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Short Communication

# Reproductive Manipulators in the Bark Beetle *Pityogenes chalcographus* (Coleoptera: Curculionidae)—The Role of *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*

**Martin Schebeck,[1,4] Lukas Feldkirchner,[1] Belen Marín,[1,2] Susanne Krumböck,[1] Hannes Schuler,[1,3,*] and Christian Stauffer[1,*]**

[1]Department of Forest and Soil Sciences, University of Natural Resources and Life Sciences, BOKU, Peter-Jordan-Straße 82/I, 1190 Vienna, Austria, [2]Current address: Avenida Puerta de Hierro 2–4, 28040 Madrid, Spain, [3]Current address: Laimburg Research Centre, Laimburg 6, 39040 Pfatten, Italy, and [4]Corresponding author, e-mail: martin.schebeck@boku.ac.at

*Equally contributing last authors.

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## Abstract

Heritable bacterial endosymbionts can alter the biology of numerous arthropods. They can influence the reproductive outcome of infected hosts, thus affecting the ecology and evolution of various arthropod species. The spruce bark beetle *Pityogenes chalcographus* (L.) (Coleoptera: Curculionidae: Scolytinae) was reported to express partial, unidirectional crossing incompatibilities among certain European populations. Knowledge on the background of these findings is lacking; however, bacterial endosymbionts have been assumed to manipulate the reproduction of this beetle. Previous work reported low-density and low-frequency *Wolbachia* infections of *P. chalcographus* but found it unlikely that this infection results in reproductive alterations. The aim of this study was to test the hypothesis of an endosymbiont-driven incompatibility, other than *Wolbachia*, reflected by an infection pattern on a wide geographic scale. We performed a polymerase chain reaction (PCR) screening of 226 individuals from 18 European populations for the presence of the endosymbionts *Cardinium*, *Rickettsia*, and *Spiroplasma*, and additionally screened these individuals for *Wolbachia*. Positive PCR products were sequenced to characterize these bacteria. Our study shows a low prevalence of these four endosymbionts in *P. chalcographus*. We detected a yet undescribed *Spiroplasma* strain in a single individual from Greece. This is the first time that this endosymbiont has been found in a bark beetle. Further, *Wolbachia* was detected in three beetles from two Scandinavian populations and two new *Wolbachia* strains were described. None of the individuals analyzed were infected with *Cardinium* and *Rickettsia*. The low prevalence of bacteria found here does not support the hypothesis of an endosymbiont-driven reproductive incompatibility in *P. chalcographus*.

**Key words:** *Wolbachia*, *Spiroplasma*, reproductive incompatibility, endosymbiont, bark beetle

A high number of terrestrial arthropods harbor maternally inherited, intracellular bacterial endosymbionts (Weinert et al. 2015). These bacteria can influence the biology of their hosts in a wide manner, e.g., by altering their reproduction (Engelstädter and Hurst 2009) or enhancing their resistance to viruses, fungi, nematodes, or parasitoids (e.g., Oliver et al. 2003, Teixeira et al. 2008, Martinez et al. 2017). Thus, endosymbionts are important players in the ecology and evolution of insects and other arthropod species (Moran et al. 2008).

One of the most common and widespread endosymbionts that can alter the reproduction of arthropods and filarial nematodes is *Wolbachia*. It can induce a variety of reproductive phenotypes in host organisms, including cytoplasmatic incompatibility (CI), male killing, parthenogenesis, or feminization (Werren et al. 2008). Among those phenotypes, CI is the most common where mating of a *Wolbachia*-infected male with a *Wolbachia*-uninfected female, or a female infected with a different *Wolbachia* strain, results in embryonic death (Hoffmann and Turelli 1997). The endosymbiont *Cardinium* also causes various reproductive phenotypes in numerous arthropod hosts (Zchori-Fein and Perlman 2004). It can induce CI, parthenogenesis, and feminization, but can also provide fitness benefits by enhancing the fecundity of its hosts (Weeks et al. 2001, Zchori-Fein et al. 2001, Hunter et al. 2003, Weeks et al. 2003, Weeks and Stouthamer 2004, Zchori-Fein et al. 2004). Another bacterial endosymbiont that can alter the biology of insect hosts is *Spiroplasma*.

It was found to act as reproductive manipulator by expressing a male-killing phenotype in *Drosophila* flies (Xie et al. 2014) and in a nymphalid butterfly (Jiggins et al. 2000). Furthermore, a *Spiroplasma* infection can protect *Drosophila* spp. against natural enemies (Jaenike et al. 2010, Xie et al. 2014). *Rickettsia* bacteria occur in various terrestrial arthropods (Weinert et al. 2015) with a wide range of effects on host biology (e.g., Sakurai et al. 2005, Zchori-Fein et al. 2006, Himler et al. 2011). They can also express phenotypes with altered reproductive behavior, such as male killing in coleopteran or parthenogenesis in hymenopteran hosts (Lawson et al. 2001, Hagimori et al. 2006).

