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Horizontal transmission of *Wolbachia* in the honeybee subspecies *Apis mellifera carnica* and its ectoparasite *Varroa destructor*

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ABSTRACT

Recent studies have revealed that the prevalence of *Wolbachia* in arthropods is attributable not only to its vertical transmission, but also to its horizontal transfer. Horizontal transmission of *Wolbachia* in the host/ parasite community was assessed between the honeybee subspecies *Apis mellifera carnica* and its ectoparasite *Varroa destructor*, because *Wolbachia* has been implicated in reproductive alterations in many insects. We first report the vectorial horizontal transmission of *Wolbachia* in these host/ parasite community, detected by diagnostic PCR amplification of the *16S* rDNA genes followed by direct sequencing. Identical sequences were found in these host/parasite communities suggesting horizontal transmission of *Wolbachia*. Interestingly, the infected bees may transmit *Wolbachia* transovarially making it abundant worldwide.

Keywords: *Wolbachia, Apis mellifera carnica, Varroa destructor,* PCR, horizontal transmission.

1. Introduction

Wolbachia are α-proteobacteria related to the Rickettsiaceae. *Wolbachia* are known to be responsible for male-killing (Hurst *et al*., 1999), parthenogenesis induction (Stouthamer & Werren, 1993; Stouthamer *et al*., 1993), and feminisation of genetic males (Rigaud, 1997) in invertebrates. In addition, many other strains increase their spread in host populations by inducing cytoplasmic incompatibility (O'Neill *et al*., 1992; Hoffmann & Turelli, 1997). *Wolbachia* have been found in numerous species of Hymenoptera, including parasitoids and ants (Stouthamer, 1997; Cook and Butcher, 1999; Shoemaker *et al*., 2000; Wenseleers *et al*., 1998; Jeyaprakash and Hoy, 2000). *Wolbachia* induces thelytoky in at least 40 species of Hymenoptera (Cook and Butcher, 1999).

At present the most common symbiotic microbe found to induce parthenogenesis is the bacterium *Wolbachia pipientis*. It was first identified as the microbe inducing parthenogenesis in parasitoid wasps of the genus *Trichogramma* (Rousset *et al.*, 1992; Stouthamer *et al.*, 1993). *Wolbachia* infect the reproductive tissues of numerous arthropods including honeybees, and are transovarialy transmitted from females to their offspring. *Wolbachia* infection is extremely widespread, having been found to infect 20%–75% of invertebrate species sampled (Jeyaprakash and Hoy 2000).

Recently, because of the prevalence of *Wolbachia* in arthropods, an increasing number of studies have examined the modes of transmission of *Wolbachia* among their arthropod hosts (West *et al.*, 1998; Vavre *et al*., 1999; Huigens *et al*., 2000, 2004; Sintupachee *et al*., 2006;

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Vaishampayan *et al*., 2007). Vertical transfer of *Wolbachia* is not the only transmission mode and other modes of transmission, including the intertaxon horizontal transmission, are known to occur in different hosts of *Wolbachia* (West *et al*., 1998; Huigens *et al*., 2004; Kittayapong *et al*., 2003; Sintupachee *et al*., 2006; Raychoudhury *et al*., 2009). While the mechanisms of such interspecific horizontal transfer of *Wolbachia* are poorly understood, the commonly suggested mechanism is that they are transferred between insect hosts and their associated parasites (Schilthuizen & Stouthamer 1997; Van Meer *et al*., 1999; Vavre *et al*., 1999; Noda *et al*., 2001).

Honeybees potentially represent an appealing insect group for studying horizontal transmission of *Wolbachia* for two main reasons. First, the studies on the high incidence of *Wolbachia* infections was revealed in social Hymenoptera, including the Cape honeybee by Wenseleers and Billen (2000) using a standard PCR protocol. Jeyaprakash *et al*., (2003) revealed the presence of *Wolbachia* in *Apis mellifera capensis, A. m. scutellata*, and their hybrids in Southern Africa. Second, the honeybee species *Apis mellifera* has numerous parasites and pathogens. The most destructive is the ectoparasitic mite, *Varroa destructor*, which is considered one of the most serious pests that could serve as vector for *Wolbachia* transfer.

Although the diversity of bacteria in the honeybee spp. *A. m. capensis* and *A. m. scutellata* have been well investigated (Jeyaprakash et al., 2003) in African honeybee, there is a lack of such investigation in the subspecies of the European honeybee, *Apis mellifera carnica* of Germany and its ectoparasite *Varroa destructor*.

V. destructor is known to be associated with honeybee pathogens and is confirmed in some cases to be vector of diseases. In order to assess the possibility of horizontal transfer of *Wolbachia* between host/parasite, we evaluated the possible acquisition of *Wolbachia* by *Apis mellifera carnica* from their ectoparasite *Varroa destructor*, which feeds on the haemolymph of brood and adult honeybees, can serve as interspecific vector of *Wolbachia* endosymbiont.

The main objective was the screening and investigation of the presence of *Wolbachia* in honeybees and its ectoparasite using molecular techniques to determine the transmission mechanisms by *V. destructor* in honeybees. Keeping all this in view and this being a new area of investigation, molecular approaches may easily be considered as a priority area.

2. Materials and Method

2.1 Colony sources

A honeybee queen of the subspecies viz. *Apis mellifera carnica*, its workers and its ectoparasite *Varroa destructor* were collected from hives at the honeybee research unit, Bremen University, Bremen, Germany. The samples were collected in 95% ethanol and stored at -80^0C in a deep freezer prior to DNA extraction.

2.2 DNA extraction and PCR protocols

DNA extraction and PCR were carried out at the Max Plank Institute for Evolutionary Biology, Ploen, Germany. Total genomic DNA was extracted from the abdomen of an individual honeybee and *Varroa destructor* using **Aquapure Genomic DNA kit** (catalog number 7326343) reagents following the procedure suggested by the manufacturer and the

genomic DNA was resuspended in 50 µl of sterile water. For PCR amplification 1 µl of genomic DNA was used.

Table 1: 16S rRNA *Wolbachia* specific primers

Name of the primer	Sequence	Expected size of product
WOB76f*	5'- TTGTAGCTGCTATGGTATAACT-3'	~1100bp
WOB1012r*	5'-GAATAGGTATGATTTCATGT-3'	

Wolbachia specific primers WOB76f and WOB1012r (Table 1), were used to amplify the *Wolbachia* present in the total genomic DNA, extracted from individual bees of the sub species *Apis mellifera carnica* and *Varroa destructor* collected from the colonies of the University of Bremen, Bremen, Germany.

Standard PCR was performed by a hot start method in a 25 µl reaction volume containing 1 µl DNA sample, 1 µl forward and reverse primers, 5 µl 10X Buffer containing 15 mM of $MgCl_2$, 1 unit of *Taq* polymerase (Roche), and 1 µl dNTPs (10mM). Deionized MilliQ water was added to a final volume of 25 µl. The PCR reaction mix was prepared in one batch and then added to each sample. A sample containing deionized water in place of template DNA was included in all reactions as a negative control. PCR amplification was done on a Master Cycler Gradient (Eppendorf) under the following thermal profile: 95^{O} C for 2min @ 1 cycle, 95^{O} C for 30sec @ 30 cycles, 40^{O} C for 30sec @ 30 cycles, 73^{O} C for 3min @ 30cycles and an extension cycle of 73^{O} C for 1min @ 1cycle.

The amplified products were detected by running a 1.5% agarose gel (TAE buffer) with a 1kb molecular weight marker. The gels were stained in ethidium bromide, observed under UV Transilluminator and then photographed (Figure. 1).

Clean laboratory practices, sealed pipette tips, and fresh reagents were used to avoid contamination. Negative controls (consisting of all components except the DNA template) were conducted on each date to detect potential contamination but positive controls were not carried out to reduce the likelihood of contamination.

2.3 DNA sequencing and Sequence deposition

Wolbachia infection in the individuals collected in this host/parasite community was detected by PCR amplification of *16S* rDNA fragments and the specificity of the amplicons was further verified by sequencing of random samples from single honeybee Queen, its workers and *Varroa destructor*. For this purpose, attempts were made to amplify the 16S rRNA gene by PCR and to sequence the amplified double-stranded DNA directly using an automated fluorescent DNA sequencer. Sequencing was carried out at the Max Plank Institute for Evolutionary Biology, Ploen, Germany. The ~1100 bp DNA samples from amplified PCR products were purified with a Prep-A-Gene PCR Clean-Up System kit (Bio-Rad) according to the manufacturer's instructions. A 500-ng amount of template (amplification product) was combined with 10 ng of primer, 2 µl of Sequence buffer, and water to 10 µl. Sequencing was carried out using Big Dye 2.0 (Applied Biosystems) end-terminal cycle sequencing, followed by separation and analysis on an Applied Biosystems 3130 DNA Analysis machine.

The partial nucleotide sequences of the honeybee Queen of the subspecies *Apis mellifera carnica*, its worker and its ectoparasite *Varroa destructor* were deposited in GenBank (EF032157.1, EF032158.1, EF032159.1) respectively using Bankit software (http://www.ncbi.nlm.nih.gov/BankIt/index.html). A BLASTN search was carried out to find related sequences in GenBank, especially from previous reports on bacteria associated with bees and other insects.

3. Results and Discussions

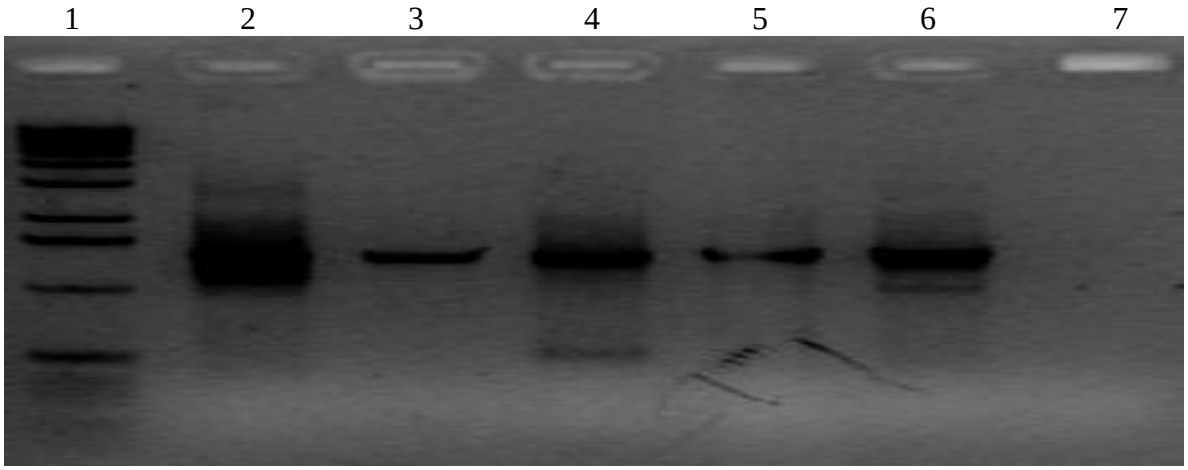

Figure 1: PCR amplification of *Wolbachia* from the Queen, workers of honeybee sub species *Apis mellifera carnica* and its ectoparasite *Varroa destructor* collected from colonies of Honeybee research unit, Bremen, Germany.

Lane 1 : Molecular weight marker
Lane 2 : Honeybee queen of sub species *Apis mellifera carnica* collected from colonies of honeybee research unit, Bremen, Germany.
Lane 3-4 : Workers of the honeybee sub species *Apis mellifera carnica* collected from colonies of honeybee research unit, Bremen, Germany.
Lane 5-6: *Varroa destructor*, ectoparasite of the honeybee worker *Apis mellifera* carnica, collected from colonies of honeybee research unit, Bremen, Germany.
Lane 7: Negative control respectively

This paper reports for the first time the presence of *Wolbachia* in the honeybee queen, its workers of subspecies *A.mellifera carnica* and its ectoparasite *Varroa destructor* collected from colonies of honeybee research unit, Bremen, Germany based on PCR and direct sequencing approach. Vertical transmission of *Wolbachia* has been previously considered to be the main mode of transmission. Here we report frequent horizontal transmission from *Varroa* mites to honeybees.

A PCR assay based on 16S primers was designed for the detection of *W. pipientis* in honeybee tissue and *Varroa destructor*, yielded an amplicon of ~1100 bp, which corresponds to 16s rDNA gene of *Wolbachia*. Analysis of honeybees and their endosymbionts, i.e. *Wolbachia* sp., indicates the presence of *Wolbachia* in *Apis mellifera carnica* and its ectoparasite *Varroa destructor*.

All the samples were tested positive (100%) (Figure 1) for *Wolbachia* infection examined by the PCR. The inability to grow these bacteria on defined cell-free medium has been the major factor underlying these uncertainties. We circumvented this problem by selective PCR amplification and subsequent direct sequencing of the symbiont 16S rRNA genes directly from infected insect tissue. The data strongly suggest that the organism has undergone a recent and extensive spread through host populations.

Apart from PCR results, the BLAST analysis of the partial sequences (EF032157.1, EF032158.1, EF032159.1) obtained from direct sequencing of random samples, revealed that all the sequences were 99% identical with each other, verified the presence of the same species of non-pathogenic *Wolbachia pipientis* belonging to α-proteobacteria in the honeybee queen, workers of the subspecies *Apis mellifera carnica* and its ectoparasite *Varroa destructor* respectively.

The presence of *Wolbachia* in the honeybee queen *Apis mellifera carnica* indicates a long-term and stable association between the two and may increase fecundity and the presence of *Wolbachia* in worker bees may have no obvious effect at all. This phenomenon needs further investigation. These bacteria may thus be specialized inhabitants of the honeybees. Therefore, these bacteria seem to constitute a specialized endogenous community in the honeybee's alimentary tract rather than bacteria accidentally taken up from the environment passing the gut.

The obligatory symbiotic relationship of *Wolbachia* in honeybees is evident from several reports of Marjorie *et al*. (2003), who established the presence of *Wolbachia* in honeybee subspecies, *Apis mellifera capensis* and *Apis mellifera scutellata.*

The usual transmission of *Wolbachia* is vertical, from the mother to her offspring through the eggs, but occasional horizontal transfers between individuals, which may or may not belong to the same species seem to occur. The transferred *Wolbachia* may then be vertically transmitted to the new host's offspring. This study found new evidence suggesting horizontal transfer in this host/parasite community. Comparison of nucleotide sequences of *Varroa destructor* and *Apis mellifera carnica* indicated that they shared infections with identical 16srDNA sequences of *Wolbachia*.

The BLAST result of the sequence (Accession number EF032157), showed 99% identity with *Wolbachia pipientis* strain from *Apis mellifera carnica* (Accession number EF032158), 99% identity with *Wolbachia* endosymbiont of *Varroa destructor* of *Apis mellifera carnica* (Accession number EF032159), 99% identity with *Wolbachia pipientis* strain from *A.diaspidis* (Accession number X87407), identity of 98% with *Wolbachia pipientis* strain EW-p (Accession number EU096232) and 98% identity with *Wolbachia* W.S.P gene(Accession Z289831) , with total query coverage of 100% and E-Value of 0.0 .

The BLAST result of the sequence (Accession number EF032158) showed 99% identity with *Wolbachia pipientis* strain from *Apis mellifera carnica* (Accession number EF032157), 99% identity with *Wolbachia* endosymbiont of *Varroa destructor* of *Apis mellifera carnica* (Accession number EF032159), identity of 98% with *Wolbachia pipientis* strain trk1/dsz (Accession number AJ306310, AJ306309, AJ306308), and 99% identity with *Wolbachia* W.S.P gene(Accession Z28983), with total query coverage of 100% and E-Value of 0.0 .

The BLAST result of the sequence (Accession number EF032159) showed 99% identity with *Wolbachia* pipientis strain from *Apis mellifera carnica* (Accession number EF032157), 99% identity with *Wolbachia* pipientis strain from *Apis mellifera carnica* (Accession number EF032158), identity of 98% with *Wolbachia* pipientis strain trk1/dsz (Accession number AJ306310, AJ306309, AJ306308), and 99% identity with *Wolbachia* W.S.P gene(Accession Z289831), with total query coverage of 100% and E-Value of 0.0 .

The BLAST results indicated that interspecific horizontal transfer occurred between two different species. Horizontal transmission is usually inferred from the presence of similar or identical *Wolbachia* strains in two unrelated host species; the strains often being determined based on one *Wolbachia* gene fragment (Werren *et al*., 1995b; Schilthuizen & Stouthamer, 1998; van Meer *et al*., 1999;Vavre *et al*., 1999). Host – parasite relationships have been cited as an important route of horizontal transfer (Werren *et al*., 1995a; West *et al*., 1998; Cook & Butcher, 1999). Some studies have found evidence suggesting transfer via host/ parasite interactions (Vavre *et al*., 1999; Noda *et al*., 2001).

Several routes of horizontal transfer have been discussed (Hurst *et al*., 1992) and there is evidence of cross-infection in predator–prey (Jolianowicz and Hoy, 1996) and host–parasitoid systems (Werren *et al*., 1995b). O'Neill *et al*. (1992) and Rousset *et al*. (1992) suggested that *Wolbachia* strains could be transmitted horizontally between insect taxa, because closely related bacteria have been found in distantly related hosts. Werren *et al*. (1995b) proposed that transmission between parasitic insects and their hosts is one vehicle for intertaxon transmission of *Wolbachia*. The *Wolbachia* strain detected in phoretic mite *Varroa destructor* was identical from *Wolbachia* symbionts of *Apis mellifera carnica,* to favour the hypothesis of horizontal transfers via this particular host-parasite assemblage system.

The *Varroa* mites of *Apis mellifera carnica* were found to harbour a *Wolbachia* strain closely related to the *Wolbachia* symbionts of *Apis mellifera carnica*, showing that horizontal transmission is taking place between them. As mites infest *Apis melliefera carnica,* these parasites could be suitable candidates for symbiont horizontal transmissions. Because the parasitic mite *Varroa destructor* feeds and moves regularly between brood and adult bees, these mites have the potential to act as either biological or mechanical vectors of *Wolbachia*. The phoretic mites are able to pierce the cuticle of honeybee larvae and feed off the haemolymph. A *Wolbachia* transfer when feeding is a possible mechanism of transfer and could be through blood-to-blood contact, because such transfers via haemolymph have been recorded in woodlice (Rigaud & Juchault, 1995). Therefore, distantly related hosts living under the same ecological conditions can share closely related symbiont strains. The mechanism for such a transfer is unknown.

Natural horizontal transfer is suggested by very similar *Wolbachia* sequences in host species that are not closely related. Evidence for horizontal transfer of *Wolbachia* between unrelated insect species has been extensively recorded (Werren *et al*., 1995; Vavre *et al*., 1999), but the routes followed by the symbionts are still poorly understood. The nucleotide sequence identity of *Wolbachia* in the predator/prey community, indicate that horizontal transmission between species must happen in nature (Rousset *et al*., 1992; O'Neill *et al*., 1992). For example, the parasitic mite, *Proctolaelaps regalis*, has been proposed as a vector for horizontal transfer of transposons between Drosophila species (Houck *et al*., 1991). In woodlice, blood-to blood contact between individuals is sufficient for horizontal transfer

(Rigaud and Juchault, 1995), however, the manner in which horizontal transfer takes place is unknown for other species.

This natural and unexpectedly frequent horizontal transfer of parthenogensis-inducing *Wolbachia* intraspecifically has important implications for the co-evolution of *Wolbachia* and their hosts. The finding of closely related symbionts in species sharing only the same habitats suggests that horizontal transfers of *Wolbachia* could occur in hymenoptera. The occurrence of closely related strains of maternally transmitted endosymbionts in distantly related insect species indicates *Wolbachia* can colonize new host species by horizontal transfer, although the mechanisms by which this occurs are unknown.

Understanding the interactions among ectoparasites, pathogens, and hosts is of importance to both applied and basic science. Deciphering the mechanisms by which microorganisms and their hosts interact at different biological levels (e.g. genetic, cellular, ecological and population) is of fundamental importance. Full understanding of the interaction between *Wolbachia* and the *Varroa* mite will provide basic information for the future development of sustainable control strategies against the mite.

It is interesting to notice that, the association of *Wolbachia* between *Varroa destructor* and *Apis mellifera carnica* is intimate involving specialized parasite and hosts. This suggests that the condition of intimate association of species for horizontal transmission of *Wolbachia* is not exclusive, although this kind of close relationship may be a more common route for transfers.

4. Conclusion

In conclusion, our data suggest that parasitism in honeybees may represent an additional mechanism of interspecies transfer of *Wolbachia*. The interacting species can exchange *Wolbachia* endosymbionts, as illustrated in the associations of hymenoptera with phoretic parasites. The finding of closely related symbionts in species sharing only the same habitats suggests that horizontal transfers of *Wolbachia* could occur in hymenopterans. *Wolbachia* to enhance its transmission would undergo both horizontal and vertical transmission. *Wolbachia* are transmitted to *Apis mellifera carnica* from a distantly related organism *Varroa destructor*, via horizontal transmission and the infected *Apis mellifera carnica* would again transmit *Wolbachia* transovarially to its offsprings via vertical transmission. Further, because parasitism is relatively common in honeybees, which are widespread and play key roles in many ecosystems, this mechanism of *Wolbachia* transmission may be significant in arthropod communities. Due to its effective transmission strategies, *Wolbachia* are widespread and abundant. This could explain, at least in part, why many species are infected by *Wolbachia*.

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