Horizontal transfer of *Wolbachia* between different Hymenopteran host-parasitoid pairs

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26th September 2011

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University of Bremen

September 2011

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To my family

“For the present, I suggest that any doubting readers should “look out of the window” and list every organism they can see. I can guarantee that most, probably all, organisms on the list are a product of symbiosis.”

Angela E. Douglas

Acknowledgements

It has been a while already since I left home to study biology in Oviedo. I always thought I would come back home after College, but life turned my plans upside down giving me the opportunity to do my degree's last year abroad. Now that I have finished my master in Germany, I would like to thank the people that have helped me through these last years.

I would first like to thank Prf. Dr. Thomas Hoffmeister, who gave me the opportunity to continue my studies in Ecology in University of Bremen, which ultimately opened for me the doors of science.

I am extremely thankful to my supervisor Dr. Martin Kaltenpoth, for teaching me everything I know about insect symbiosis. Thank you for making of the lab such a nice atmosphere, for encouraging us to develop as critical scientists and to provide us with all the resources (technical but also personal) we need to do our best.

I would also like to thank Dr. Oliver Niehuis, Thomas Schmitt and Mareike Wurdack for providing some of the samples I have used in this project and Dr. Johannes Kroiss for the great wasp pictures that he has lend me.

My time in the Institute would not have been as productive, comfortable and happy if it wasn´t for my colleagues Hassan, Laura, Sabrina, Sailen, Taras, Tobi, Uli and Woife, who have become not only my co-workers but friends. Thank you for those long talks, advices, cakes, neigbor-joining dinners and so many other things; also for guiding me through science.

And last but not least, I would like to thank Jorge and my big family. Specially my dad, who is a passionate of science and showed me the first microorganism I ever saw; my mum, who has always encouraged me to pursue my dreams, even when they are away from home; and my sister, who is always there, no matter the distance, for long and nocturnal talks. Thank you for your wise advices, economical support and for cheering my up when I was down.

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Summary

Wolbachia is one of the most common endosymbionts on Earth, infecting up to 70% of all insect species. They are maternally inherited through the egg cytoplasm, and manipulate their host reproductive system in such a way that increases the proportion of infected females among the host's progeny. Therefore, *Wolbachia* is easily transmitted from generation to generation; driving itself into populations. Arthropod-infecting *Wolbachia* do not usually show congruent phylogenies with their insect hosts, suggesting that horizontal transfer can also occur, although the mechanism by which it takes place is not very well known.

Here we present a system in which horizontal transfer of *Wolbachia* can be assessed using different Hymenopteran host-parasitoid pairs as a model. Host females are mass provisioning their offspring with insect prey. Parasitoids lay an egg by the host larva, and when the chrysidid larva hatches, it exploits not only the host progeny but also the stored resources. Thus, parasitoids would acquire *Wolbachia* by feeding on the host larvae. If this is the case, *Wolbachia* strains harbored by hosts and parasitoids belonging to the same pair would be identical or very similar, being more genetically related to each other than to strains belonging to hosts or parasitoids that are not associated with each other.

In order to compare *Wolbachia* strains, we have performed a Multilocus Typing System approach to genotype strains for four different genes (*wsp, ftsZ, coxA* and *fbpA*). Analysis of the phylogenetic relationships between different strains showed similar *Wolbachia* strains in hosts and parasitoids of five pairs (*Philanthus triangulum-Hedychrum rutilans, Cerceris interrupta-Hedychrum chalybaeum, Astata boops-Hedychridium roseum, Odynerus spinipes-Pseudospinolia neglecta-Chrysis viridula* and *Cerceris sabulosa – Hedychrum gerstaeckeri*). Statistical analysis of average sequence distances between *Wolbachia* strains belonging to the same host-parasitoid pairs were - for three out of four genes -significantly lower than between strains of unrelated hosts or parasitoids (*wsp:* p=0.009, *coxA:* p=0.013, *fbpA:* p=0.049, *ftsZ:* p=1), suggesting that horizontal transfer has occurred frequently.

Our study further supports horizontal transfer as a frequent and important source of *Wolbachia* infection between different taxa in natural populations.

1. Introduction

1.1. Insect-bacteria symbiosis

Insects are the most abundant and diverse animal class on Earth (May 1988), with an estimated number of up to 30 million species (Erwin 1982), representing 90% of all existing metazoan species.

Symbiotic interactions between insects and microorganisms are extremely common and they range from antagonistic (parasitism) to neutral (commensalism) and beneficial (mutualism). The high diversity and ecological success of insects may be related to mutualistic relationships with bacteria, since these interactions can be an important source of evolutionary novelty (Maynard-Smith 1989). Many insect-associated microorganisms play a fundamental role in host nutrition, providing them with vitamins (Blewett *et al*. 1944) or essential amino acids (Douglas 1998), and allowing their host to exploit otherwise inaccessible food resources and to occupy new ecological niches. However, in more recent times, other types of symbiotic relationships have gained interest. *Hamiltonella defensa*, a gamma-proteobacterium, is able to protect its aphid host against wasp parasitization, and in several insects Actinobacteria have been found to play a key role in host defense against pathogens by producing secondary metabolites with antibiotic effects (Currie *et al*. 1999; Kaltenpoth *et al.* 2005, Scott et al. 2008, Kroiss et al. 2010). There are now many examples of microorganisms defending their hosts (Brownlie et al. 2009), suggesting that symbiont-mediated protection is more frequent than previously thought.

Theory predicts that heritable symbionts providing benefits to their hosts should be maintained through generations, and those producing costs should be eliminated (Hoffmann *et al.* 1997). However, some vertically transmitted symbionts such as *Cardinium* and *Wolbachia* do not usually provide fitness benefits and yet they manage to invade host populations. They usually do so by manipulating their host reproductive system. Sex-ratio distorter bacterium can persist and spread within host populations by increasing the fitness of infected hosts in comparison with uninfected, increasing sex ratio toward one of the sexes (the sex that will ultimately produce more infected offspring capable of spreading the infection), and they can also decrease the fitness of uninfected hosts by preventing reproduction between insects harboring different strains.

1.2.Wolbachia

1.2.1. Biology of Wolbachia

Wolbachia are intracellular α-proteobacterium closely related to the Rickettsiales group (Figure 1a). It is one of the most widespread obligate endosymbionts, infecting a wide range of filarial nematodes (Sironi *et al*. 1995; Bandi *et al*. 1998) and arthropods (Werren 1997). It was first described by Hertig and Wolbach (Hertig M *et al*. 1924), who reported the presence of rod-shaped

bacteria in the reproductive tissues of the mosquito *Culex pipiens*. Later, however, Wolbachia was described to be distributed throughout the insect body.

Phylogenetic studies on the basis of different genes (*ftsZ, wsp* and *16s rRNA*) have classically divided *Wolbachia* into eight different supergroups (A-H). While groups C and D are very common in filarial nematodes, the remaining ones mainly occur in arthropods, with groups A and B being predominantly found in insects (Figure 1b).

Wolbachia infects between 16%-75% of all insect species (Werren 1995a; Jeyaprakash *et al* 2000; Hilgenboecker *et al.* 2008) and have been found in each of the major insect orders: Coleoptera, Diptera, Hemiptera, Hymenoptera, Lepidoptera and Orthoptera (Werren *et al*. 1995a). More conservative estimations hypothesized that between 1 and 5 million insect species are infected (Werren *et al.* 1995b). However, not all the species or individuals are equally infected with *Wolbachia*. Rates of infection range from 3-100% (Arthofer *et al.* 2009). Infections with different strains within a population (multiple infections), as well as within one single individual (superinfections) are frequently found (Breeuwer *et al.* 1992; Zhou *et al* 1998; Werren *et al*. 2000).

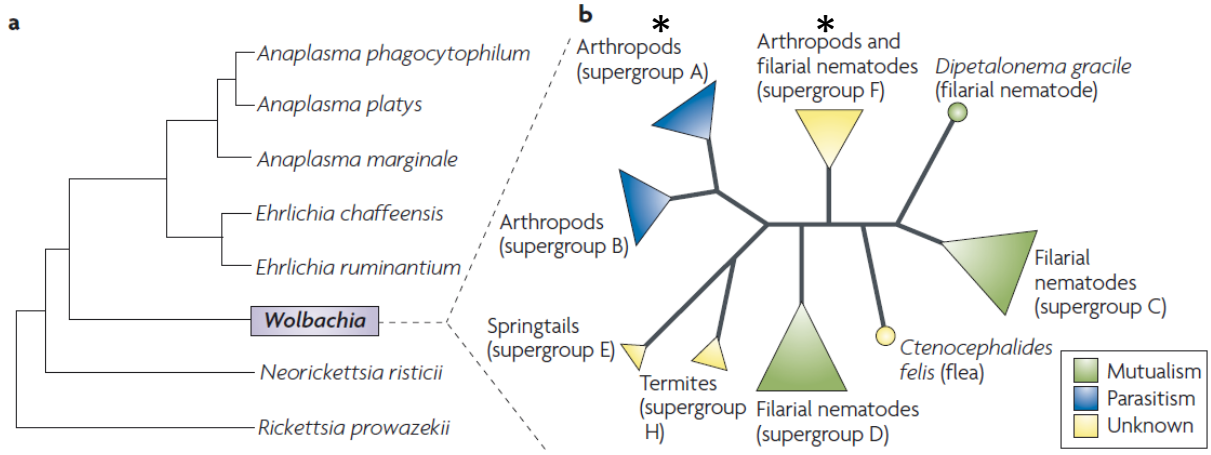

Figure 1 a. Phylogenetic relationship of *Wolbachia* in relation to other closely related Rickettsiales. b. Phylogenetic tree of the main *Wolbachia* supergroups. The size of the triangles depicts strain diversity within each lineage. Circles denote lineages represented by a single strain. Different colors show different patterns of symbiosis. (*) Supergroups in which recently also mutualistic *Wolbachia* have been found. Modified from Werren et al. 2008.

1.2.*2*. Transmission of Wolbachia

Symbionts are transmitted to new hosts by different mechanisms ranging from strict horizontal (infectious) transmission, to strict vertical (heritable) transmission (Hoffmann *et al.* 1997). Heritable microorganisms depend on the survival and reproduction of their hosts and this leads to the conclusion that natural selection would favour those microbes that increase host fitness. By contrast, horizontally transmitted symbionts do not strictly require reproduction of their hosts and therefore, they do not necessarily need to increase hot fitness and might evolve a parasitic interaction.

1.2.2.1 Vertical transmission

Wolbachia is transmitted vertically, from mother to offspring through the egg cytoplasm. Nematode-associated *Wolbachia* are usually mutualistic, and there are also some insect species in which it acts as a mutualistic partner, e.g. in the bedbug *Cimex lectularius Wolbachia* provides B

vitamins (Hosokawa *et al*. 2009) and some strains can protect *Drosophila melanogaster* against a viral pathogen (Texeira *et al.* 2008). In most insect species, however, *Wolbachia* manipulates the host reproductive system by inducing one of several different phenotypes. The most common effect is cytoplasmic incompatibility (Breeuwer *et al.* 1992; O´Neill *et al.* 1992), in which sperm of infected males is not compatible with eggs of females infected with different *Wolbachia* strains uninfected females. A second phenotype induced is thelytokous parthenogenesis (Stouthamer *et al.* 1993), which has only been found in species where males develop from unfertilized eggs (arrhenotokous). Infected females produce daughters from unfertilized eggs (thelytoky) instead of sons. A third phenotype is feminization in which *Wolbachia* induces developing male embryos to turn into females due to the hyperthrophy of the androgenic gland or interfering with the sex-determination pathway forcing the development into females (Rousset *et al.* 1992). The last phenotype induced is male killing, in which death of males occurs through lethal feminization, i.e. genetic males are feminized and die during larval development (Hurst *et al.* 1999).

All of these phenotypes result in an increased production of infected females among the progeny. Since *Wolbachia* is transmitted through the egg cytoplasm and male sperm does not have any, males are dead ends for *Wolbachia's* transmission. Therefore, *Wolbachia* benefits of this increment in infected females, enhancing its own transmission and driving easily into the population.