Bark beetles (Coleoptera: Curculionidae: Scolytinae) are key players in forest ecosystems, and some species can have huge economic and ecological impacts on their environment (Grégoire et al. 2015, Raffa et al. 2015). Numerous scolytines express symbiotic relationships with other organisms, such as arthropods, fungi, or bacteria (Hofstetter et al. 2015, Wegensteiner et al. 2015). Associations with bacterial endosymbionts have been described for various bark beetles, but knowledge of effects on host biology is often limited. For example, infections with *Wolbachia* and *Rickettsia* can be related to sex determination or oogenesis in bark beetles, however, background on underlying mechanisms is scarce (e.g., Peleg and Norris 1972a,b, Stauffer et al. 1997, Vega et al. 2002, Zchori-Fein et al. 2006, Arthofer et al. 2009, Kawasaki et al. 2010, 2016). The influence of *Spiroplasma* and *Cardinium* on the reproduction of scolytines is largely unknown.

*Pityogenes chalcographus* (L.) (Coleoptera: Curculionidae: Scolytinae) is a common and widespread bark beetle in the Palearctic. It is oligophagous utilizing various conifers of the family Pinaceae; however, the main host tree in its European range is Norway spruce, *Picea abies*. *Pityogenes chalcographus* prefers thin-barked parts of trees, such as upper parts of the trunk or branches. Usually, this beetle infests weakened hosts, for example, trees damaged by wind or snow, or after logging operations. *Pityogenes chalcographus* is polygamous where one male can mate with up to seven females and one female beetle can deposit about 10 to 26 eggs. After subcortical development young beetles emerge to infest new host trees. This bark beetle can produce up to three generations per year (Schwerdtfeger 1929, Postner 1974).

Reproductive incompatibilities in scolytines are rare. *Pityogenes chalcographus* is unique among bark beetles studied, in that reproductive incompatibilities among certain European populations have been detected for this species (Führer 1976, 1977). Another remarkable example in this weevil subfamily is the North American mountain pine beetle, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), where reproductive incompatibilities around the Great Basin Desert have been described (Bracewell et al. 2017).

In *P. chalcographus*, mating experiments with males and females from certain European populations revealed partial, unidirectional crossing incompatibilities, reflected in a reduced number of offspring in allochthonous (males and females from different populations) compared to autochthonous (males and females from the same population) crosses. A northeastern and a central European lineage of this beetle were proposed and processes of incipient speciation were suggested (Führer 1976, 1977). Knowledge of the background of these findings is lacking; however, bacterial endosymbionts as potential factors have been hypothesized (Riegler 1999, Avtzis 2006, Arthofer et al. 2009).

Previous studies reported a low *Wolbachia* prevalence in *P. chalcographus* (Riegler 1999, Avtzis 2006). Arthofer et al. (2009) found *Wolbachia* only in low titers in European individuals and no geographic infection pattern was observed. *Pityogenes chalcographus* might harbor an ancient *Wolbachia* infection that might have been lost over time making it unlikely that this endosymbiont infection affects the reproduction of this bark beetle (Arthofer et al. 2009, 2010). Moreover, evidence suggests a strong, positive relationship between the density of *Wolbachia* in a host and the level of CI that occurs (Bordenstein and Bordenstein 2011) supporting the hypothesis that low-titer and low-prevalence *Wolbachia* in *P. chalcographus* likely does not result in reproductive incompatibilities. Knowledge on the presence and potential role of other secondary endosymbionts present in this bark beetle is lacking.

The aim of this study was to screen for endosymbionts in *P. chalcographus* that are described to alter the reproduction of arthropods and might be involved in reproductive peculiarities reported by Führer (1976, 1977). Thus, we screened this beetle for the presence of *Cardinium*, *Rickettsia*, and *Spiroplasma* using polymerase chain reaction (PCR). In addition, we aimed to detect non-low-titer *Wolbachia* to supplement previous findings. Subsequently, endosymbiont infections were confirmed and characterized by sequencing positive PCR products. We studied 226 individuals from 18 European populations to find drivers of reproductive alterations in this bark beetle. This is the first study on the prevalence of *Cardinium*, *Rickettsia*, and *Spiroplasma* in *P. chalcographus* and provides novel insights in the biology of this important beetle species.

