was fed with leaves of alder trees, while *G. tanaceti*, a polyphagous species, was provided with leaves of Chinese cabbage and *Achillea millefolium*. The eggs laid in the laboratory were collected and stored at –20°C until preparation. Eggs of the elm leaf beetle (*Xanthogaleruca luteola*) were obtained from adult beetles collected near Montpellier, France and kept frozen under the same conditions as mentioned above. The scale insect *Dactylopius* spec. was collected from its host plant *Opuntia* spec. at the Canarian island Lanzarote, Spain and stored in 96% ethanol.

DNA extraction

The hardened, dark extrachorion covering the egg masses of *G. tanaceti* was removed manually prior to DNA extraction of eggs. Egg masses of *A. alni* and *X. luteola* do not possess such an extrachorion. Prior to DNA extraction, 30-50 eggs of each species were surface-sterilised with 10% sodium hypochlorite for 1 min and rinsed twice with 2 ml sterile water. The GenomicPrep Cells and Tissue DNA Isolation Kit (Amersham Biosciences, Buckinghamshire, UK) was used. Eggs were crushed with a sterile glass pestle. Glass beads (0.5 mm; BioSpec Products, Bartlesville, USA) were used to completely destroy all cells. All buffer solutions were provided with the kit. After vigorous mixing eggs and buffer, the mixture was incubated for 1 h at 65°C. A proteinase K digestion was incubated over night. Protein precipitation was conducted twice according to the instruction manual of the kit by using 100 µl of the protein precipitation solution for the second step. DNA was stored at -20°C. Extraction of DNA from the scale insect was done with the same protocol except the first washing step with 10% NaOCl.

The number of different samples analysed for the presence of *Wolbachia* were $N = 5$ for *G. tanaceti*, $N = 5$ for *X. luteola*, $N = 5$ for *A. alni* and $N = 3$ for *Dactylopius* spec..

PCR and sequencing

All primers were obtained from Tib Molbiol, Berlin, Germany. All PCR reactions were performed in 50 µl volume containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 200 µM concentrations of each deoxynucleotide triphosphate (dNTP) and 1 U *Taq* polymerase (AmpliTaq, Applied Biosystems, Roche, Canada) with minor changes in the salt concentrations mentioned for the respective primer pairs.

An approximately 0.9 kb fragment from the 16S rDNA gene was amplified using the slightly modified primers 99f (5´-TTG TAG CYT GCT ATG GTA TAA CT-3´) and 994r (O'Neill et al. 1992) specific for *Wolbachia*. Amplifications were done as described above with additional 1 mM $MgCl_2$, 30 pmol of each primer and 10 ng of template DNA. The following cycling conditions were used: initial denaturation for 5 min at 95°C followed by 30 cycles with 95°C for 1 min, 58°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min.

To amplify a 0.6 kb fragment of the bacterial *wsp* gene, the primer pair *wsp*F and *wsp*R (Kondo et al. 2002) was used. Amplification reactions were performed as described before with additional 1.5 mM $MgCl_2$, 50 pmol of each primer and 30 ng of template DNA. The PCR cycling conditions were as described by Kondo et al. (2002) with a final extension step at 72°C for 10 min.

For amplifying the *ftsZ* gene, two different primer pairs were used. For PCR with DNA from *A. alni* and *Dactylopius* spec. the primers *ftsZf1* and *ftsZr1* (Werren et al. 1995b) amplifying a 1 kb fragment were used. Amplification reactions were performed as mentioned above with 25 pmol of each primer and 30 ng of template DNA. The cycling conditions were as follows: initial denaturation for 3 min at 94°C followed by 35 cycles with 94°C for 1 min, 55°C for 1 min, 72°C for 1.5 min and a final extension step at 72°C for 10 min. For the amplification of the *ftsZ* gene from *Wolbachia* bacteria present in *G. tanaceti* a second primer pair, *fts*F and *fts*R (Kondo et al. 2002), generating a DNA fragment of 0.75 kb was used. PCR reaction was done with the general protocol with additional 1.5 mM $MgCl_2$, 50 pmol of each primer and 30 ng of template DNA. The

cycling conditions were as follows: initial denaturation for 4 min at 95°C followed by 35 cycles with 95°C for 1 min, 50°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 10 min.

PCR products were ligated and transformed using the TOPO TA cloning kit (Invitrogen, Carlsbad, California) following the instruction manual. Up to 10 white colonies were picked and the insert was directly amplified with M13 primers. Plasmids with an insert according to the expected size were chosen for sequencing on a 3130x Genetic Analyser (Applied Biosystems, ABI-Hitachi).

Phylogenetic analysis

For sequence assembling, the software program Chromas 1.55 (Technelysium Pty Ltd., Australia) was used. Sequences of all three *Wolbachia* genes analysed here (from *Wolbachia* found in *G. tanaceti*, *A. alni* and *Dactylopius* spec.) were included in the existing alignments presented by Czarnetzki and Tebbe (2004) and Shoemaker et al. (2002). For the 16S rDNA, the *ftsZ* and the *wsp* genes fragments of 880 bp, 632 bp and 509 bp in lengths were used. Due to a high variability and therefore problems for a confident alignment, the third hypervariable region of the *wsp* gene was excluded (Zhou et al. 1998). For all three genes, trees based on neighbour-joining (NJ) method, maximum likelihood (ML) and maximum parsimony (MP) were constructed using PAUP 4.0b10 (Swofford 2000). Distance matrixes were calculated using the Kimura-2-parameter model. For bootstrap analysis, 100 replicates were chosen for the NJ and MP trees. The proteobacterium *Anaplasma marginale* was used as an outgroup for the 16S rDNA and the *ftsZ* alignment. The *wsp* gene tree was midpoint-rooted.

Results

Detection and sequencing of Wolbachia *specific genes*

PCR with primers *ftsZf1* and *ftsZr1* (Werren et al. 1995b) yielded no PCR product with template DNA of *Wolbachia* in the anthraquinone-containing beetle eggs of *G. tanaceti*. Therefore the alternative primer pair *fts*F and *fts*R (Kondo et al. 2002) was chosen. With these primers a partial region of the targeted gene with the PCR product size (approx. 750 bp) that was expected by selected primer pairs was amplified. In the course of the

cloning procedure for each of the three genes, two distinctive nucleotide sequences were found only for *Wolbachia* in the anthraquinone-containing scale insect, *Dactylopius* spec..

Wolbachia were detected both in anthraquinone-containing eggs of the tansy leaf beetle *G. tanaceti* (Galerucinae, tribe Galerucini) and in anthraquinone-free eggs of the alder leaf beetle *A. alni* (Galerucinae, tribe Sermylini). They were found in all samples analysed ($N = 5$ in *G. tanaceti* and $N = 5$ in *A. alni*). While the three *Wolbachia* specific genes (16S rDNA, *ftsZ* and *wsp*) were amplified from anthraquinone-containing eggs of the tansy leaf beetle *G. tanaceti*, no *Wolbachia* were detected in the anthraquinone-containing eggs of a galerucine species of the same tribe, the elm leaf beetle *X. luteola* (Galerucinae, tribe Galerucini). In the anthraquinone-rich scale insect *Dactylopius* spec., *Wolbachia* specific PCR products were also detected for each of the three genes in all samples analysed ($N = 3$).

Phylogenetic analysis using 16S rDNA, fts*Z and* wsp *genes*

The phylogenetic tree based on the 16S rDNA gene analysis revealed no clear differentiation and no significant bootstrap support, especially for separation of the supergroups A and B (data not shown).

However, well supported taxonomic groups (100% bootstrap values for A-F) were obtained when the faster evolving *ftsZ* gene was used (Fig. 1). As evident from the gene genealogies of *ftsZ* and *wsp*, *Wolbachia* present in the galerucine beetles *G. tanaceti* (anthraquinone-containing) and *A. alni* (anthraquinone-free) cluster among members of the supergroup A (Fig. 1 and 2). In contrast, the different sequences of the *ftsZ* and *wsp* genes from the same individual of the scale insect *Dactylopius* cluster in supergroup A and B. These results are supported by high bootstrap values. For the 16S rDNA only a single sequence type was found for the latter species (data not shown). The *Wolbachia* sequences detected in the galerucine leaf beetles *Diabrotica virgifera* (anthraquinone-free) and *G. tanaceti* (with anthraquinone) are next neighbours, while the *Wolbachia* strains of the anthraquinone-containing scale insect *Dactylopius* spec. are distantly related to *G. tanaceti* (Fig. 1). Tree topologies did not change independent of the construction method (MP or ML) used (data not shown). Thus, only the trees based on the distance method are shown.

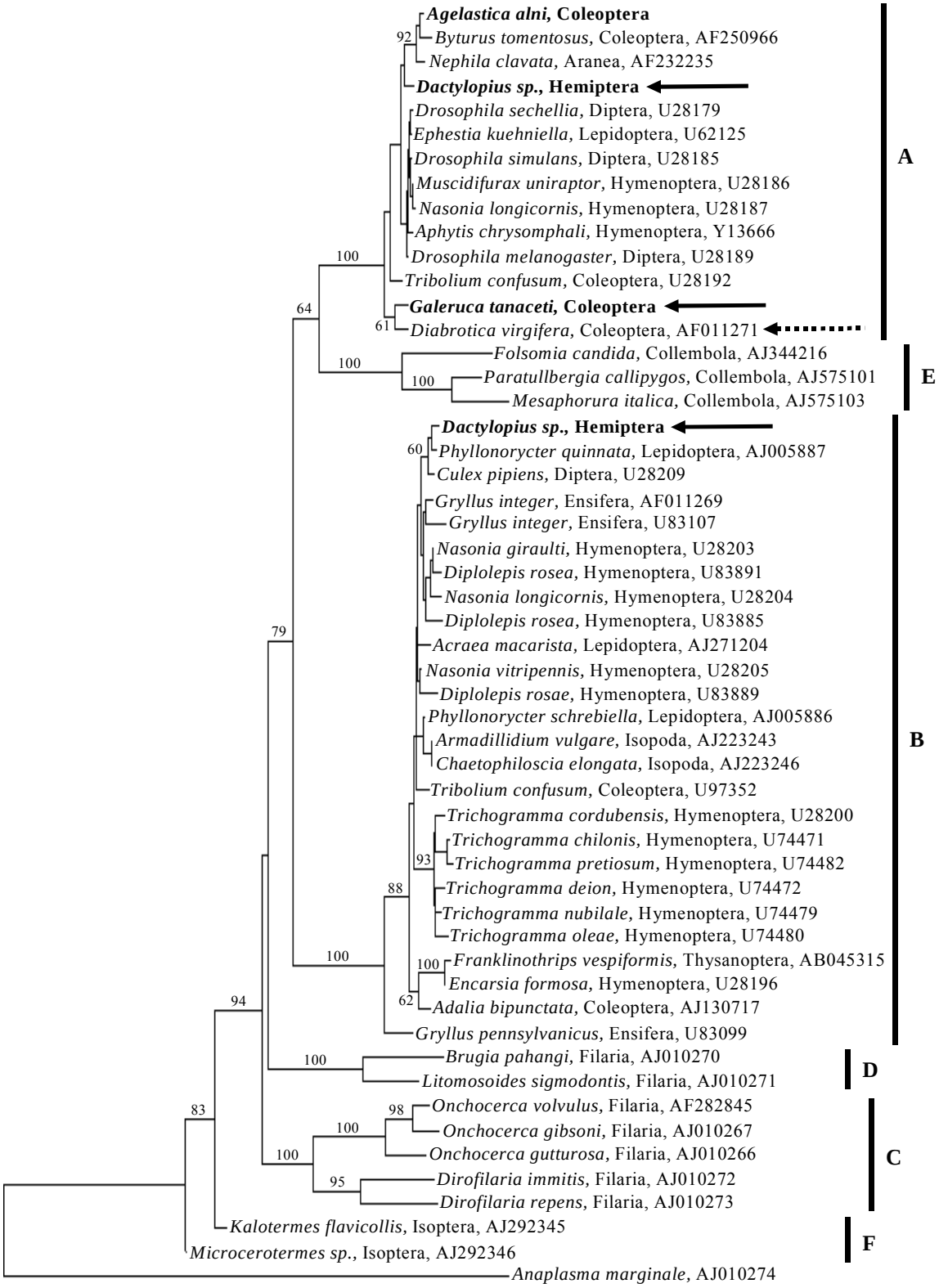

Figure 1. Neighbour-joining phylogram of *Wolbachia* strains using *ftsZ* gene sequences. *Anaplasma marginale* was used as outgroup. Bootstrap values (100 replicates) for nodes are indicated (above 50). Taxon labels refer to the host species from which the *Wolbachia* strain was isolated. The *Wolbachia* strains from hosts analysed in this study are indicated in bold. Arrows mark species containing known antimicrobial/ cytostatic components (bold: anthraquinones or anthraquinone glycosides; dashed: cucurbitacins). The six supergroups (A-F) are indicated by vertical bars. Sequences for the following organisms were deposited at EMBL database: *Dactylopius* spec. (AM180552 and AM180555), *A. alni* (AM180553) and *G. tanaceti* (AM180554).

Discussion

Wolbachia were found to be present in leaf beetle eggs containing 1,8-dihydroxylated anthrones (chrysarobin and dithranol) and anthraquinones (chrysophanol and chrysazin). Also the scale insect *Dactylopius* spec. containing carminic acid as anthraquinone glycoside harboured *Wolbachia*. The presence of antimicrobially and cytostatically active anthraquinones did obviously not protect the insects and their eggs from an infection with *Wolbachia*.

How can *Wolbachia* circumvent the antimicrobial activity of anthraquinones? The phenolic hydroxy groups enable anthraquinones to affect very different cellular targets by formation of hydrogen and ionic bonds with proteins, thus affecting e.g., enzymes, receptors or ion channels (Wink and Schimmer 1999). For example, anthraquinones have been shown to affect nucleic acid synthesis in *Bacillus subtilis* (Levin et al. 1988). However, the membranes enveloping *Wolbachia* might provide protection from attacks by multi-targeting anthraquinones. *Wolbachia* is not only enclosed by two bacterial membrane layers, but also by a third, outer layer of host origin (Louis and Nigro 1989; Stouthamer et al. 1999). The antimicrobial activity of anthraquinones and anthrones towards *Wolbachia* will depend on the ability of these components to permeate the membranes. A considerable amount of anthraquinones and anthrones in the eggs of *G. tanaceti* are present in a bound form, while about less than half of the quantities occur free (Pankewitz and Hilker 2006). When subjecting egg extracts of *G. tanaceti* to acidic hydrolysis, the free anthraquinone aglyca were obtained, however, the components to which the anthraquinones bind have not been identified so far. When considering the concentrations of only the free anthraquinones and anthrones in the eggs of *G. tanaceti*, these are still within the range of antimicrobially active concentrations (Le Van 1984; Kambizi et al. 2004; Pankewitz and Hilker 2006). The free aglyca of the anthraquinones present in Galerucini have intermediate polarity. The anthraquinone emodin that is very similar to chrysophanol and chrysazin has been shown to weaken hydrophobic interactions in membranes due to its amphiphilic character. Only when emodin was enclosed in liposomes, it easily could pass membranes and display antimicrobial effects (Alves et al. 2004). Whether chrysophanol and chrysazin present in Galerucini eggs are able to interfere or even to pass the *Wolbachia* membranes, needs to be addressed in future studies.

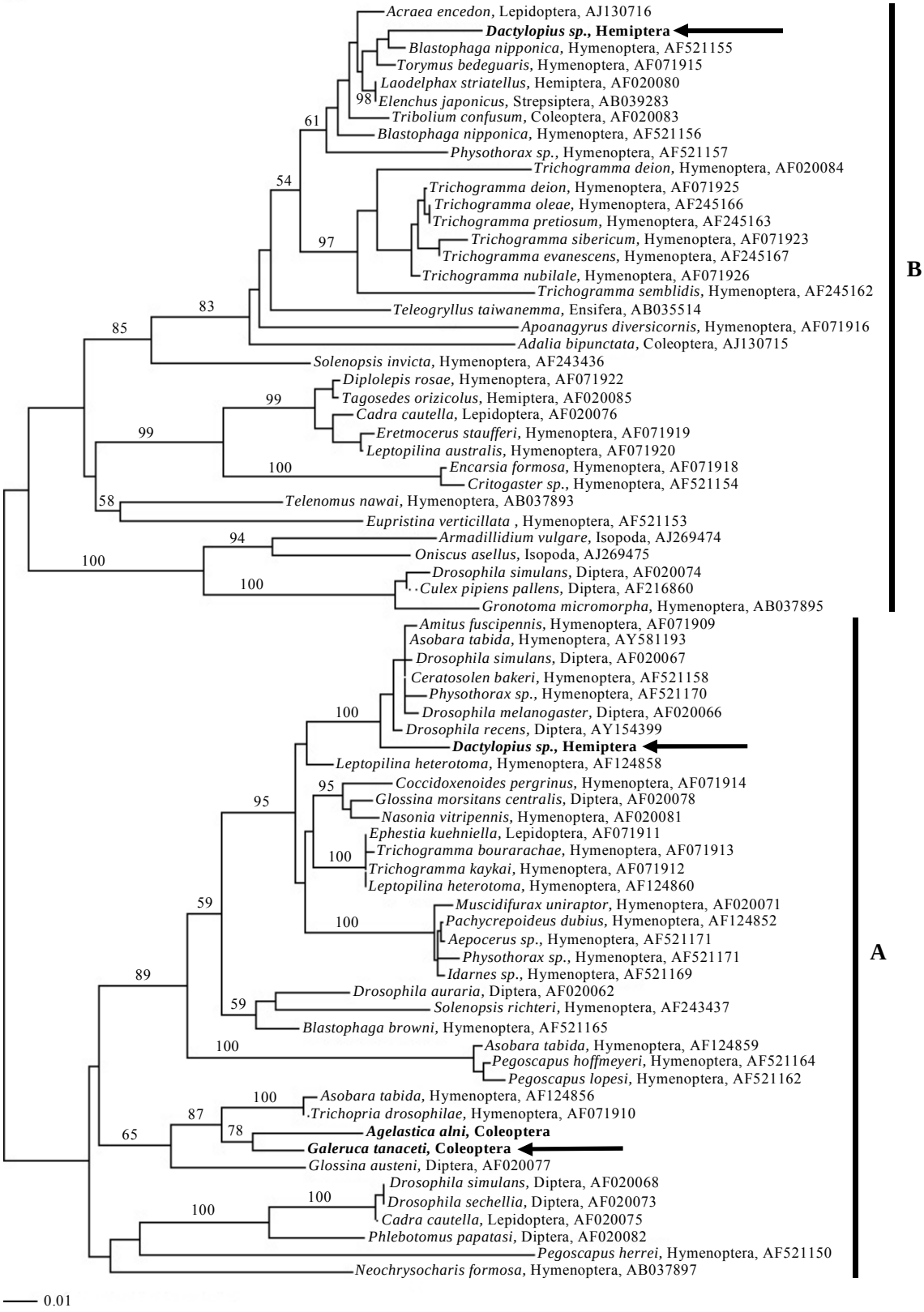

Figure 2. Neighbour-joining phylogram of *Wolbachia* strains based on *wsp* gene sequences. The tree was midpoint-rooted. Bootstrap values (100 replicates) for nodes are indicated (above 50). Taxon labels refer to the host species from which the *Wolbachia* strain was isolated. The *Wolbachia* strains from hosts analysed in this study are indicated in bold. Arrows mark species containing known antimicrobial/ cytostatic components (bold: anthraquinone or anthraquinone glycosides). The supergroups A and B are indicated by vertical bars. Sequences for the following organisms were deposited at EMBL database: *Dactylopius* spec. (AM180556 and AM180557), *A. alni* (AM180558) and *G. tanaceti* (AM180559).

The susceptibility of *Wolbachia* to other antibiotics than anthraquinones varies greatly, from high susceptibility to antibiotics like doxycycline and rifampin to very low susceptibility to, e.g., gentamicin or thiamphenicol (Fenollar et al. 2003). The target of these antibiotics are enzymes involved in translation or transcription (Finch et al. 2003). Both doxycycline and rifampin possess phenolic OH-groups like anthraquinones, but their stronger lipophily might enable them to pass membranes much faster than can be expected from free anthraquinones (Kenny and Strates 1981; Riond and Riviere 1988; Alves et al. 2004).

Wolbachia are also present in insects containing other cytotoxic natural products than anthraquinones. For example, *Wolbachia* were found in eggs of the cucumber leaf beetle *Diabrotica virgifera* (Giordano et al. 1997), which contain cytotoxic cucurbitacins (Ferguson et al. 1985; Teuscher and Lindequist 1994). The eggs of the coccinellid *Adalia bipunctata* harbour *Wolbachia* (Hurst et al. 1999) in spite of the presence of alkaloids, the homotropane adaline and the piperidone adalinine (Lognay et al. 1996), which are toxic for amphibians, reptiles and birds (Teuscher and Lindequist 1994). However, the activity of these natural products against microorganisms has not been tested in detail.

Since phylogenetical distant strains of *Wolbachia* were found to be able to infect anthraquinone-containing insects like *G. tanaceti* and *Dactylopius*, our data suggest that resistance against anthraquinones is very likely a common trait that is widespread in different *Wolbachia* genotypes. Since recombination was detected in the *wsp* gene, phylogenetic analyses based on this gene may be questioned (Baldo et al. 2005). However, no recombination of the *ftsZ* gene is known so far (Baldo et al. 2006). In our study, both the phylogenetic analyses based on *wsp* and *ftsZ* sequences place *Wolbachia* in anthraquinone-containing insects in supergroups A and B. Therefore, our hypothesis that anthraquinone resistance is a character of a unique genotype of *Wolbachia* can most likely be rejected. In the recently sequenced genome of *Wolbachia* from *Drosophila melanogaster* (*w*Mel) only gene sequences for a putative penicillin-binding protein and a putative multi-drug resistance protein were detected (Wu et al. 2004). Anthraquinone resistance, however, may be encoded on mobile elements distributed by horizontal gene transfer like the antimicrobial resistance in other bacteria (Gill et al. 2005) and thus is not a prerequisite of any *Wolbachia* strain.

The chosen cloning procedure allowed to obtain different sequence types for the *Wolbachia wsp* and *ftsz* genes in a single individual of the scale insect *Dactylopius* spec., clustering in supergroups A and B. With the primers used for the 16S rDNA gene, only A-type *Wolbachia* were detected in *Dactylopius* spec.. Due to the low resolving power of the latter gene (O'Neill et al. 1992), no further search for B supergroup *Wolbachia* was conducted. Our findings indicate that two different *Wolbachia* strains are able to infect *Dactylopius*. To explain the presence of *Wolbachia* genes belonging to different supergroups in one individual, several hypotheses have been suggested: (i) *Wolbachia* is present with two different copies of one gene (gene duplication events) (Breeuwer et al. 1992), (ii) two different *Wolbachia* strains are present in a single individual (double infection) (Breeuwer et al. 1992) or (iii) recombination events between strains of different supergroups occurred, e.g., by horizontal transfer (Malloch and Fenton 2005). Evidence for genetic exchange of *Wolbachia* was found in two *Byturus* raspberry beetles (Malloch and Fenton 2005). These beetles harbour a single copy of the *Wolbachia ftsZ* gene belonging to supergroup A, whereas the copy of the *wsp* gene is grouped with type B sequences (Malloch and Fenton 2005). This did not match the situation in *Dactylopius* spec., where the *ftsZ* and the *wsp* gene sequences both cluster with sequences from supergroup A and B. Also, the two-copies-one-gene hypothesis is unlikely because it has been shown by Braig et al. (1998) that the *wsp* gene occurs in a single copy within the *Wolbachia* genome. But a recent study sequencing the genome of *Wolbachia* from *Drosophila melanogaster* (*w*Mel) found a duplication in the *wsp* gene: Additional to the *wsp* gene, two paralogs were detected in the genome suggesting that multiple copies can co-exist, but they are different from *wsp* (Wu et al. 2004). With the wsp specific primers used in our study, we cannot have amplified such paralogs. Thus, the most parsimonious explanation for two different copies of the *wsp* and *ftsZ* genes in *Dactylopius* is double infection. In fact, double infections with two *Wolbachia* strains seem to be quite common. For example, a study on 154 Panamanian insects revealed a *Wolbachia* double infection rate of 35% (Werren et al. 1995a).

No *Wolbachia* bacteria were present in the eggs of the elm leaf beetle *X. luteola*, an anthraquinone-containing species also belonging to the taxon Galerucini like *G. tanaceti* does. Several other studies show that in closely related potential host species one may harbour *Wolbachia*, while the other one is not infected, as was demonstrated in different *Drosophila* species and nematodes or in two sibling species of butterflies (Casiraghi et al.

2004; Miller and Riegler 2006; Narita et al. 2006). Two explanations were suggested for these findings: (i) the host ancestor has been infected by *Wolbachia* and one of the derived species kept the infection, the other one lost it (vertical transmission); (ii) each species gains the bacteria individually, while the ancestor was not infected (horizontal transmission) (Casiraghi et al. 2004). The fact that the *Wolbachia* sequences detected in *G. tanaceti* cluster with the *Wolbachia* sequences of other Galerucinae (*ftsZ* gene: with *D. virgifera*, Luperini, Galerucinae; *wsp* gene: with *A. alni*, Sermylini, Galerucinae) may argue for transfer from an ancestral Galerucinae species and a single loss of infection in the close relative *X. luteola*. Future studies on *Wolbachia* in Galerucinae need to be undertaken to confirm this hypothesis.

Acknowledgement

We thank Torsten Meiners (Freie Universität Berlin, Germany) for providing elm leaf beetles and Janine Fröhlich (Humboldt University Berlin, Germany) for technical assistance. Funding for this work was provided by the Deutsche Forschungsgemeinschaft, Gr 1467/6-1, 2 to Yvonne Gräser and Hi 416/16-1, 2 to Monika Hilker.

References

Alves, DS, Pérez-Fons, L, Estepa, A, Micol, V (2004). Membrane-related effects underlying the biological activity of the anthraquinones emodin and barbaloin. Biochem. Pharmacol. 68:549-561.

Baldo, L, Lo, N, Werren, JH (2005). Mosaic nature of the *Wolbachia* surface protein. J. Bacteriol. 187:5406-5418.

Baldo, L, Bordenstein, S, Wernegreen, JJ, Werren, JH (2006). Widespread recombination throughout *Wolbachia* genomes. Mol. Biol. Evol. 23:437-449.

Blum, MS, Hilker, M (2002). Chemical protection of insect eggs. In: Hilker, M, Meiners, T (eds), Chemoecology of Insect Eggs and Egg Deposition. Blackwell Publishing, Berlin, Oxford, pp 61-90.

Braig, HR, Zhou, W, Dobson, SL, O'Neill, SL (1998). Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180:2373-2378.

Breeuwer, JAJ, Stouthamer, R, Barns, SM, Pelletier, DA, Weisburg, WG, Werren, JH (1992). Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Mol. Biol. 1:25-36.

Casiraghi, MC, Bain, O, Guerrero, R, Martin, C, Pocacqua, V, Gardner, SL, Franceschi, A, Bandi, C (2004). Mapping the presence of *Wolbachia pipientis* on the phylogeny of filarial nematodes: Evidence for symbiont loss during evolution. Int. J. Parasitol. 34:191-203.

Cudlin, J, Blumauerova, M, Steinerova, N, Mateju, J, Zalabak, V (1976). Biological activity of hydroxyanthraquinones and their glucosides towards microorganisms. Folia Microbiol. 21:54-57.

Czarnetzki, AB, Tebbe, CC (2004). Detection and phylogenetic analysis of *Wolbachia* in Collembola. Environ. Microbiol. 6:35-44.

Eisner, T, Nowicki, S, Goetz, M, Meinwald, J (1980). Red cochineal dye (carminic acid): Its role in nature. Science 208:1039-1042.

Eisner, T, Ziegler, R, McCormick, JL, Eisner, M, Hoebeke, ER, Meinwald, J (1994). Defensive use of an acquired substance (carminic acid) by predaceous insect larvae. Experientia 50:610-615.

Fenollar, F, Maurin, M, Raoult, D (2003). *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. Antimicrob. Agents. Ch. 47:1665-1671.

Ferguson, JE, Metcalf, RL, Fischer, DC (1985). Disposition and fate of cucurbitacin B in five species of Diabroticites. J. Chem. Ecol. 11:1307-1321.

Finch, R, Greenwood, D, Norrby, SR, Whitley, RJ (2003). Antibiotic and Chemotherapy: Anti-infective Agents and Their Use in Therapy. Churchill Livingston, Edinburgh.

Gálvez, J, Gomez-Lechón, MJ, Garcia-Domeneche, R, Castell, JV (1996). New cytostatic agents obtained be molecular topology. Bioorg. Med. Chem. Lett. 6:2301-2306.

Gill, SR, Fouts, DE, Archer, GL, Mongodin, EF, DeBoy, RT, Ravel, J, Paulsen, IT, Kolanay, JF, Beanan, M, Dodson, RJ et al. (2005). Insights on evolution of virulence and resistance from the complete genome analysis of an early methicillin-resistant *Staphylococcus aureus* strain and a biofilm-producing methicillin-resistant *Staphylococcus epidermis* strain. J. Bacteriol. 187:2426-2438.

Giordano, R, Jackson, JJ, Robertson, HM (1997). The role of *Wolbachia* bacteria in reproductive incompatibilities and hybrid zones of *Diabrotica* beetles and *Gryllus* crickets. Proc. Natl. Acad. Sci. U.S.A. 94:11439-11444.

Hilker, M, Köpf, A (1995). Evaluation of the palatability of chrysomelid larvae containing anthraquinones to birds. Oecologia 100:421-429.

Hilker, M, Schulz, S. (1991). Anthraquinones in different developmental stages of *Galeruca tanaceti* (Coleoptera; Chrysomelidae). J. Chem. Ecol. 17:2323-2332.

Hilker, M, Eschbach, U, Dettner, K (1992). Occurence of anthraquinones in eggs and larvae of several Galerucinae (Coleoptera: Chrysomelidae). Naturwissenschaften 79:271-274.

Holden, PR, Brookfield, JFY, Jones, P (1993). Cloning and characterization of an *ftsZ* homologue from a bacterial symbiont of *Drosophila melanogaster*. Mol. Gen. Genet. 240:213-220.

Howard, DF, Phillips, DW, Jones, TH, Blum, MS (1982). Anthraquinones and anthrones: Occurrence and defensive function in a chrysomelid beetle. Naturwissenschaften 69:91-92.

Hurst, GDD, Jiggins, FM, Schulenberg, JHG, Bertrand, D, West, SA, Goriacheva, II, Zakharov, IA, Werren, JH, Stouthamer, R, Majerus, MEN (1999). Male-killing *Wolbachia* in two species of insect. Proc. R. Soc. Lond. B 266:735-740.

Izhaki, I (2002). Emodin - a secondary metabolite with multiple ecological functions in higher plants. New Phytol. 155:205-217.

Jeyaprakash, A, Hoy, MA (2000). Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty-three arthropod species. Insect Mol. Biol. 9:393-405.

Kambizi, L, Sultana, N, Afolayan, AJ (2004). Bioactive compounds isolated from *Aloe ferox*: A plant traditionally used for the treatment of sexually transmitted infections in the Eastern Cape, South Africa. Pharm. Biol. 42:636-639.

Kenny, MT, Strates, B, (1981). Metabolism and pharmacokinetics of the antibiotic rifampin. Drug Metab. Rev. 12:159-218.

Kondo, N, Ijichi, N, Shimada, M, Fukatsu, T (2002). Prevailing triple infection with *Wolbachia* in *Callosobruchus chinensis* (Coleoptera: Bruchidae). Mol. Ecol. 11:167-180.

Kunze, A, Witte, L, Aregullin, M, Rodriguez, E, Proksch, P (1996). Anthraquinones in the leaf beetle *Trirhabda geminata* (Chrysomelidae). Z. Naturforsch. C 51:249-252.

Koukou, K, Pavlikaki, H, Kilias, G, Werren, JH, Bourtzis, K, Alahiotis, SN (2006). Influence of antibiotic treatment and *Wolbachia* curing on sexual isolation among *Drosophila melanogaster* cage populations. Evolution 60:87-96.

Le Van, T (1984). Emodin a fungal metabolite and the effects of emodin on the growth of some soil microorganisms. Acta Agrar. Silv., Ser. Agrar. 23:235-242.

Levin, H, Hazenfrantz, R, Friedman, J, Perl, M (1988). Partial purification and some properties of the antibacterial compounds from *Aloe vera*. Phytoter. Res. 1:1-3.

Lo, N, Casiraghi, M, Salatis, E, Bazzocchi, C, Bandi, C (2002). How many *Wolbachia* super-groups exist. Mol. Biol. Evol. 19:341-346.

Lognay, G, Hemptinne, J-L, Chan, YY, Gaspar, CH, Marlier, M, Braekman, JC, Daloze, D, Pasteels, JM (1996). Adalinine, a new piperidine alkaloid from the ladybird beetles *Adalia bipunctata* and *Adalia decempunctata*. J. Nat. Prod. 59:510-511.

Louis, C, Nigro, L (1989). Ultrastructural evidence of *Wolbachia* Rickettsiales in *Drosophila simulans* and their relationship with unidirectional cross-incompatibility. J. Invert. Pathol. 54:39-44.

Malloch, G, Fenton, B (2005). Super-infections of *Wolbachia* in byturid beetles and evidence for genetic transfer between A an B super-groups of *Wolbachia*. Mol. Ecol. 14:627-637.

Manojlovic, NT, Solujic, S, Sukdolak, S, Krstic, LJ (2000). Isolation and antimicrobial activity of anthraquinones from some species of the lichen genus *Xanthoria*. J. Serb. Chem. Soc. 65:555-560.

Miller, WJ, Riegler, M (2006). Evolutionary dynamics of *w*Au-like *Wolbachia* variants in neotropical *Drosophila* spp.. Appl. Environ. Microb. 72:826-835.

Narita, S, Nomura, M, Kato, Y, Fukatsu, T (2006). Genetic structure of sibling butterfly species affected by *Wolbachia* infection sweep: Evolutionary and biogeographical implications. Mol. Ecol. 15:1095-1108.

Oh, HW, Kim, MG, Shin, SW, Bae, KS, Ahn, YJ, Park, HY (2000). Ultrastructural and molecular identification of a *Wolbachia* endosymbiont in a spider, *Nephila clavata*. Insect Mol. Biol. 9:539-543.

O'Neill, SL, Hoffmann, AA, Werren, JH (1997). Influential Passengers. Inherited Microorganisms and Arthropod Reproduction. Oxford University Press, Oxford.

O'Neill, SL, Giordano, R, Colbert, AM, Karr, TL, Robertson, HM (1992). 16S rRNA phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. U.S.A. 89:2699-2702.

Pankewitz, F, Hilker, M (2006). Defensive components in insect eggs: Are anthraquinones produced during egg development? J. Chem. Ecol. 32:2067-2072.

Riond, JL, Riviere, JE (1988). Pharmacology and toxicology of doxycycline. Vet. Hum. Toxicol. 30:431-443.

Shoemaker, DD, Machado, CA, Molbo, D, Werren, JH, Windsor, DM, Herre, EA (2002). The distribution of *Wolbachia* in fig wasps: Correlations with host phylogeny, ecology and population structure. Proc. R. Soc. Lond. B 269:2257-2267.

Stouthamer, R, Breeuwer, JAJ, Hurst, GD (1999). *Wolbachia pipientis*: Microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53:71-102.

Swofford, DL (2000). PAUP. Phylogenetic analysis using parsimony (and other methods). Version 4.0b8. Sinauer Associates, Sunderland.

Teuscher, E, Lindequist, U (1994). Biogene Gifte. Gustav Fischer Verlag, Stuttgart.

Van Meer, MM, Witteveldt, J, Stouthamer, R (1999). Phylogeny of the arthropod endosymbiont *Wolbachia* based on the *wsp* gene. Insect Mol. Biol. 8:399-408.

Werren, JH (1998). *Wolbachia* and speciation. In: Howard, D, Berlocher, S (eds), Endless Forms: Species and Speciation. Oxford University Press, New York, pp 245-260.

Werren, JH, Windsor, DM, Guo, L (1995a). Distribution of *Wolbachia* among Neotropical arthropods. Proc. R. Soc. Lond. B 262:197-204.

Werren, JH, Zhang, W, Guo, L (1995b). Evolution and phylogeny of *Wolbachia*: Reproductive parasites of arthropods. Proc. R. Soc. Lond. B 261:55-63.

Wink, M, Schimmer, O (1999). Modes of action of defensive secondary metabolites. In: Wink, M (ed), Functions of Plant Secondary Metabolites and their Exploitation in Biotechnology. Academic Press, pp 17-133.

Wu, M, Sun, LV, Vamathevan, J, Riegler, M, Deboy, R, Brownlie, JC, McGraw, EA, Martin, W, Esser, C, Ahmadinejad, N et al. (2004). Phologenomics of the reproductive parasite *Wolbachia pipientis w*Mel: A streamlined genome overrun by mobile genetic elements. PLOS Biol. 2:327-341.

Zhou, W, Rousset, F, O'Neill, S (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B 265:509-515.