1.2.2.2 Horizontal transmission

Previous studies have shown that *Wolbachia* strains infecting arthropods diverged around 50 million years ago, whereas the arthropods diverged 200 million years earlier (Werren *et al.* 1995a), suggesting that the colonization of different arthropods must have involved horizontal transmission events. This hypothesis is supported by several studies reporting non-congruent phylogenies of *Wolbachia* with its hosts (Vavre *et al* 1999) and the fact that *Wolbachia* isolates from closely related hosts usually do not cluster together in phylogenetic trees (Cook *et al* 1999). Considering *Wolbachia* undergoing strict vertical transmission, there should be coevolution between *Wolbachia* and its host, and thus, congruent phylogenies would be expected (Casiraghi *et al.* 2001).

Although the mechanism of horizontal transmission in *Wolbachia* is still unknown, several hypotheses have been discussed. It has been shown that *Wolbachia* transfer can occur in nature by hemolimph contact after injury (Rigaud *et al.* 1995), which is supported by studies that experimentally transferred *Wolbachia* fron one individual to another by microinjections (Frydmann *et al.* 2006). A second hypothesis discusses the acquisition of *Wolbachia* after predation (Houck *et al.* 1991), although this has yet to be proven in nature. The third option is that parasitoids may play a role in horizontal transfer (Werren *et al.* 1995a). Parasitoids lay an egg that develops within the host larvae or feeds on them. This intimate relationship could result in the transmission of the bacterium from an infected host to its parasitoid by predation. However, in the case of endoparasites the host can respond to the parasitism by mounting an immune response and survive, so horizontal transfer may also be possible from parasitoid to host. Maintenance of *Wolbachia* depends on the rates of acquisition and loss of infections within species relative to the horizontal transfer rates between species (Werren *et al.* 2008).

1.3. Hymenopteran hosts and parasitoids

Some *Crabronidae* wasps species are host for parasitic wasps belonging to the *Chrysididae* family, which are specialists regarding their host, i.e. they often parasitize only one host species. Host-parasitoid interactions are usually very intimate, being a perfect setting for horizontal transmission of microorganisms.

Crabronid wasp females are mass-provisioning their offspring with prey. At some point in the host development, the parasitoid oviposits next to the host egg. The *Chrysidid* larva will hatch first and feed not only on the stored food resources but also on the host larva, spin its cocoon and develop into an adult. *Wolbachia* horizontal transfer may occur by feeding on the host larva. This life cycle (Figure 3) may vary -depending on the species- in some features, such as the timing in which the parasitoid enters the nest, how they enter the brood cell, as well as the oviposition method.

Figure 2. a. *Philanthus triangulum* life cycle. Host female provides the egg with paralyzed insects as food resources. The larvae feeds on the prey, spins its cocoon and emerge as an adult wasp. b. Host and parasitoid life cycle. At a certain point in host development, the parasitoid lays an egg near the host larvae. The parasitoid larva will feed on the stored insects as well as on the host larvae. Modified from Köhler *et al*. 2011.

1.4. Multi Locus Sequence Typing (MLST)

Historically, *Wolbachia's* mitochondrial *16s rRNA* and nuclear *ftsZ* (Filamenting Temperature-Sensitive Mutant Z, which codifies for a protein involved in cell division) genes have been widely used for phylogenetic studies, and have shown that *Wolbachia* clusters in eight different supergroups. However, it has been more difficult to develop a method to type intragroup strains (Paraskevopoulos *et al.* 2006). The *wsp* gene (which codifies for a *Wolbachia* surface protein) has also been widely used for typing *Wolbachia* strains (Zou *et al*. 1998); however, this single locus approach is not reliable due to extensive recombination of *wsp* among strains (Baldo *et al.* 2005) and a strong disruptive selection (Werren *et al.* 2001). *16s rRNA*, by contrast, evolves too slowly and, therefore, is also not suitable for this study.

MLST is a powerful strain typing method to differentiate between bacterial strains, using the sequences of a number of different housekeeping genes. Internal fragments of each gene with an average length of 450-500bp are used because these can be accurately sequenced. In MLST the number of nucleotide differences between sequences does not matter and they are considered to be different if they differ in one nucleotide or more. Bacterial housekeeping genes are variable enough differentiate strains.

In order to genotype Wolbachia five different housekeeping genes are usually used: *ftsZ, gatB* (codifying the subunit b of glutamyl-tRNA amidotransferasa)*, coxA* (which codifies for the subunit I of cytochrome c oxidase) *, hcpA* and *fbpA* (which codify for a highly conserved protein in bacteria with an unknown function and for fructose-bisphosphate aldolasa respectively). It also uses *wsp* gene in order to be able to compare phylogenies. Recombination is not a major problem with this approach, since it uses a combination of genes as molecular markers to type strains, and one recombinant event is unlikely to affect more than one marker (Baldo *et al.* 2006).

MLST is useful in studying bacterial phylogenies and tracing global movement of infection types within insect populations; it helps to associate infections with geographic regions, host ecology, and specific phenotypic effects on hosts (Raychoudhury *et al.* 2009).

1.5. Objectives of the project

Publications on *Wolbachia* have been continuously increasing during the last twenty-five years (Figure 2). Recent projects have mainly focused on the evaluation of *Wolbachia´*s host range, full range of effects on host reproduction, how this bacterium alters its host´s reproduction system on a molecular level, population dynamics of *Wolbachia* infections within and between populations and species, and coevolution of *Wolbachia* with their arthropod hosts (Cook et al. 1999). However, not many analyses have been focusing on Wolbachia´s horizontal transfer.

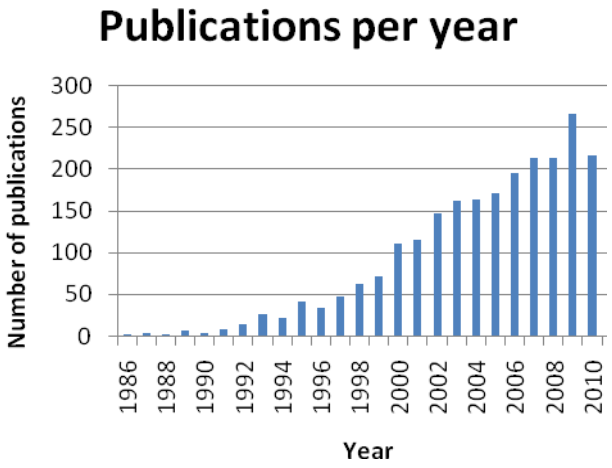

Figure 3 Annual publications of research articles about *Wolbachia* from 1986 to 2011. 2011 data only until August. Data were generated using Web of Knowledge (http://apps.webofknowledge.com) using *Wolbachia* as the search term.

Infections with *Wolbachia* need close relationships since they cannot grow outside the cells (Heath *et al.* 1999; Vavre *et al.* 1999; Noda 2001). Therefore, a good approach for studying horizontal transfer is to do it in host-parasitoid interactions since it is an intimate relationship in which this mode of transmission is likely to occur.

A number of studies have failed in finding horizontal transfer between aphids or Lepidoptera and their parasitoids (West *et al.* 1998) or between the guilds associated with the wasp *Diplolepsis rosae* (Schilthuizen *et al.* 1998). However, other surveys have been successful in showing horizontal transfer of *Wolbachia* between Diptera and the *Nasonia* complex (Raychoudhury *et al.* 2008) and from *Drosophila simulans* to its parasitoids (Heath *et al.* 1999; Vavre *et al.* 1999).

The aim of this project was to test whether there is evidence for horizontal transfer of *Wolbachia* within different Hymenopteran host-parasitoid pairs. We have studied this by phylogenetic comparison of *Wolbachia* strains infecting Hymenopteran hosts and their *Chrysididae* parasitoids and performed subsequent phylogenetic and statistical analyses. We expect *Wolbachia* strains from hosts and parasitoids belonging to the same pair to be very similar, being more closely related to each other than to hosts or parasitoids with which they have no interaction. Therefore, bacterial strains belonging to the same pair should cluster together in the phylogenetic trees. In the absence of horizontal transfer phylogenetically related hosts, on the other hand, usually share more similar *Wolbachia* strains than more distantly related arthropods, indicating vertical transmission (Baldo *et al.* 2008; Russel *et al.* 2009). Thus, if there was no horizontal transfer but strict vertical transmission between our hosts and chrysidid parasitoids, we expect strains belonging to parasitoids to be more similar to each other than to those of any host; since they are phylogenetically closely related i.e. they belong to the same family.

2. Methods and material

2.1. Collection, dissection, DNA extraction and Wolbachia screening

Male and female specimens of different *Chrysididae* parasitoid and their hosts species (Table 1) were collected in different locations in Germany, during the period between June 1998 and August 2011 by Thomas Schmitt, Oliver Niehuis, Mareike Wurdack and Martin Kaltenpoth. They were identified, preserved in 70% ethanol and stored at -20°C.

Table 1 Different Chrysididae parasitoids and hosts pairs used in this study. Host and parasitoid species without a symbiotic partner where also screened for Wolbachia. Hosts with two parasitoids form a tripartite association. Arrows join hosts with their respective parasitoids. Numbers in brackets indicate the number of screened individuals for *Wolbachia*.

Family	Hosts		Parasitoids
Crabronidae	*Cerceris interrupta* (2)	→	*Hedychrum chalybaeum* (4)
Crabronidae	*Cerceris arenaria* (6)	→	*Hedychrum nobile* (9)
Apidae	*Osmia adunca* (2)	→	*Chrysura austriaca* (4)
			Chrysis viridula (1)
Vespidae	*Odynerus spinipes* (12)	→	
			Pseudospinolia neglecta (3)
Crabronidae	*Astata boops* (1)	→	*Hedychridium roseum* (4)
Crabroninae	*Lindenius pygmaeus* (2)	→	*Hedychridium krajiniki* (1)
Crabronidae	*Trypoxylon figulus* (1)	→	*Trichrysis cyanea* (4)
Vespidae	*Microdynerus timidus* (2)	→	*Chrysis gracillima* (1)
Sphecidae	*Miscophus bicolor* (2)	→	*Chrysis cortii* (4)
Crabronidae	*Philantus triangulum* (3)	→	*Hedychrum rutilans* (9)
Crabronidae	*Cerceris quinquefasciata* (2)	→	
Crabronidae	*Cerceris rybiensis* (8)		*Hedychrum gerstaeckeri* (9)
Crabronidae	*Cerceris sabulosa* (2)	→	
			Chrysis ignita (2)
			Chrysis cyanea (1)

Insects were dissected; the abdomens were crushed with liquid nitrogen and subjected to DNA extraction following the Epicentre DNA isolation protocol (see Appendix). At least one individual from each species was left as a voucher specimen. Samples that after extraction failed to amplify in PCRs with insect-specific primers were purified again using the Soil DNA Purification Kit from Epicentre (see Appendix).

Presence of *Wolbachia* was tested by diagnostic PCR with the *Wolbachia*-specific primer pairs wspF1/R1 and wsp81F/wsp691 (amplifying products of 603bp and 610bp respectively) (Table 2). PCRs were carried out in 12.5µl final reaction volumes containing 6.4µl of water, 1.25µl of Buffer

VWR 10x , 0.25µl $MgCl_2$ (25mM), 1.5 µl of dNTPs (2mM), 2µl of each primer (10pmol/µl), 0.1µl Taq polymerase (5u/µl, VWR) and 1µl DNA. Samples were amplified in a WVR thermal cycler using the following temperature profile for wspF1/R1: a first step at 94°C for 2 min. followed by 37 cycles at 94°C for 30 sec., 59°C for 45 sec., 72°C for 90 sec., and a final extension at 72°C for 10 min. The thermal profile for wsp81F/691 was: a first step at 95°C during 2 min, followed by 35 cycles of 94°C for 30 sec, 50°C for 30 sec, 72°C for 1 min, and a final extension of 72°for 5 min (as described in Duran *et al.* 2008). Samples yielding a PCR product of the expected size were scored as positive for *Wolbachia*, whereas species that failed to amplify were provisionally considered negative.

Samples showing no amplification were subjected to a second PCR using the eubacterial primers fD1 and rP2 (Table 2.) Reactions were initiated by incubation at 94°C for 3 min, which was followed by 35 cycles of 94°C for 40 sec, 65°C for 40 sec, and 72°C for 40 sec, a final elongation step at 72°C for 4 min, and a final hold at 4°C. If they were negative for eubacterial DNA, they were amplified with insect-specific primers targeting 28s rRNA; 28s 3665F and 28s 4749R (Table 2.) to check if the DNA extraction had been successful. Temperature profile was 3min at 94°C, 35 cycles at 94°C for 40s, 63°C for 40s and 72°C for 40s, and a final extension of 4min at 72°C. Samples yielding a PCR product of the expected size for eubacterial and/or insect-specific primers were considered true *Wolbachia*-free individuals. By contrast, samples that failed to amplify with both bacterial and insect primers were not included in further analyses.

In order to investigate the possibility that *Wolbachia* can be acquired from the insects on which the host and parasitoid larvae feed on, the prey bees (*Apis mellifera*) of one host species (*Philanthus triangulum)* were tested for *Wolbachia* following the same procedure above mentioned.

2.1.1. Wolbachia supergroups

All positive samples for *Wolbachia* were tested with A and B *Wolbachia* supergroup-specific primers (Table 2.): 16SWOLBF1 forward primer was used with each one of the A-specific 16WOLBRA1 and B-specific 16WOLBRB1 reverse primers. The temperature profile was: 95°C during 3 min, 35 cycles of 95°C for 30s, 58°C for 1min and 72°C for 2 min, followed by a final extension of 10 min.

All PCR reactions, except for the B-type specific ones, included a positive control consisting of DNA from a *Philanthus triangulum* sample known to harbor *Wolbachia,* and all included a negative control (Millipore water). All PCR products were subjected to electrophoresis in a gel stained with Gel red 10.000x in water.

2.1.2. *Wolbachia* prevalence

Two different statistical tests were performed to analyze whether one of the groups, either hosts or parasitoids, were more prone to *Wolbachia* infection than the other. A two-tailed Fisher's exact test was performed (using BIAS software) to test for differences in the number of *Wolbachia*-

infected and –uninfected species between hosts and parasitoids. A Mann Whitney-U test was performed in R software to test for differences in infection rates between hosts and parasitoids.

Table 2. General, MLST and *Wolbachia* supergroup specific primers.

Primer name	Sequence (5´-3´)	Target gene	Target taxa	Ann. Temp (°C)	Reference
fD1	AGAGTTTGATCCTGGCTCAG	*16s rRNA*	Eubacteria	65	Weisburg et al. 1991
rP2	ACGGCTACCTTGTTACGACTT	*16s rRNA*	Eubacteria	65	Weisburg et al. 1991
16SWOLBF1	AGTCCTGGCTAACTCCGTGCCA	*16s rRNA*	*Wolbachia*	58	Van Borm et al. 2001
16WOLBRA1	GGGATTRGCTTAGCCTCGCGAC	*16s rRNA*	*Wolbachia* A supergroup	58	Van Borm et al. 2001
16WOLBRB1	TAGCTTAGGCTTGCGCACCTTG	*16s rRNA*	*Wolbachia* B supergroup	58	Van Borm et al. 2001
wsp_F1	GTCCAATARSTGATGARGAAAC	*wsp*	*Wolbachia*	59	Baldo et al. 2006
wsp_R1	CYGCACCAAYAGYRCTRTAAA	*wsp*	Wolbachia	59	Baldo et al. 2006
wsp81F	TGGTCCAATAAGTGATGAAGAAAC	*wsp*	Wolbachia	50	Braig et al. 1998
wsp691	AAAAATTAAACGCTACTCCA	*wsp*	Wolbachia	50	Braig et al. 1998
28s_3665F	AGAGAGAGTTCAAGAGTACGTG	*28s rDNA*	Insects	63	Danforth-Homepage
28s_4749R	GTTACACACTCCTTAGCGGA	*28s rDNA*	Insects	63	Danforth-Homepage
ftsZf1	GTTGTCGCAAATACCGATGC	*ftsZ*	Wolbachia	55	Baldo et al. 2006
ftsZr1	CTTAAGTAAGCTGGTATATC	*ftsZ*	Wolbachia	55	Baldo et al. 2006
ftsZ_BspecF1	AAAGATAGCCATATGCTCTTT	*ftsZ*	*Wolbachia* B supergroup	59	Baldo et al. 2006
ftxZ_BspecR1	CATTGCTTTACCCATCTCA	*ftsZ*	*Wolbachia* B supergroup	59	Baldo et al. 2006
ftsZ_AspecF1	AAAGATAGTCATATGCTTTTC	*ftsZ*	*Wolbachia* A supergroup	55	Baldo et al. 2006
ftsZ_AspecR1	CATCGCTTTGCCCATCTCG	*ftsZ*	*Wolbachia* A supergroup	55	Baldo et al. 2006
ftsZ_F1	ATYATGGARCATATAAARGATAG	*ftsZ*	*Wolbachia*	54	Baldo et al. 2006
ftsZ_R1	TCRAGYAATGGATTRGATAT	*ftsZ*	*Wolbachia*	54	Baldo et al. 2006
fbpA_F1	GCTGCTCCRCTTGGYWTGAT	*fbpA*	*Wolbachia*	59	Baldo et al. 2006
fbpA_R1	CCRCCAGARAAAAYYACTATTC	*fbpA*	*Wolbachia*	59	Baldo et al. 2006
fbpA_BspecF1	GTTAACCCTGATGCTTACGAT	*fbpA*	*Wolbachia* B supergroup	58	Baldo et al. 2006
fbpA_BspecR1	CCRCCAGARAAAAYYACTATTC	*fbpA*	*Wolbachia* B supergroup	58	Baldo et al. 2006
fbpA_AspesF1	TTAACCCTGATGCTTATGAC	*fbpA*	*Wolbachia* A supergroup	55	Baldo et al. 2006
gatB_F1	GAKTTAAAYCGYGCAGGBGTT	*gatB*	*Wolbachia* B supergroup	61,8	Baldo et al. 2006
gatbB_R1	TGGYAAYTCRGGYAAAGATGA	*gatB*	*Wolbachia* B supergroup	61,8	Baldo et al. 2006
gatB_BspecF1	TAAGAATCGCAAGAATTCAC	*gatB*	*Wolbachia* B supergroup	62	Baldo et al. 2006
gatB_AspecF1	TTTAGAGCAAGATGCAGGRAAGAGCG	*gatB*	*Wolbachia* A supergroup	64	Baldo et al. 2006
coxA_F1	TTGGRGCRATYAACTTTATAG	*coxA*	*Wolbachia*	54	Baldo et al. 2006
coxA_R1	CTAAAGACTTTKACRCCAGT	*coxA*	*Wolbachia*	54	Baldo et al. 2006
coxA_BspecF1	ATACCCACCTYTRTCGCAAA	*coxA*	*Wolbachia* B supergroup	54	Baldo et al. 2006
coxA_AspecF1	ATACCCACCTTTATCACAGG	*coxA*	*Wolbachia* A supergroup	56	Baldo et al. 2006
hcpA_F1	GAAATARCAGTTGCTGCAAA	*hcpA*	*Wolbachia*	54	Baldo et al. 2006
hcpA_R1	GAAAGTYRAGCAAGYTCTG	*hcpA*	*Wolbachia*	54	Baldo et al. 2006

2.2. *Wolbachia* Multilocus Sequence Typing (MLST)

2.2.1. PCR amplification and sequencing

Five different housekeeping genes which codify for essential proteins (Table 2) were used to genotype *Wolbachia* strains: the nuclear genes *gatB, ftsZ, hcpA, fbpA* and the mitochondrial gene *coxA. Wsp*, which codifies for a surface protein involved in the symbiont-host interaction, was also used. They are degenerated primers to be general for the arthropod *Wolbachia* supergroups. PCRs were carried out in 12.5µl final reaction volumes containing 6.4µl of water, 1.25µl of Buffer VWR 10x , 0.25µl $MgCl_2$ (25mM), 1.5 µl of dNTPs (2mM), 2µl of each primer (10pmol/µl), 0.1µl Taq polymerase (5u/µl, VWR) and 1µl DNA. Reactions were initiated by incubation for 2min at 94°C, followed by 37 cycles of 94°C for 30 sec, the optimal annealing temperature (Table 2.) for 45sec, and 72°C for 90 sec, there was a final elongation step of 72°C for 10 min. PCR products were purified using the PeqGold MicroSpin Cycle-PureKit (Peqlab) purifying kit.

One individual from each species that was positive for all the MLST genes was chosen and the genes sequenced. If there were two individuals of the same species that were positive for all the genes and were caught in different locations, both were sequenced. Every sample was sequenced in both directions and then blasted in Basic Local Alignment Search Tool (BLAST). Single infections were assumed if direct sequencing of PCR products was successful without prior cloning.

2.2.2. Cloning

Samples suspected to harbor more than one *Wolbachia* strain, due to double signals after sequencing, were cloned following the StrataClone PCR Cloning Kit (Stratagene) protocol. The amplicons were inserted in StrataClone SoloPack (Stratagene) competent cells following the manufacturer´s protocol (see Appendix). They were plated onto petri dishes with LB-ampicillin agar (see Appendix) spread with 40 µl of 2% X-Gal, and incubated at 37°C. After approximately 12 hours they were stored at 4°C to stop bacterial growth for approximately half an hour. White colonies were picked and subjected to amplification with the primer pair M13 fwd/rev (Table 2.). Temperature profile was as follows: an initial step of 3 min at 94°C, 32 cycles of 94°C during 40 sec, 65°C for 60 sec and 72°C for 60sec, and a final step of 72°C for 4 min. PCR products were sequenced bidirectionally with primers M13fwd (5´TGTAAAACGACGGCCAGT3´) and M13rev (5´-CAGGAAACAGCTATGAC-3´).

2.3. Phylogenetic analysis

Sequences were manually edited. Translation-alignments for each gene were built with other *Wolbachia* sequences belonging to different Hymenoptera, obtained from MLST website (http://pubmlst.org/wolbachia/). Alignments were checked by eye, and ambiguously aligned regions were excluded. Single gene phylogenetic trees were built using both distance (neighbor-joining (NJ)) and maximum likelihood (ML) algorithms. Trees were assessed using 1000 bootstrap replicates. Sequence editing, alignment and phylogenetic tree construction were done in Geneious 5.3.4 software.

2.4. Statistical analysis

Statistical analyses were performed to test for horizontal transfer and cospeciation between insects and *Wolbachia*. If there is horizontal transmission of *Wolbachia* between hosts and parasitoids we expect *Wolbachia* strains harboured by both partners to be identical or very similar. Therefore, we analyzed whether *Wolbachia* strains harbored by host and their respective parasitoids were more closely related to each other than expected by chance. In addition, we also analyzed the opposite option, strict vertical transmission. In this case, *Wolbachia* strains would be more similar between the chrysidids, which belong to the same family, than between the hosts, which are unrelated. Therefore, we analyzed whether differences among *Wolbachia* strains harbored by the *Crhysidids* were smaller than differences among hosts.

In order to do this, we built a distance matrix in which strain differences between pairs of individuals were noted, i.e. all individuals were compared against all individuals and the differences between them noted. Distances ranged from 0 (identical) to 1 (completely different). To test for the hypotheses permutation tests were run in R 2.12.2 using DAAG package.

3. Results

3.1. DNA extraction and *Wolbachia* prevalence

45 individuals were excluded from the analyses because they failed to yield 28s rDNA products, from which 30 were parasitoids. 38 samples yielded products of the correct size for insect specific primers but did not amplify any bacterial DNA when screened with eubacterial primers and were also excluded. Of the 96 individuals amplifying with both insect and bacterial primers, 31 were positive for bacteria, but did not show *Wolbachia*, being recorded as true *Wolbachia*-free samples. 65 were infected with *Wolbachia*, representing 67.7% of all samples and 59.3% of all species. Among infected species, rates of infection ranged from 62 to 100 % (Figure 5). 13 species presented high infection frequencies (>90%), 3 harbored medium infection frequencies, whereas no low ones (<10%) were detected (Tables 3 and 4).

As in earlier studies, honeybees (*Apis mellifera*, prey of *Philanthus triangulum*) were tested with *wsp* primer pairs and did not yield any product, being recorded as *Wolbachia*-negative.

3.1.1 Differences between hosts and parasitoids.

61.53% (8/13) of host species and 57.14% (8/14) of parasitoids were infected. Both groups were equally prone to *Wolbachia* infection (Fisher's exact test, p=0.83). Likewise, infection rates across species did not differ significantly between hosts and parasites (Mann-Whitney-U test, df=15, U=35, p=0.70)

Table 3 Infection statuses of hosts and rate of infection, as well as the number of strains sequenced. Geographic origin: Berlin (B), Alzenau (A), Ihringen (Ih), and Jena (J).

			Males		Females			Different
Host species	Geographic origin	Infected	Total	Infected	Total	Infected	Rate of infection (in %)	strains sequenced
Astata boops		+	1	1			100	1
Cerceris arenaria	B, A	-	5	0	1	0	0	
Cerceris interrupta	Ih	+			2	2	100	1
Cerceris quinquefasciata	A, B, Ih	-	1	0	1	0	0	
Cerceris rybyensis	B, J, A, Ih	+	2	1	6	4	62.5	1
Cerceris sabulosa	Ih	+			2	2	100	3
Lindenius pygmaeus		+			2	2	100	1
Microdynerus timidus		-			2	0	0	
Miscophus bicolor		+	2	2			100	1
Osmia adunca	Ih	+			2	0	0	0
Philanthus triangulum	Ih	+	1	1	2	2	100	1
Trypoxylon figulus		-			1	0	0	
Odynerus spinipes		+	7	7	5	5	100	1

Table 4. **Table showing sequenced genes for each species. Host-parasitoid pairs are grouped and shaded in the same colour.**

Parasitoid species	Geographic origin	Infected	Males Total	Males Infected	Females Total	Females Infected	Rate of infection (in %)	Different strains sequenced
Chrysis cortii		-	2	0	2	0	0	
Chrysis cyanea		+			1	1	100	3
Chrysura austriaca		-	1	0	3	0	0	
Chrysis gracillima		-			1	0	0	
Chrysis ignita		-			2	0	0	
Chrysis viridula		+			1	1	100	1
Hedychrum chalybaeum		+	2	2	2	2	100	1
Hedychrum gerstaeckeri	B	+	2	2	7	7	100	2
Hedychridium krajniki		-			1	0	0	
Hedychrum nobile	A, B	+	2	2	7	7	100	1
Hedychridium roseum		+	1	1	3	3	100	1
Hedychrum rutilans	B	+	2	2	7	4	88.8	3
Pseudospinolia neglecta		+			3	2	66.66	1
Trichrysis cyanea		-	1	0	3	0	0	

3.1.2. Double infections

Of the 16 species found to harbor *Wolbachia* in this study, 4 harbored more than one *Wolbachia* strain (Tables 3 and 4). Therefore, the frequency of double infections within infected species was 25%. All strains belonged to the A supergroup, with the exception of *Chrysis cyanea* which harbored three B supergroup strains.

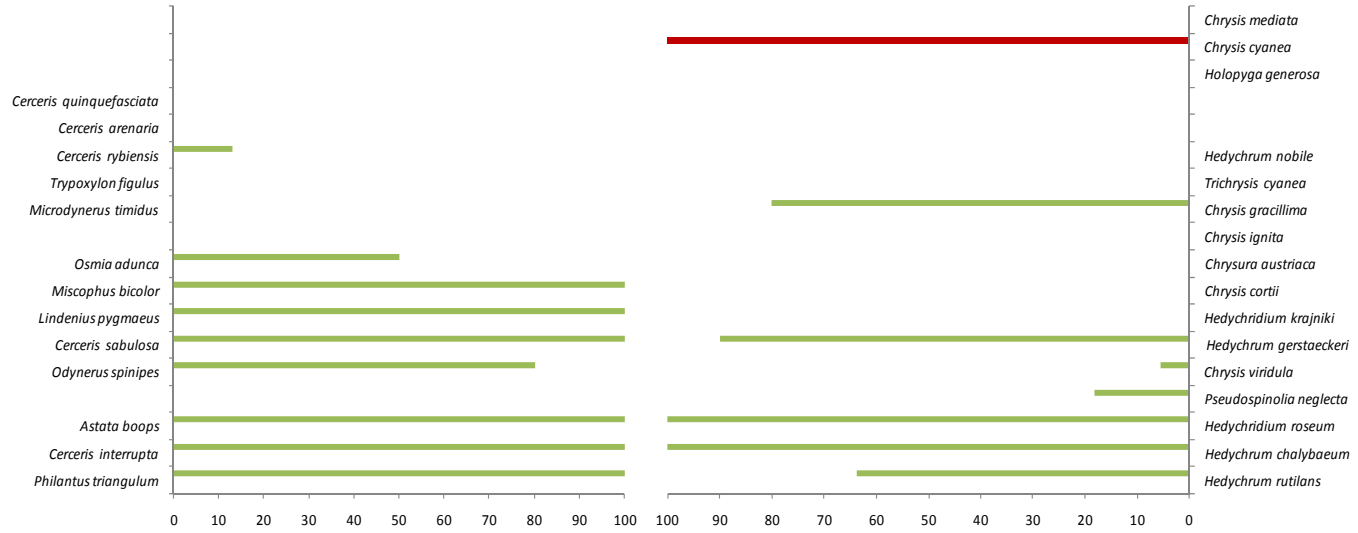

Figure 4. **Graph depicting *Wolbachia* infection frequencies in hosts (left) and parasitoids (right). Each host is presented against its parasitoid. *Pseudospinolia neglecta*, *Chrysis viridula* and *Odynerus spinipes* form a tripartite relationship. Green bars represent A supergroup *Wolbachia* strains, the red one represents the only case of a B supergroup strains found in this study.**

3.2. MLST

For MLST, not all five genes could be successfully amplified and sequenced for every sample. There were some positive hosts whose parasitoid was not positive for *Wolbachia* and vice versa. Therefore, from the 11 host-parasitoid pairs that were initially screened for *Wolbachia*, only five could be used to perform the comparative phylogenetic analyses. However, all samples were taken into account when analyzing rates of infection.

Table 5. Table showing sequenced genes for each species. Host-parasitoid pairs are grouped and shaded in the same color.

Species	wsp	ftsZ	coxA	fbpA
Hedychrum rutilans	+	+	+	+
Philanthus triangulum	+	+	+	+
Hedychrum chalybaeum	+	+	+	+
Cerceris interrupta	+	+	+	+
Hedychridium roseum	+	+	+	+
Astata boops				+
Pseudospinolia neglecta	+			
Chrysis viridula	+			
Odynerus spinipes	+	+		
Hedychrum gerstaeckeri	+	+	+	+
Cerceris sabulosa	+	+		+
Lindenius pygmaeus	+	+	+	+
Miscophus bicolor		+	+	
Hedychrum nobile	+	+	+	+
Chrysis cyanea	+	+	+	+

Wsp was the gene that overall was amplified and sequenced most successfully across species, followed by *ftsZ, fbpA* and *coxA*. The other genes *hcpA* and *gatB* were excluded from the analyses, due to amplification failure in a great number of samples.

Two genes were amplified for *Pseudospinolia neglecta* and *Chrysis viridula*, however, due to sequencing problems, only one (*wsp*) was sequenced. Only one gene (*fbpA*) of *Astata boops* was amplified and sequenced.

Thus, the following pairs were used for comparative phylogenetic and statistical analysis: *Philanthus triangulum – Hedychrum rutilans, Cerceris interrupta – Hedychrum chalybaeum, Astata boops – Hedychridium roseum, Odynerus spinipes - Pseudospinolia neglecta – Chrysis viridula* and *Cerceris sabulosa – Hedychrum gerstaeckeri.*

3.3. Phylogenetic analysis

Single gene phylogenetic trees were constructed. For all genes both NJ and ML trees look very similar. In all except the *wsp* tree, three different groups can be distinguished. Supergroups A and B contain several different *Wolbachia* strains belonging to different insects, whereas the F supergroup shows only one harbored by *Apoica pallens*, which was used to root the tree.

Philanthus triangulum and *Hedychrum rutilans* cluster together in all four trees. *Cerceris interrupta* and *Hedychrum chalybaeum* cluster together in three trees (*ftsZ, coxA and fbpA*), whereas they cluster separated from each other in the *wsp* tree. Both pairs *Astata boops* - *Hedychrum roseum* and *Odynerus spinipes – Pseudospinolia neglecta – Chrysis viridula*, cluster together in the trees in which they could be included (*wsp* and *fbpA* respectively). On the contrary, *Cerceris sabulosa* and *Hedychrum gerstaeckeri* only appear close to each other in the *coxA* tree, whereas they cluster separately in all the others.

Surprisingly, all pairs –with the exception of *C.sabulosa* and *H. gerstaeckeri*- clustered together in the *fbpA* tree, indicating that they all harbor very similar *Wolbachia* strains.

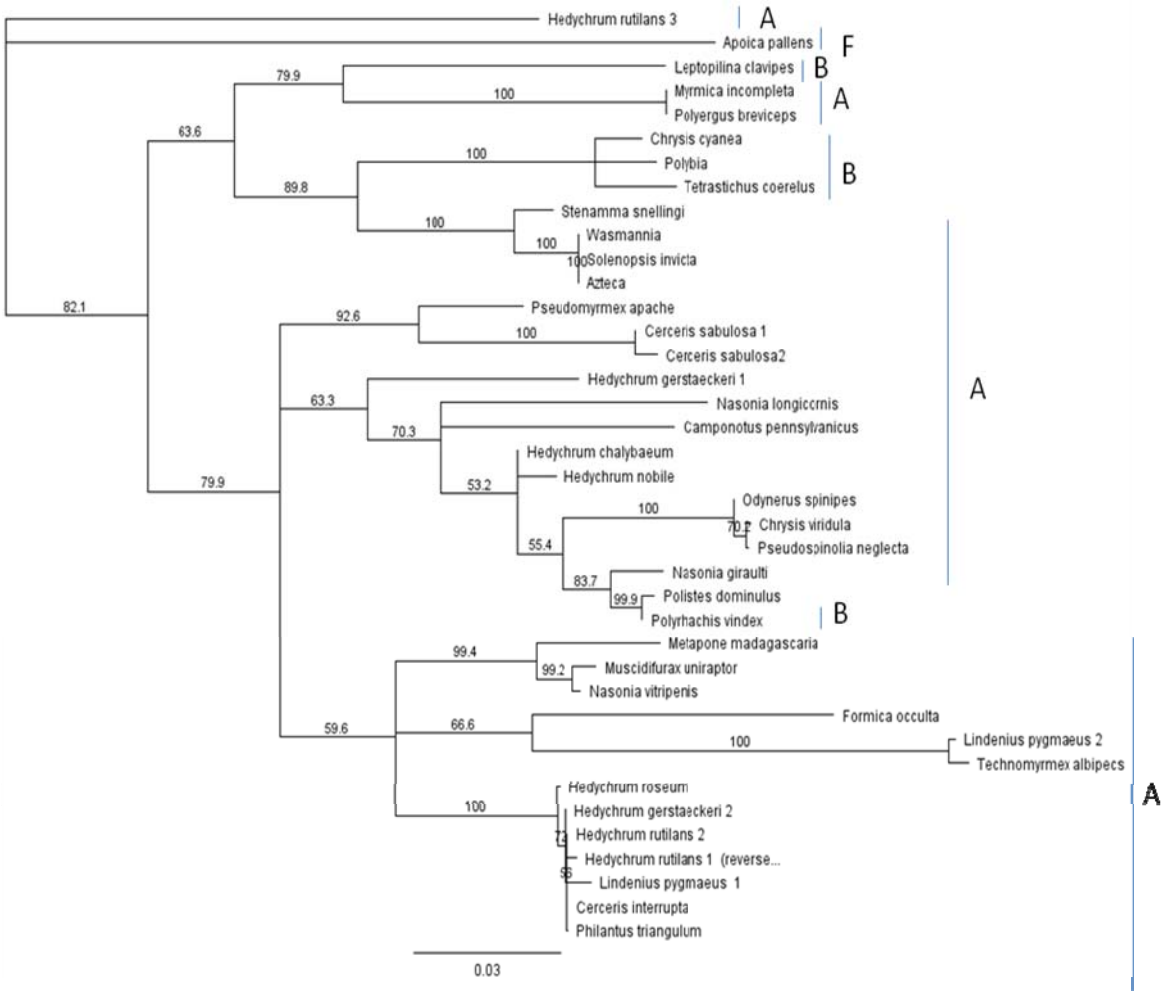

Figure 5. **Wsp NJ phylogenetic tree based on a 603bp translation-alignment. Bootstrap of 1000 replicates.**

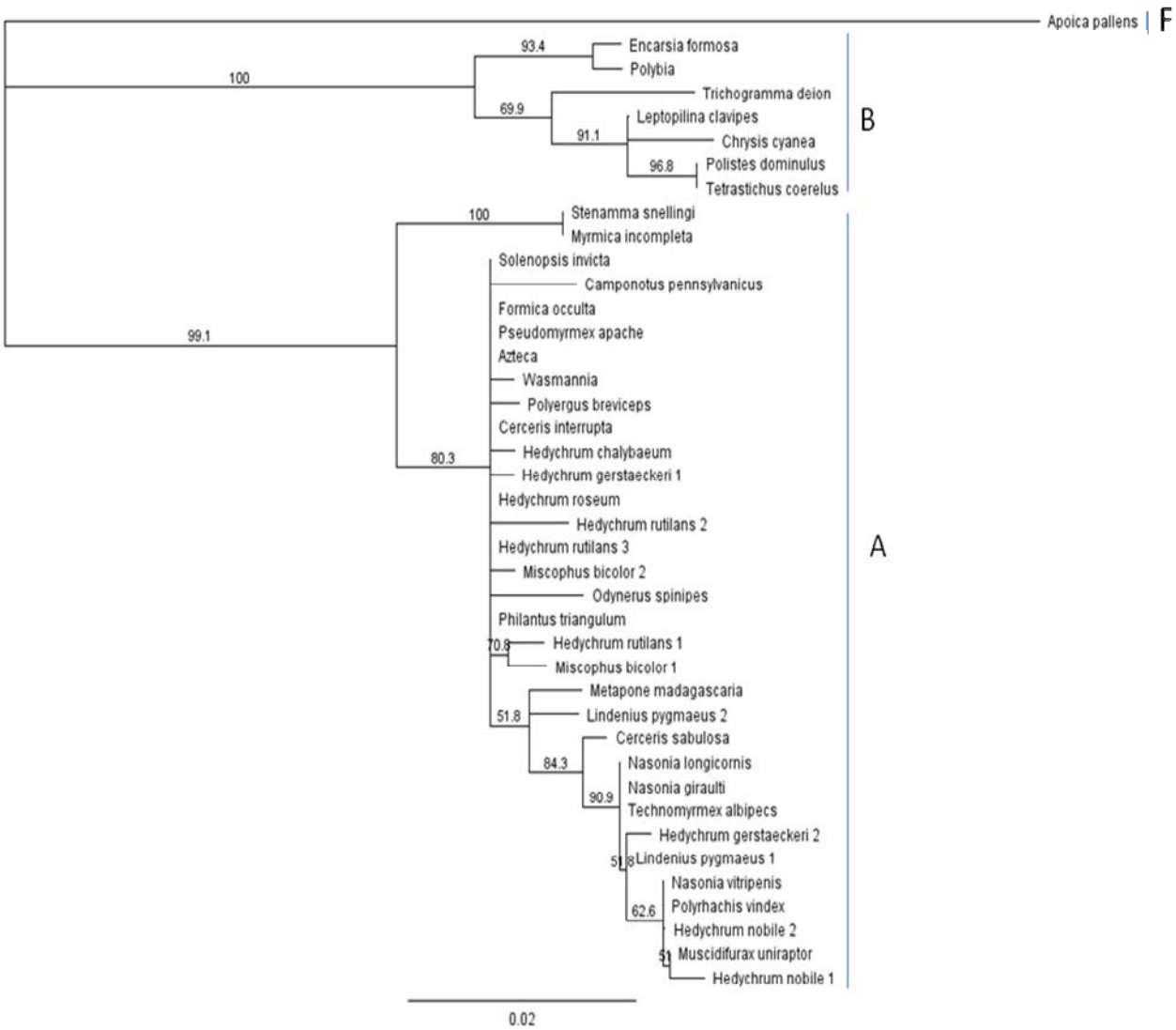

Figure 6. **FtsZ phylogenetic tree based on a 481 bp translation-alignment. Bootstrap of 1000 replicates.**

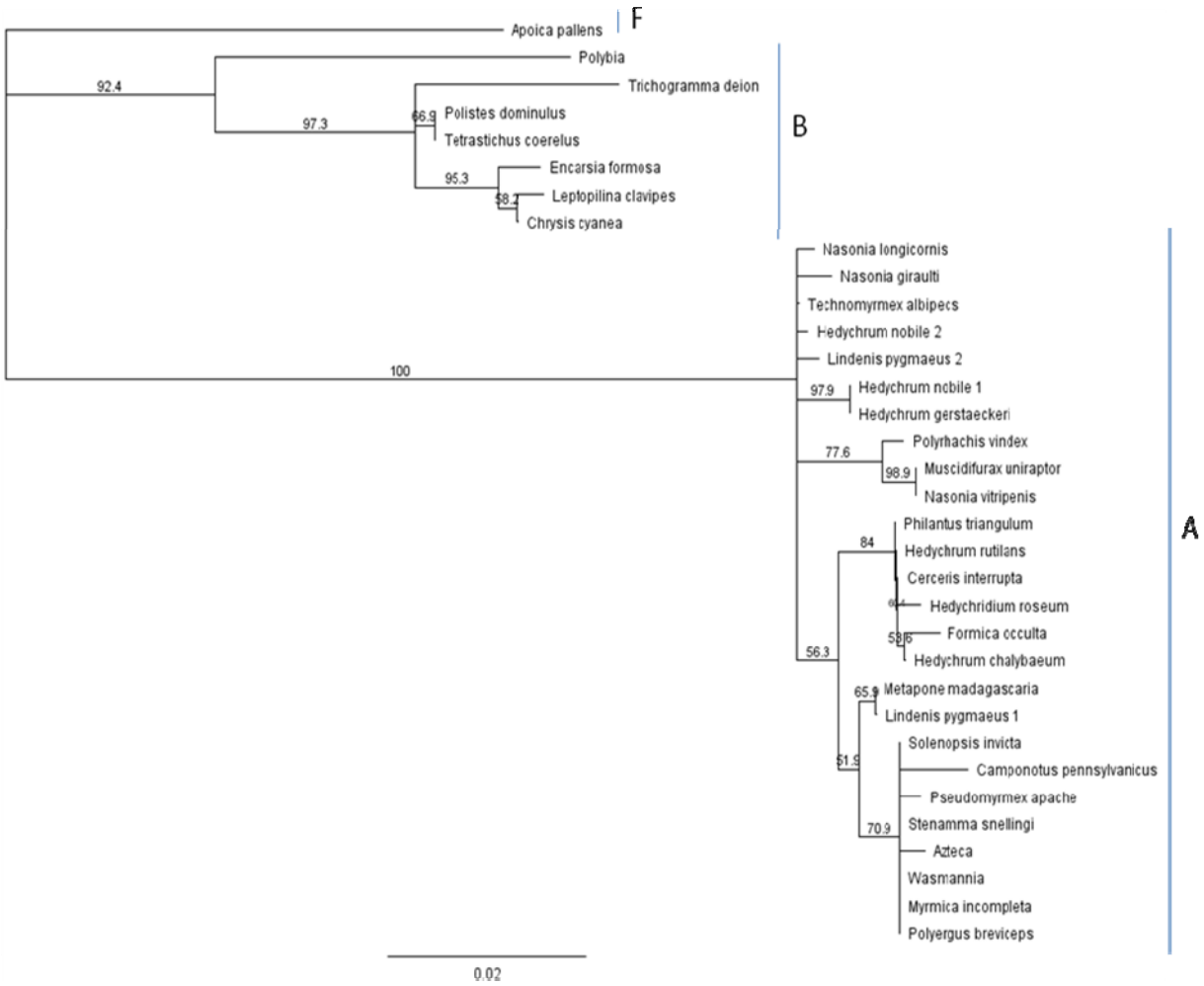

Figure 7. CoxA phylogenetic tree based on a 449 bp translation-alignment. Bootstrap of 1000 replicates.

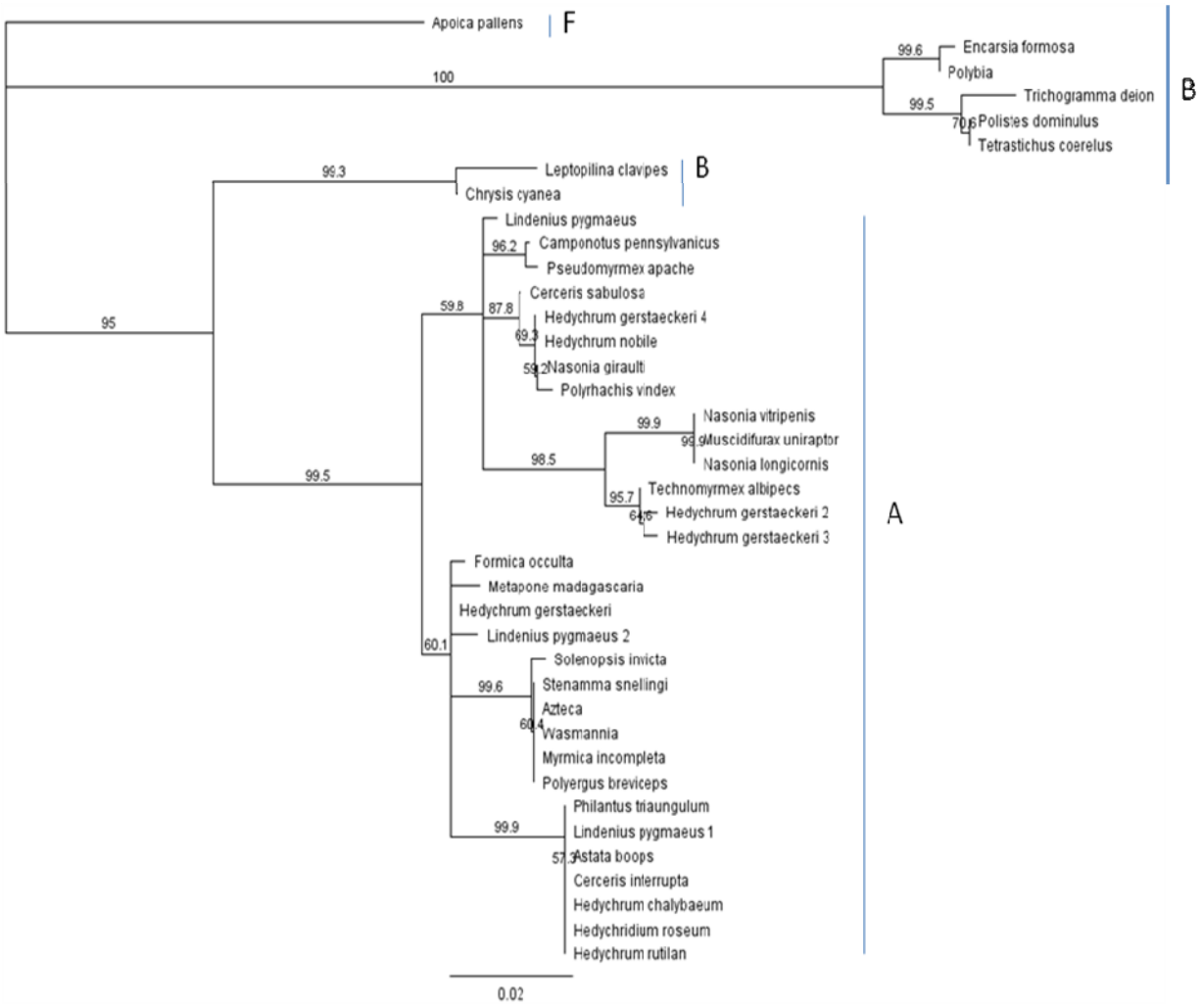

Figure 8. **FbpA NJ phylogenetic tree based on a 467 bp translation-alignment. Bootstrap of 1000 replicates.**

3.4. Statistical analysis

Statistical analysis testing for horizontal transfer and strict vertical transmission were performed for each gene. For three out of four genes, average distances of *Wolbachia* strains in host-parasitoid pairs were significantly lower than of *Wolbachia* strains from hosts and parasitoids that are not associated with each other (permutation tests; *wsp:* p=0.009, *coxA:* p=0.013, *fbpA:* p=0.049, *ftsZ:* p=1), suggesting that horizontal transmission of *Wolbachia* from hosts to parasitoids has occurred frequently. There was no evidence for cospeciation between insect hosts and *Wolbachia*, as *Wolbachia* strains of *Chrysidid* parasitoids were not more closely related to each other than were *Wolbachia* strains of the phylogentically much more diverse hosts (permutation tests; p-value>0.3 for all four genes).

4. Discussion

4.1. *Wolbachia* prevalence

We evaluated *Wolbachia* infection in different Hymenoptera host-parasitoid pairs in order to compare strains and analyzed whether there is horizontal transfer of *Wolbachia* from host to parasitoids in those pairs.

DNA extraction did not work in a number of samples, being parasitoids a major part of them. DNA failure was probably due to technical problems. Surprisingly, some samples were successfully amplified with insect- but not bacteria-specific primers. It is unlikely that these individuals are bacteria-free, as there should be at least bacterial DNA from the gut microbiota or from the insect surface. Thus, the apparent lack of bacteria is more likely to be caused by a procedure failure. Samples that did not amplify insect or bacterial DNA were not included in the dataset. This procedure is conservative with regard to the hypotheses tested.

Some samples presenting insect and bacterial DNA failed in amplifying *Wolbachia* and were recorded as *Wolbachia*-free. Yet, this result has to be interpreted carefully given that failure in amplification with *Wolbachia*-specific primers may be due to several reasons, e.g. *Wolbachia* might be absent in the insect, primers might not be specific enough, or they might be too specific and fail to amplify different *Wolbachia* strains. *Wolbachia* detection might have also been problematic due to low densities within individuals, in which bacterial titre might have been below the PCR detection threshold (Jeyaprakash *et al*. 2000).

Despite this, we found 59.3% of all studied species to be infected. It has been reported that *Wolbachia* presents a "most-or-few" infection pattern, with *Wolbachia* infections being either very high (>90%) or very low (<10%) (Hilgenboecker *et al*. 2008). In our study only high and medium infection frequencies were detected, although medium ones were rare. This result is not surprising since our analyses are based on screening few individuals per species, so low-frequency infections will appear as uninfected or as medium to high infection rates. Taking this into account, our study has shown 61.5% of hosts and 57.1% of parasitoids to be infected, with the rate of infection not being significantly different between these two groups. This is unexpected since parasitoids have usually higher infection frequencies than hosts (Vavre *et al.* 1999), probably due to their life style. Ectoparasitoids can acquire *Wolbachia* vertically, horizontally from their usual hosts as well as from occasional ones and also from other unknown sources, whereas hosts can only acquire it vertically or from an unknown source, since there are almost no surviving hosts after parasitism.

Differences in rates of infection between species could be due methodological or ecological reasons. Methodological reasons mainly refer to bacterial titre and PCR sensitivity, i.e. bacterial densities might be lower in some species than in others and therefore be more difficult to detect. It could also be caused by a sampling bias, where more infected individuals could have been caught from one species just by chance, although this is very unlikely. Ecological reasons depend on host and insect biology. Some species may be, for instance, resistant to *Wolbachia*. Despite the great amount of literature on aphids, there are not many reported *Wolbachia* infections. It has been

proposed that these rare infections in aphids may be due to resistance to *Wolbachia* driven by aphid´s primary and secondary symbionts (Valero *et al.* 2004).). A second option is that some species might be more prone to acquiring and maintaining infections than others (Werren *et al.* 2000). Another possibility is that some species while being equally prone to infection may be more exposed to *Wolbachia* than others (West et al. 1998). For example, gregarious woodlice species are more liable to infection by hemolimph contact after injury due to aggregation than solitary ones (Rigaud *et al.* 1995). *Wolbachia´s* vertical transmission is not 100% effective; it depends on the mother infection status, and is not identical for different strains, which could also lead to differences in rates of infections (Narita *et al.* 2007). This is an ecological reason that will influence methodological detection of *Wolbachia*, since imperfect vertical transmission leads to low bacterial densities (Dutton *et al.* 2004). Besides, other factors such as host and bacterial genotype or environmental as temperature, can influence bacterial load (Mouton *et al.* 2007).

Our study is probably underestimating the real number of infected species and individuals, since not all populations of a given species, or individuals within a population might be infected with *Wolbachia*, given that vertical transmission is not perfect (Cook *et al.* 1999). Thus, results from screening strategies that are based on few samples per species, are biased towards an underestimate of infected species (Hilgenboecker *et al.* 2008).

4.2. MLST and phylogenetic analysis

Several genes from 14 different species were amplified and sequenced. *Pseudospinolia neglecta*, *Cerceris rybyensis* and *Chrysis viridula* were amplified with two genes but only one could be sequenced, due to difficulties in the sequencing process. Surprisingly, only one *Wolbachia* gene was successfully amplified for *Astata boops*. Reasons for the failure of amplification of *wsp, ftsZ* and *coxA* genes might be the same as for normal *Wolbachia*-screening PCR mentioned above. However, there is a second possibility. This individual might be in fact *Wolbachia*-free and harbor the *fbpA* gene as a result of an earlier horizontal gene transfer from *Wolbachia* to the insect genome. Horizontal gene transfer between symbionts and hosts was thought to be very rare, but recent studies have shown that it is more common than expected (Dunning-Hotopp *et al.* 2007). *Drosophila ananassae*, for instance, has inserted in its genome almost the whole *Wolbachia* chromosome (Dunning-Hotopp *et al.* 2007).

All the sequenced strains belonged to *Wolbachia's* A supergroup with the exception of *Wolbachia* strains harbored by *Chrysis cyanea*, which belonged to supergroup B. It has been previously reported to exist differences between insect orders in their relative frequencies of A or B infections, being supergroup A the most common in Hymenoptera (Werren et al. 2000), indicating different abilities of the supergroups to infect specific taxa, or differences in maintaining such infections. In our study there were no superinfections with strains belonging to different supergroups, although 4 species harbored more than one strain. This is unexpected since superinfections with A and B strains are very common in insects (Werren et al. 1995a) and occur significantly more frequently than expected by chance (Werren et al. 1995). A misdetection of double infections might happen if one of the strains is present in a very low density, being imperceptible for the screening methods used. Since our MLST analysis has been performed on just one individual per species, we ignore if there are multiple infections at a population level.

Phylogenetic analyses

Phylogenetic trees for *ftsZ, coxA* and *fbpA* are consistent in the information they provide, showing groups A and B clearly clustering separately. In the *wsp* tree, however, species belonging to A and B supergroups appear intermingled. This might be caused by one or several factors: (i) *wsp* is a highly variable gene due to the high rate of recombination, (ii) horizontal gene transfer might have occurred, or (iii) because the third position in each codon is saturated. In protein-coding genes, the third position in a codon is often highly variable, which do not normally change the amino acid that it codifies. However, if in two different individuals, the same third nucleotide in a condon suffer a first different mutation (i.e. changing from A to T in the first individual and from A to G in the second), and a second mutation that leads to the same new nucleotide (this is from T to A and from G to A), the amino acid produced after that codon in both individuals remains the same although, the sequence history or sequence evolution is completely different.

Horizontal transfer

Our study shows evidence for horizontal transfer from hosts to their Hymenopteran parasitoids. Most of the host-parasitoid pairs for which we could perform the phylogenetic analysis harbor identical or extremely similar strains. There is some variability in some genes across species, but overall it is very low.

Philanthus triangulum and *Hedychrum rutilans* cluster together in all four trees. *Odynerus spinipes*- *Pseudospinolia neglecta- Chrysis viridula* appear close to each other in the *wsp* tree, which was the only one that could be built due to failure in amplification for other genes. *Astata boops* and *Hedychridium roseum* harbor identical *Wolbachia* strains in the *fbpA* tree. *Cerceris interrupta* and *Hedychrum chalybaeum* cluster together in two trees (*ftsZ* and *fbpA*), and are reasonably close in the *coxA* tree, whereas they cluster far from each other in the *wsp* tree. This could be explained, again, albeit with difficulties, by the variability in *wsp* gene. Another possibility is a low-density-strain misdetection. If a second strain was present in any of the insects at a very low density, it is feasible that it was amplified by chance only once, for the *wsp* gene. Strain differences would have been large enough to cluster distantly in the tree. For the rest of genes, the same strain would have been sequenced and therefore cluster together. The parasitoid could have acquired that second *Wolbachia* strains from an untested occasional host (Vavre *et al*. 1999).

Cerceris sabulosa cluster together with its parasitoid *Hedychrum gerstaeckeri* in only one tree (*fbpA*) and far from each other in the others. *C. gerstaeckeri* has two different hosts, *Cerceris rybyensis* and *Cerceris sabulosa*, which usually do not share geographical location. However, in this study we have used individuals from the three species coming from the same geographical area. Therefore, it is highly likely that the *Chrysidid* parasitizes both species in that particular area and can harbor *Wolbachia* strains belonging to both hosts. We cannot prove this, since amplification of *C. rybyensis* was unsuccessful, but it remains open for further investigation. If strains belonging to each of the hosts occur in the parasitoid at different densities, it is possible that we have not been able to amplify and sequence strains that they might share with *C. sabulosa* in more than one case.

Surprisingly, there is one case in which host and parasitoid that do not belong to same pair cluster together. This is the case for *C. gerstaeckeri* and *P. triangulum* in the *wps* and *ftsZ* trees, in which they appear to harbor closely related *Wolbachia* strains. Although our experience leads us to think

that parasitoids are specialists in terms of hosts (Oliver Niehuis, personal communication), these last two facts might indicate that we have overestimated parasitoid specificity.

Unexpectedly, all tested pairs clustered together in the *fbpA* tree, suggesting that they harbor similar *Wolbachia* strains. Unrelated hosts harboring identical *Wolbachia* strains have been previously reported (Shoemaker *et al.* 2002), although the mechanism by which horizontal transfer between hosts occurs is unknown.

Support for some of the phylogenetic tree branches is not very high; however, since our statistical test was performed on the basis of the distance matrix, it does not affect our results. The statistical analysis testing for horizontal transmission was significant for three out of four genes, meaning that *Wolbachia* strains harbored by host and parasitoids from the same pair are more similar to each other than expected by chance, which points to horizontal transfer. The non-significant value for the *ftsZ* gene might be due to the low resolution in the phylogenetic trees, since there are many soft polytomies. To strengthen our hypothesis, we have also tested a different possibility, that the sequences of Wolbachia belonging to *Chrysidids* are more similar among each other than the strains harbored by hosts among themselves, which would indicate vertical transmission. This was not significant in any case, showing that *Wolbachia* strains harbored by *Chrysidids* are as different among each other as they are with the strains harbored by their hosts. We have, therefore, evidence supporting the hypothesis that there is horizontal transfer of *Wolbachia* from host to parasitoid.

It can be argued that *Wolbachia* might be acquired by consuming food resources, given that *Chrysidid* parasitoids exploit not only the host larvae bur also the stored insects. We have ruled out this possibility for *Philanthus triangulum* and *Hedychrum rutilans*, since all tested bees were *Wolbachia*-free. This correlates with previous studies reporting European honeybees to be *Wolbachia*-free. However, we cannot discard this possibility for the remaining pairs since we have not been able to analyze other species' food resources. If they are *Wolbachia*-positive, it cannot be stated with certainty that transfer of *Wolbachia* has occurred from host to parasitoid or from food to both host and parasitoid. However, both cases would constitute examples of horizontal transfer. In the absence of further experiments to confirm the infection status of food resources for some of the samples, we can affirm that our data strongly suggests horizontal transfer is a probable manner in which *Chrysidid* wasps might acquire *Wolbachia* by feeding on their infected hosts, as it has been reported for other parasitoid species (Werren *et al*. 1995b).

This study further supports horizontal transfer as a method by which *Wolbachia* maintains itself in populations and invade new host species broadening the already impressive range of insects it can infect. It also supports the idea of host-parasitoid interactions being one of the major sources of interespecific infection (Vavre *et al*. 1999; Huigens *et al.* 2004; Heath *et al.* 1999). Other routes in which *Wolbachia* might be horizontally transmitted are predator-prey interactions and cannibalism (Cook *et al.* 1999).

However the horizontal transfer mechanism occurs, *Wolbachia* has to cross several different tissues in order to invade an organism and be transmitted to the next generation. In the case of the *Chrysidid* exoparasitoids, *Wolbachia* has to cross from the gut lumen to the ovaries, in order to be vertically transmitted to the next generation. It is unknown how *Wolbachia* moves outside the gut, but a mechanism has been proposed for its movement in the ovaries. It has been shown that

Wolbachia reaches the germline through the somatic stem cell niche in *Drosophila melanogaster´s* germarium, and they are very abundant in the somatic stem cell niche, which suggests that this location helps to efficient vertical transmission (Frydman *et al.* 2006). It has been suggested that *Wolbachia* uses the host´s microtubule cytoskeleton and transport system to aggregate themselves during the middle stages of oogenesis in the future oocyte (Ferree *et al.* 2005).

Applications

Wolbachia can be used as a biocontrol agent. Since it can cause parthenogenesis, introducing thelytokous strains in parasitoids would be very useful in pest control. They are cheap and easily produced, since sex-ratio is not a problem; released individuals and their progeny will be females, which will develop killing hosts; and new generations are easily produced, since there is no need for males (Cook *et al*. 1999).

Wolbachia´s horizontal transfer has several applications in control of insect-borne diseases. Some *Wolbachia* strains have lost the ability to replicate at the same time as their host and shorten their host (*Drosophila melanogaster*) life span in more than 50% (Min et al. 1997). It has also been reported, that these strains also reduce lifespan of female mosquitoes (Brownstein et al. 2003). Since pathogens need some time to replicate themselves within the mosquito vector body before reaching the salivary glands, this lifespan reduction might help in controlling diseases such as dengue and malaria. Therefore, artificial horizontal transfer of *Wolbachia* is carried out in the lab from *Drosophila melanogaster* to female mosquitoes in order to fight diseases.

5. Conclusion

In this study, we screened Hymenopteran host-parasitoid pairs for the presence of *Wolbachia* and investigated the possibility of interspecific horizontal transfer. Five host-parasitoid pairs were identified in which both partners harbor similar *Wolbachia* strains at least in one case. Previous studies highlighted that the fact that both hosts and parasitoids belonging to the same pair harbor very similar or identical *Wolbachia* strains, strongly suggests that horizontal transmission has occurred. Our survey shows further evidence of horizontal transfer of *Wolbachia* between different taxa in natural populations. It is important to note that this mechanism may be more common than previously thought, since horizontal transfer was detected despite a low number of available host-parasitoid pairs.

Species or guilds previously reported as not undergoing horizontal transfer of *Wolbachia* may indeed experience this transmission but not at extremely high frequencies and bacterial densities for infection establishment.

We are confident that further studies surveying larger number of individuals per species, and using more sensitive approaches, will be successful in finding more evidence for horizontal transfer events of *Wolbachia* among insect hosts.

6. References

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7. Apendix: Phylogenetic trees

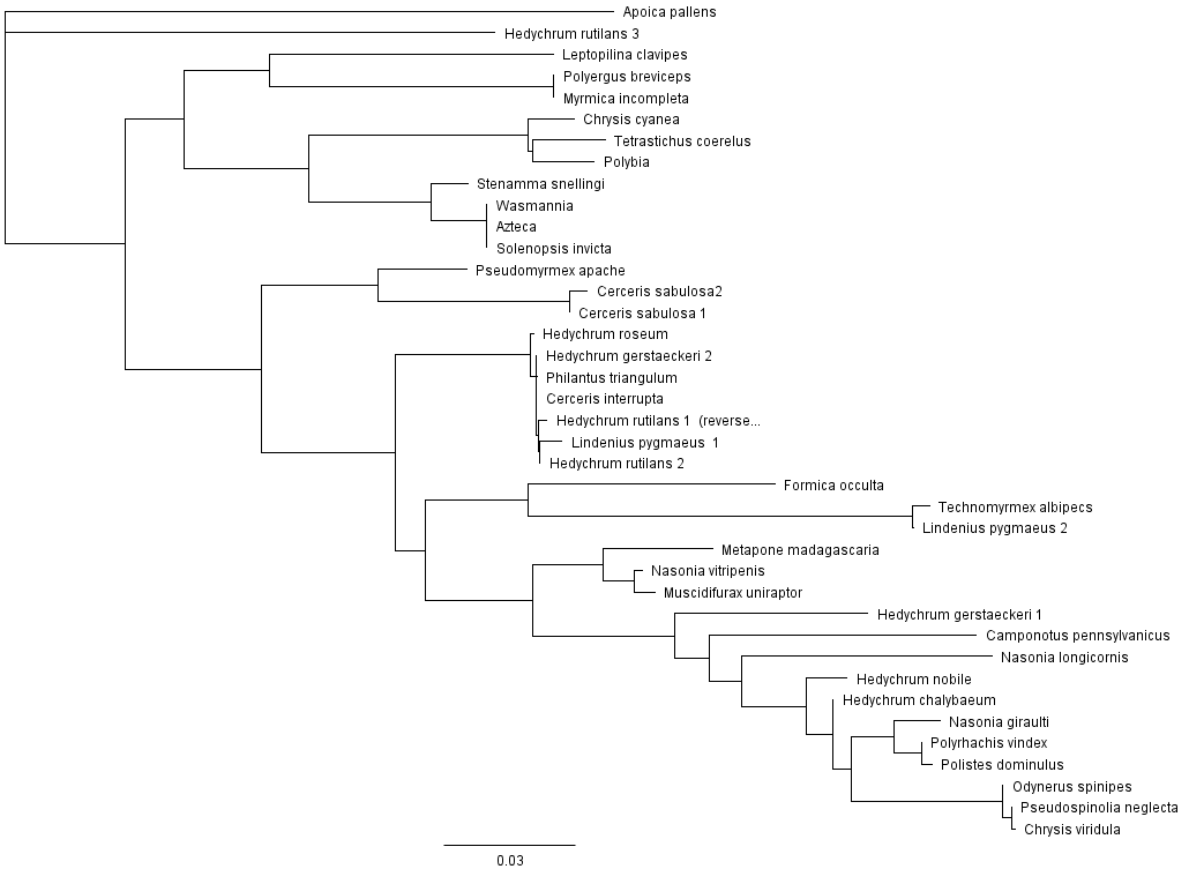

Figure 2 ML phylogenetic tree based on a 601bp translation-alignment of the *wsp* gene. Bootstrap of 100 replicates.

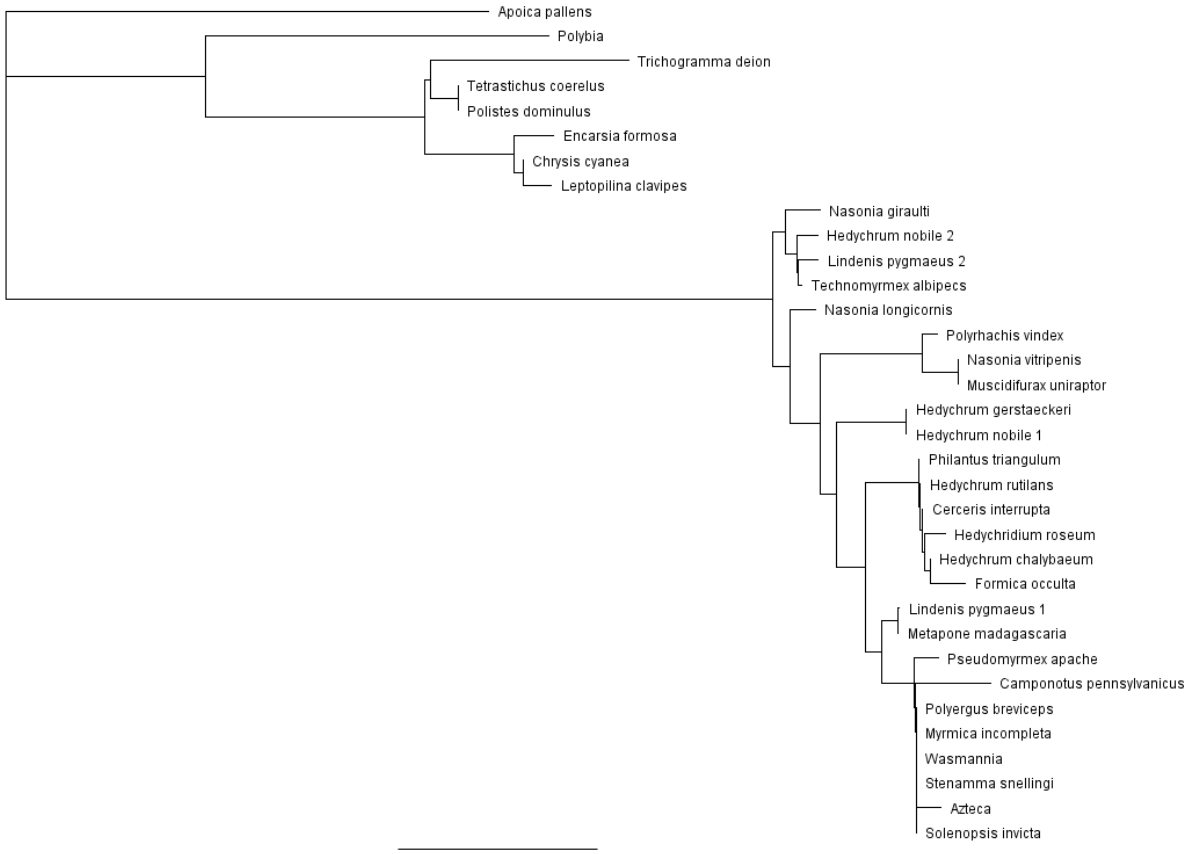

Figure 3 ML phylogenetic tree based on a 481bp translation-alignment of the *ftsZ* gene. Bootstrap of 100 replicates.

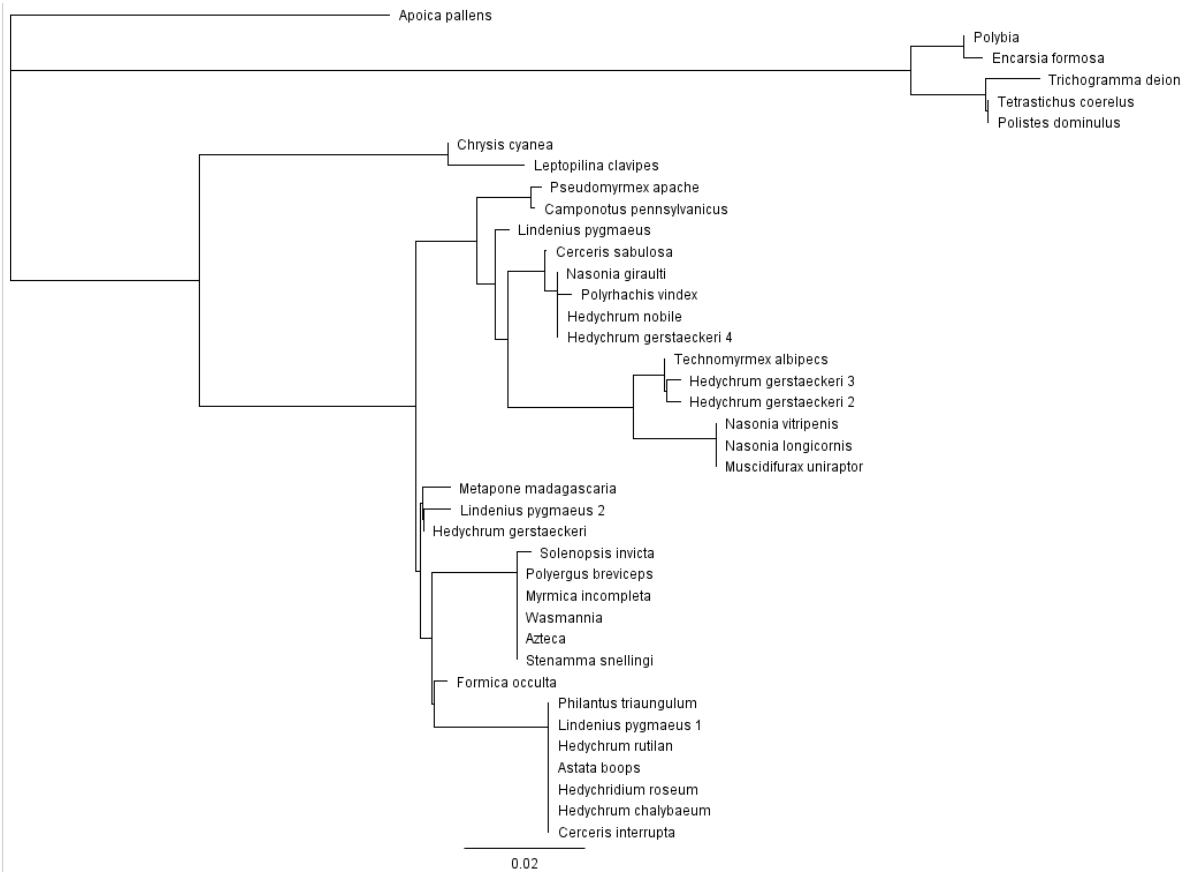

Figure 4 ML phylogenetic tree based on a 449bp translation-alignment of the *coxA* gene. Bootstrap of 100 replicates.

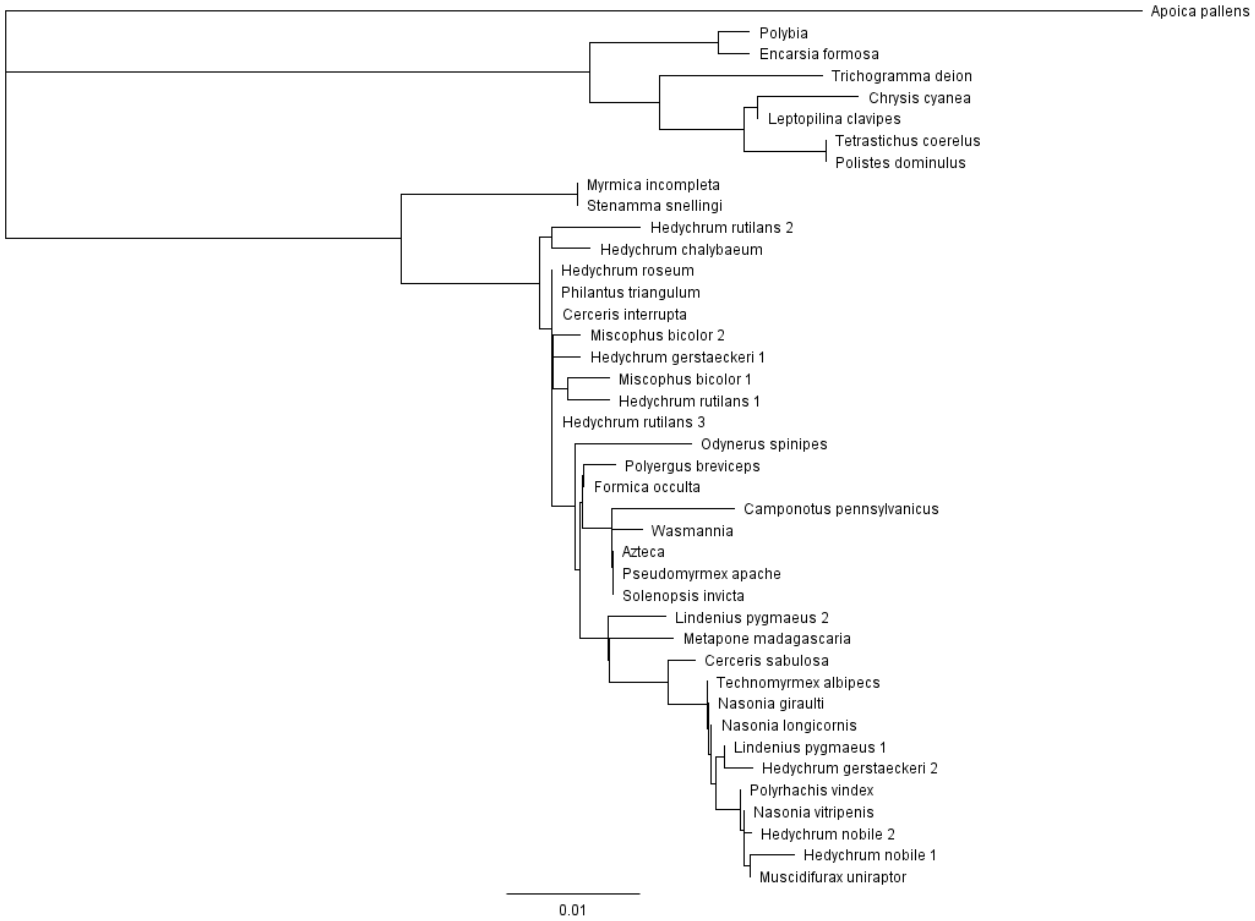

Figure 5 ML phylogenetic tree based on a 467bp translation-alignment of the *fbpA* gene. Bootstrap of 100 replicates.

8. Protocols

DNA extraction protocol: Epicentre MasterPure™ DNA isolation

1. Add liquid nitrogen and grind to homogenize the sample
2. Add 300 µl of Tissue and Cell Lysis Solution
3. Add 4 µl of lysozym (100mg/ml) if needed
4. Add 5 µl ProK (10mg/ml)
5. Incubate at 60-65°C for 15 min
6. Place samples on ice for 3-5 min
7. Add 150 µl of MPC Protein Precitpitation Reagent
8. Vortex vigorously (10 sec)
9. Pellet the debris by centrifugation (10 min., 10000rpm)
10. Transfer the supernatant to a clean Eppendorf cup (discard the pellet)
11. Add 500 µl of isopropanol
12. Invert the tube 30-40 times to mix
13. Store at -20°C overnight (or at east 1h)
14. Pellet the DNA by centrifugation (10 min., 14000rpm)
15. Carefully remove and discard the supernatant
16. Add 200 µl of cold 70% EtOH
17. Mix for 10 sec.
18. Centrifuge for 5 min. at 14000 rpm
19. Carefully remove and discard the supernatant
20. Let the samples dry under the hood (ca. 1 hour)
21. Resuspend the DNA with 50-100 µl of Low TE
22. Store samples at -20°C or -70°C

Polymerase Chain Reaction

Mastermix for 12,5 µl reaction (11,5 µl reaction mix +1 µl template)

- 6,4 µl H2O
- 1,25 µl Buffer
- 0,25 µl MgCl2
- 1,5 µl dNTPs
- 1 µl Primer1 (10pmol/µl)
- 1 µl Primer2 (10pmol/µl)
- 0,1 µl Taq

Gel for electrophoresis (1 gel)

1. Add 0,75 g agarose to 50 ml of TBE
2. Heat until the mixture is transparent and clear
3. Add 5 µl GelRed
4. Pour in a mold and let solidify for 20-30 min.

PeqGold MicroSpin Cycle-PureKit (PeqLab)

1. Determine the volume of the reaction.
2. Add 125 µl volume of Buffer CP to 12,5 µl PCR reaction.
3. Apply solution to PerfectBind MicroSpin TM DNA column assembled in 2 ml collection tube.
4. Centrifuge at 10,000 x g for 2 min. at room temperature.
5. Discard liquid.
6. Place column into clean 1,5 ml. tube.
7. Elute DNA with 20 µl DNA Elution Buffer.
8. Centrifuge 1 min. at 8000 x g.

Soil DNA Purification Kit Protocol Epicentre

1. Preparation
    a. Spin columns
        i. Add 550 µl of Inhibitor Removal Resin to each empty Spin Column to be used. Centrifuge for one minute at 2000 x g to pack the column.
        ii. Decant flow-through and place the column in the same collection tube.
        iii. Add another 550 µl of Inhibitor Removal Resin to each packed column. Centrifuge for 2 min at 2000 x g.
        iv. Move the column to a clean 1.5 ml collection tube.
    b. Pellet Wash Solution:
        i. For 50 extractions Kit: Add 45 ml of ethanol to the Pellet Wash Solution before first use.
        ii. For 5 Extraction Kit: Add 4.5 ml of ethanol to the Pellet Wash Solution before first use.
2. Purification
    a. Extract DNA.
    b. Carefully transfer 100-150 µl of the supernatant directly onto the prepared Spin Column (from section 1).
    c. Centrifuge for 2 min at 2000 x g into the 1.5 ml tube. Discard the column.
    d. Add 6 µl of DNA Precipitation Solution (stored at -20°C), vortex briefly. Incubate the tube at room temperature for 5 minutes.
    e. Centrifuge for 5 minutes at maximum speed. Carefully decant the supernatant.
    f. Wash the pellet with 500 µl of Pellet Wash Solution (prepared in section 1). Invert to mix, then spin for 3 minutes at maximum speed. Carefully decant the supernatant.
    g. Repeat the wash and spin.
    h. Resuspend the pellet in 300 µl of EB Buffer.

Purification for sequencing with EtOH-precipitation

1. PCR-product
2. +1/10 Vol. 3M NaAc (Room temperature)
3. +2 Vol. 100% EtOH (Room temperature)
4. Let the mixture precipitate for 30-60 min. at room temperature
5. Centrifugate at 14.000rpm, discard the supernatant
6. Wash with 70% EtOH (same volume as the precipitation-volume)
7. Let it at room temperature for 10-15 min
8. Centrifuge at 14.000rpm, discard the supernatant
9. Let dry under the hood
10. Suspend in Merck water

Sequencing preparation

1. Use strip tubes
2. Determine the compound of DNA in the sample with Nanodrop
3. PCR product:
    a. < 500 bp: 25ng/per preparation
    b. >500 bp: 30-40 ng/per preparation
4. Primer: 0,5 µl (10 pmol/ µl)
5. Merck water: refill until 6 µl

Preparation of media and reagents

1. LB agar (per liter)
    a. 10 g of NaCl
    b. 10 g of tryptone
    c. 5 g of yeast extract
    d. 20 g of agar
    e. Add deioniyed H2O to a final volume of 1 liter
    f. Adjust pH to 7.0 with 5N NaOH
    g. Autoclave

h. Pour into petri dishes (~ 25 ml/100mm plate)

2. LB-Ampicillin Agar (per liter)

    a. 1 liter of LB agar, autoclaved

    b. Cool to 55°C

    c. Add 10 ml of 10mg/ml filter- sterilized ampicillin

    d. Pour into petri dishes (~25 ml/100mm plate)

Cloning: StrataClone PCR Cloning Kit (Stratagene)

1. Prepare the insert DNA by PCR using Taq DNA polymerase or an enzyme blend qualified for PCR cloning applications
2. Prepare the ligation reaction mixture by combining the following components. Add the components in the order given below and mix gently by repeated pipetting.
3. 3 µl StrataClone Cloning Buffer

    2 µl of PCR (5-50 ng, typically a 1:10 dilution of a robust PCR reaction)

    1 µl Strata Clone Vector Mix amp/kan
4. Incubate at room temperature for 5 minutes, then place the reaction on ice.
5. Add 1 µl of the cloning reaction mixture to a tube of thawed StrataClone SoloPack competent cells. Mix gently (do not mix by repeated pipetting)
6. Incubate the transformation mixture on ice for 20 – 30 min
7. Heat-schock the transformation mixture at 42°C for 45 seconds (exactly)
8. Incubate the transformation mixture on ice for 2 minutes (exactly)
9. Add 250 µl of LB medium (pre-warmed to 42°C). allow the cells to recover at 37°C with agitation for at least 1 hour (incubate for 1,5-2 hours before plating on kanamycin plates)
10. Plate 50 µl and 100 µl of the transformation mixture on LB-ampicillin or LB-kanamicyn plates that have been spread with 40 µl of 2% X-gal
11. Incubate the plates overnight at 37°C
12. Pick white or light blue colonies for plasmid DNA analysis. Do not pick dark blue colonies