## Materials and Methods

### Sample Collection and DNA Extraction

*Pityogenes chalcographus* was collected from breeding systems of infested trees or felled logs of Norway spruce between 1994 and 2009. In total, 226 individuals from 18 European locations were studied (Table 1), covering a wide part of the beetle's range. To avoid biases due to the maternal inheritance of endosymbionts only one beetle per breeding system was collected and specimens were stored in absolute ethanol at –20°C. DNA was extracted from whole beetles using the 'GenElute Mammalian Genomic DNA miniprep kit' (Sigma-Aldrich, St. Louis, MO), following the manufacturer's instructions.

### PCR Screening for Endosymbionts

PCR screening of *P. chalcographus* for all four endosymbionts was performed in a total volume of 10 µl using 1× Y-buffer (2 mM, incl. $MgCl_2$), 1 mg/ml BSA, 800 µM dNTP-mix, 0.2 µM symbiont-specific forward and reverse primer, 0.5 U peqGold Taq-DNA-Polymerase 'all inclusive' (Peqlab/VWR, Erlangen, Germany), and 1.0 µl template DNA.

To detect *Cardinium* we used the primers CLO-F1 and CLO-R1, targeting a ~450 bp fragment of the *16S rDNA* gene (Weeks et al. 2003). PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 45 s, followed by a final extension step at 72°C for 7 min. *Cardinium*-harboring *Bemisia tabaci* (Gennadius) (Hemiptera: Aleyrodidae) whiteflies were used as positive controls.

*Rickettsia* screening was done using the primers 528F and 1044R, targeting a ~500 bp fragment of the *16S rDNA* gene (Chiel et al. 2009). PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 60°C for 45 s, 72°C for 45 s, followed by a final extension step at 72°C for 7 min. We used *Rickettsia*-infected individuals of *B. tabaci* as positive controls.

Samples were screened for *Spiroplasma* using the primer pair SpixoF/SpixoR, amplifying a ~800 bp fragment of the *16S rDNA* gene (Duron et al. 2008). PCR was performed at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 52°C for 45 s, 72°C for 1 min, followed by a final extension step at 72°C for 7 min. *Spiroplasma*-harboring *Dermanyssus gallinae* (De Geer) (Mesostigmata: Dermanyssidae) mites were used as positive controls.

**Table 1.** Overview on *Pityogenes chalcographus* samples screened for the prevalence of the bacterial endosymbionts *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*: population origin, coordinates, year of collection, sample size (*n*), and endosymbiont infection. + endosymbiont-infected samples (with number of infected individuals per population), – endosymbiont-uninfected samples

| | | | | *Cardinium* | *Rickettsia* | *Spiroplasma* | *Wolbachia* |
|---|---|---|---|---|---|---|---|
| Population | Coordinates | Year | *n* | +/– | +/– | +/– | +/– |
| Austria, Kalkalpen | 47°56′N, 14°22′E | 2004 | 12 | – | – | – | – |
| Austria, Murau-Neumarkt | 47°07′N, 14°01′E | 2004 | 7 | – | – | – | – |
| Bosnia-Herzegovina, Ingman Mountain | 43°52′N, 18°25′E | 2004 | 28 | – | – | – | – |
| Croatia, Sarborsko | 44°59′N, 15°28′E | 2009 | 26 | – | – | – | – |
| Finland, Jarvenpää | 60°28′N, 25°06′E | 2004 | 10 | – | – | – | – |
| Finland, Kangashäkki | 62°36′N, 25°44′E | 2004 | 13 | – | – | – | + (2) |
| France, Massif Central | 45°01′N, 3°05′E | 2004 | 5 | – | – | – | – |
| Germany, Harz - Hasselfelde | 51°45′N, 11°00′E | 2004 | 5 | – | – | – | – |
| Greece, Drama | 41°08′N, 24°09′E | 2004 | 12 | – | – | + (1) | – |
| Italy, Abetone | 44°08′N, 10°39′E | 2009 | 6 | – | – | – | – |
| Italy, Asiago | 45°52′N, 11°30′E | 2004 | 9 | – | – | – | – |
| Italy, Pavullo | 44°20′N, 10°50′E | 2004 | 30 | – | – | – | – |
| Italy, Tolmezzo | 46°24′N, 13°01′E | 2004 | 6 | – | – | – | – |
| Lithuania, Kaunas | 55°02′N, 24°12′E | 1994 | 5 | – | – | – | – |
| Lithuania, Vilnius | 54°04′N, 25°20′E | 2004 | 12 | – | – | – | – |
| Norway, Rora | 63°50′N, 11°22′E | 2004 | 13 | – | – | – | – |
| Poland, Beskid Slaski | 50°06′N, 18°32′E | 2004 | 5 | – | – | – | – |
| Sweden, Overkalix | 66°19′N, 22°50′E | 2004 | 22 | – | – | – | + (1) |
| Total | | | 226 | | | + (1) | + (3) |

*Wolbachia* infection was studied using the primer pair 81F/691R, targeting a ~600 bp fragment of the *wsp* (*Wolbachia* surface protein) gene (Braig et al. 1998, Zhou et al. 1998). PCR conditions were 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 45 s, 72°C for 45 s, followed by a final extension step at 72°C for 8 min. *Wolbachia*-infected specimens of the tephritid fly *Rhagoletis cerasi* (L.) (Diptera: Tephritidae) were used as positive controls.

PCR products of all endosymbionts were separated by electrophoresis on a 2% agarose gel stained with GelRed nucleic acid dye (Biotum, Hayward, CA). To avoid false positive results, PCR in all samples where an endosymbiont infection was detected were replicated.

### Characterization of Endosymbionts

PCR products of positive samples were Sanger sequenced by a commercial provider (Eurofins Genomics, Ebersberg, Germany). Sequence chromatograms were carefully screened for the presence of ambiguous sites using ChromasLite version 2.1 (Technelysium, Australia). Sequences were edited in GeneRunner version 5.0 (www.generunner.net) and a BLAST search was done using the 'blastn' algorithm in GenBank. Sequences were aligned to the top BLAST hits using ClustalX version 2.0 (Larkin et al. 2007). All genotypes were confirmed by sequencing three independent PCR products. Representative sequences for each strain were submitted to GenBank.

## Results

### Detection of Endosymbionts using PCR

Screening of *P. chalcographus* for the presence of the endosymbionts *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia* revealed that these bacteria were present in less than 2% of the individuals studied (Table 1). Only one out of 12 *P. chalcographus* from the Greek population Drama was infected with the bacterium *Spiroplasma* (Fig. 1). *Wolbachia* was found in three beetles: in two out of 13 individuals from the Finnish population Kangashäkki and in one out of 22 samples from the Swedish population Overkalix (Table 1, Fig. 2). No *Cardinium* and no *Rickettsia* infections were detected (Table 1). Individuals with an endosymbiont infection had amplicons in the same size range as the positive controls (Figs. 1 and 2).

### Characterization of Endosymbionts

Sequence chromatograms of all four positive samples had unambiguous single peaks, suggesting infections with only one bacterial strain per individual. The *Spiroplasma* infection detected in a *P. chalcographus* individual from Drama/Greece (GenBank accession number: MH232090) has 99% homology to a *Spiroplasma* found in the weevil *Curculio sikkimensis* (Heller) (Coleoptera: Curculionidae) (AB545038, Toju and Fukatsu 2011), differing by four mutations.

Genotyping of *Wolbachia* from three infected individuals resulted in three different strains: One *P. chalcographus* individual from Overkalix/Sweden (MH232091) shows 100% identity with the strain *w*Cha-B that was described for *P. chalcographus* previously (DQ993183, Arthofer et al. 2009). Two beetles from Kangashäkki/Finland were infected by two new *Wolbachia* strains: One strain (MH232093) has 99% homology to the previously described strain *w*Cha-B (DQ993183, Arthofer et al. 2009), differing by two mutations; this new strain was named *w*Cha-B2. The other strain (MH232092) shows 99% homology to a *Wolbachia* infection from *Chilo suppressalis* (Walker) (Lepidoptera: Crambidae) (HQ336511), differing by four mutations; this new strain was named *w*Cha-B3.

## Discussion

Here, we screened the bark beetle *P. chalcographus* for the presence of *Cardinium*, *Rickettsia*, *Spiroplasma*, and *Wolbachia*. These endosymbionts are known to manipulate the reproduction of several arthropod hosts and could explain previously described unidirectional reproductive incompatibilities in this beetle. We found a low prevalence of the four endosymbionts. A single beetle from a Greek population carried the bacterium *Spiroplasma*. To the best of our knowledge, this is the first time that *Spiroplasma* has been described in a bark beetle.

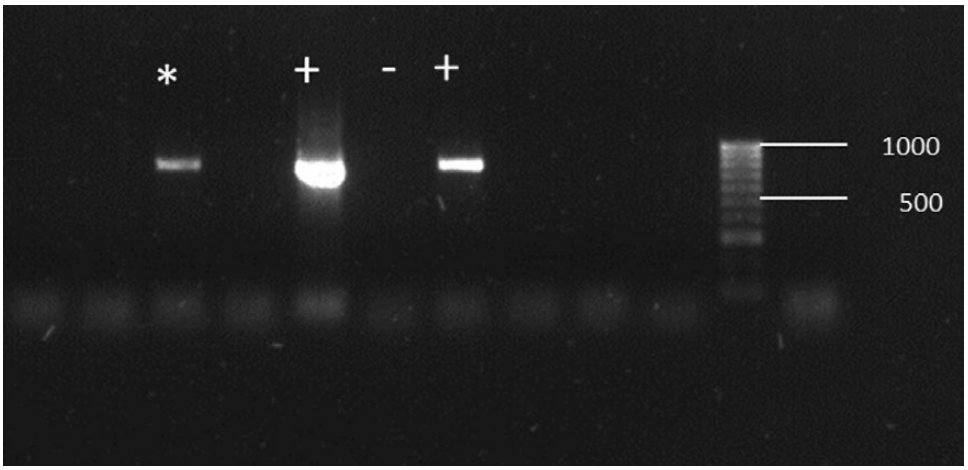


**Fig. 1.** Detection of *Spiroplasma* in *Pityogenes chalcographus* using PCR and electrophoretic separation on a 2% agarose gel stained with GelRed. **Spiroplasma*-infected individual, + positive control, – negative control. Numbers indicate fragment size.

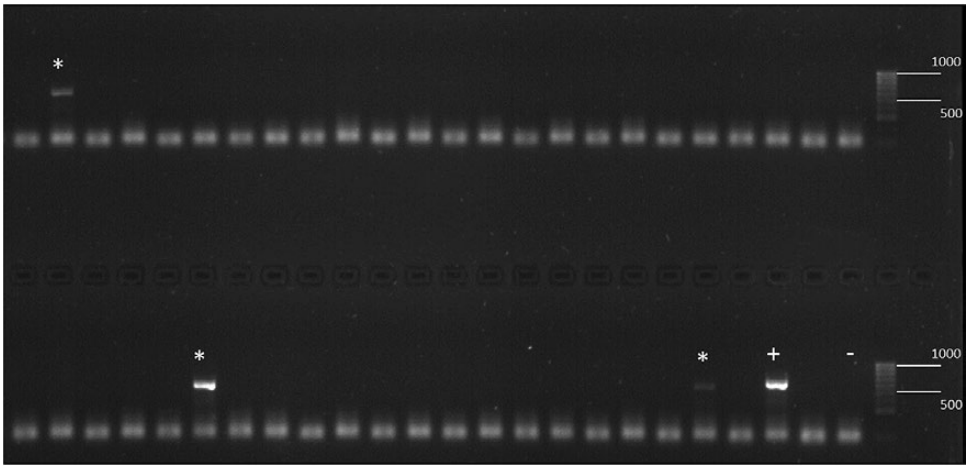


**Fig. 2.** Detection of *Wolbachia* in *Pityogenes chalcographus* using PCR and electrophoretic separation on a 2% agarose gel stained with GelRed. **Wolbachia*-infected individual, + positive control, – negative control. Numbers indicate fragment size.

Three individuals from two Scandinavian populations were infected with *Wolbachia*, two of them with two new *Wolbachia* strains. The low prevalence of *Wolbachia* and *Spiroplasma* does not support the hypothesis that heritable bacterial endosymbionts are causing reproductive incompatibilities in *P. chalcographus*.

Our results confirm previously published data that *P. chalcographus* is rarely infected with *Wolbachia* (Riegler 1999, Avtzis 2006, Arthofer et al. 2009) and that this symbiont might not be an important player in the reproductive biology of this bark beetle. Moreover, *Wolbachia* in *P. chalcographus* is present mainly in low titers (Arthofer et al. 2009). Since CI is correlated with *Wolbachia* density (Bordenstein and Bordenstein 2011) it is unlikely that low-titer infections result in reproductive incompatibilities in this beetle (Arthofer et al. 2009, 2010).

*Spiroplasma* was detected only in one single individual. Therefore, we assume that it is unlikely that this bacterium expresses a phenotype with an altered reproductive mode that affects evolutionary processes in *P. chalcographus* on a broad geographic scale. *Spiroplasma* infections are described for various insect orders, such as Diptera, Lepidoptera, and Coleoptera (Jiggins et al. 2000, Weinert et al. 2007, Jaenike et al. 2010, Xie et al. 2014). Our results support the assumption (Duron et al. 2008, Weinert et al. 2015) that many relationships between *Spiroplasma* and arthropods are still undiscovered.

None of the individuals in this study harbored an infection with *Cardinium* or *Rickettsia*. A screening of the whole microbiome of *P. chalcographus* using Next Generation Sequencing techniques (e.g., