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Prevalence of *Wolbachia* in the European Honeybee, *Apis mellifera carnica*

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Abstract: Intracellular endosymbiont, *Wolbachia* spp., have been previously reported in many different orders of insects, but not in the European honeybee sub species *Apis mellifera carnica*. We first report the presence of *Wolbachia* spp. in the honeybee sub spp. *Apis mellifera carnica* of the order Hymenoptera. Using standard polymerase chain reaction (PCR) targeting of the 16S ribosomal RNA gene, we screened for *Wolbachia* spp. in honeybee Queen and workers collected from different parts of Germany. Results of the study revealed 100% infection status of *Wolbachia* in *Apis mellifera carnica* collected from different parts of Germany, confirming that the *Wolbachia spp.* were invariably present in the honeybee Queen and the workers screened.

Key words: *Wolbachia* % *Apis mellifera carnica* % 16S ribosomal RNA gene % PCR

INTRODUCTION

The "-proteobacterium *Wolbachia pipientis* is probably the most common endosymbiont in the world, infecting an estimated 25- 76% of all insect species, as well as many other arthropod and filarial nematode species [1, 2]. This alphaproteobacteria was first described by Hertig & Wolbach (1924) in the mosquito *Culex pipiens*. Later, Hertig (1936) named them *Wolbachia pipientis*.

Wolbachia pipientis is well known for inducing a variety of reproductive abnormalities in the diverse arthropod hosts which include- altering sex ratio, feminization, induction of cytoplasmic incompatibility, inducing thelytoky [5-8], male killing and parthenogenesis in at least 40 species of Hymenoptera [8] to enhance its own inheritance from mother to daughter. *Wolbachia* has been found in numerous species of Hymenoptera, including parasitoids and ants [2, 8-11]. It can also confer fitness benefits on its host [12] and increase host resistance to infection with a range of pathogens [13-17].

Wolbachia's remarkable biology and its potential application for control of vector-borne diseases such as dengue fever, malaria or filariasis [16, 18] have led to numerous genome sequencing projects aimed at understanding *Wolbachia*'s evolution and the genetic basis of the phenotypes it induces.

Although routinely transmitted vertically, *Wolbachia* has also undergone extensive intertaxon transmission, even between different orders of insects. These vertically transmitted symbionts may thus affect the expression and regulation of reproductive conflict in insect societies. Rather little effort has been undertaken to estimate the total diversity of this endosymbiont associated with these social insects. The limits of their distribution are currently unknown. The studies available today suggest that surveys of this kind are rewarding because, a number of interesting specific associations between social insects and microbial symbionts have recently been discovered and analyzed for their co-evolutionary interactions with molecular tools. Despite their common occurrence and major effects on host biology, little is currently known about the molecular mechanisms that mediate the interactions between *Wolbachia* and their invertebrate hosts.

Because *Wolbachia* are fastidious bacteria that cannot be cultured outside host cells, it can be detected using modern molecular techniques such as PCR. Molecular biological techniques are dramatically changing our view of microbial diversity in almost any environment that has so far been investigated. In a PCR-based screening study, Werren *et al.*, [19] found that over 16% of insect species in their sample were infected with *Wolbachia*. In a study using similar methods, West *et al*.,

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[20] revealed that 22% of British insects were infected. The latter screening study has indicated that the prevalence may even be underestimated and that *Wolbachia* infection levels are as high as 76% of all insect species [21].

Although the diversity of *Wolbachia* infection in the honeybee spp. *A. m. capensis* and *A. m. scutellata* have been well investigated [21] in African honeybee, there is a lack of such investigation in the subspecies of European honeybee *Apis mellifera carnica* of Germany. Until now, there are no reports on the existence of *Wolbachia* in the subspecies of European honeybee *Apis mellifera carnica*. To address this issue, PCR based screening was carried out for the first time in the Queen and workers of honey bee subspecies *Apis mellifera carnica* collected from Bremen and Ploen, Germany. Keeping all these in view, it was thought to screen the bee species for the presence or absence of *Wolbachia* by means of PCR.

MATERIALS AND METHODS

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

DNA Extraction: DNA extraction and PCR amplification was carried out at the Max Planck Institute for Evolutionary Biology, Ploen, Germany. Single honeybee Queen and worker bees were used for DNA extraction. The whole abdomen was cut off from the bees using a new single-edged razor blade for each bee and the internal tissues were removed for extraction of genomic DNA. Total genomic DNA was extracted from the abdomen of an individual bee 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.

Wolbachia specific primers (eub primers in Table 1) were used to amplify the 16S rRNA gene of the bacteria present in the total genomic DNA, extracted from each of Queen and worker honeybee.

Table 1: 16S rRNA *Wolbachia* specific primers

Name of the primer	Sequence
Eu27.F	5'- gagagtttgatcctggctcag-3'
Eu1495.R	5'- ctacggctaccttgttacga-3'

PCR Protocols: 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°C for 2min @ 1 cycle, 95°C for 30sec @ 30 cycles, 40°C for 30sec @ 30 cycles, 73°C for 3min @ 30cycles and an extension cycle of 73°C for 1min @ 1cycle.

The amplified products were detected by running a 1.5 percent agarose gel (TAE buffer) with a 1kb molecular weight marker. Gels were stained with ethidium bromide and bands visualized under UV illumination (Fig. 1 and Fig. 2).

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.

RESULTS AND DISCUSSION

The result of PCR (Fig. 1 and Fig. 2) using the *Wolbachia* primers (Table 1) yielded 100% amplification in all samples of *Apis mellifera carnica* collected from different parts of Germany, yielding an amplicon of ~1100 bp which corresponds to *Wolbachia*. The result of the study showed that the amplifiable bacteria were invariably present in the honeybee Queen (Fig. 1, Lane-2) and its workers, collected from different colonies of Bremen, Germany (Fig. 1, Lane 2-12). The primers invariably amplified the 16s region of the prokaryotes which is highly conserved for *Wolbachia*. Lane-13 showed negative for PCR, indicating there was no contamination in the PCR carried out.

The result of PCR (Fig. 2, Lane 2-12) using the *Wolbachia* primers (Table 1) yielded 100% amplification in all samples of *Apis mellifera carnica* collected from Ploen, Germany, yielding an amplicon of ~1100 bp. The result of the study showed that the amplifiable bacteria were invariably present in the honeybee workers, collected from Ploen, Germany. The primers invariably amplified the 16s region of the prokaryotes which is

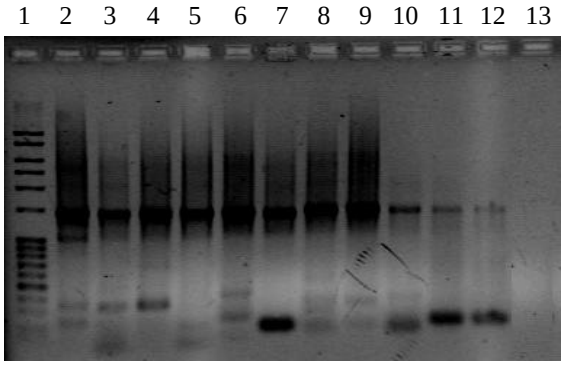

Fig. 1: PCR amplification using *Wolbachia* 16srRNA from the Queen, workers of the honeybee subspecies *Apis mellifera carnica*, collected from colonies of honeybee research unit, Bremen, Germany

Lane 1 Mol. wt marker,
2 Honeybee Queen collected from honeybee research unit, Bremen University, Germany.
3-12 Worker honeybees collected from different colonies of honeybee research unit, Bremen University, Germany.
13 Negative Control respectively

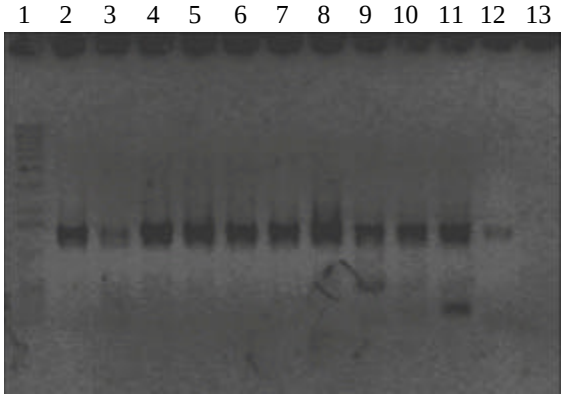

Fig. 2: PCR amplification using *Wolbachia* 16srRNA from the workers of honeybee subspecies *Apis mellifera carnica* collected from different colonies of Ploen, Germany

Lane 1 Mol. wt marker,
2-12 Worker honeybees of the subspecies *Apis mellifera carnica* collected from different colonies from Ploen, Germany.
13 Negative Control respectively

highly conserved. Lane-13 showed negative for PCR, clearly indicating there was no contamination in the PCR carried out.

A PCR assay based on 16S primers was designed for the detection of *W. pipientis* in honeybee tissue and initial screening of honeybees indicates that 100% of the samples screened were positive for *Wolbachia* infection and may induce parthenogenesis in the Hymenopteran insects. The presence of *Wolbachia* in *Apis mellifera carnica* indicates a long-term and stable association between the two and may increase fecundity or have no obvious effect at all.

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 of the symbiont 16S rRNA genes directly from infected insect tissue.

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. It is evident from the PCR results (Fig. 1, Lane-2) that *Wolbachia* is present in honeybee Queen and in its workers (Fig. 1, Lane-3-12) indicating that vertical transmission of *Wolbachia* is more common. The honeybee Queen and the workers of the sub spp. *Apis mellifera carnica* were found to harbour a *Wolbachia* strain closely related to the *Wolbachia* symbiont, showing that vertical transmission is taking place between them. The finding of closely related symbiont in species sharing only the same habitats suggests that vertical transfers of *Wolbachia* could occur more often in hymenopteran insects.

This obligatory symbiotic relationship is evident from several reports of Marjorie *et al*., [22], who established the presence of *Wolbachia* in honeybee species, *Apis mellifera capensis* and *Apis mellifera scutellata*.

Through this preliminary survey of naturally occurring *Wolbachia* infection types in European honeybee *Apis mellifera carnica* have been identified. However, *Wolbachia* infection in other species and races of European and Asian honeybee needs to be investigated to understand the variation in *Wolbachia* infection. Such studies will provide basic descriptive information about the *Wolbachia* and its interaction between the hosts.

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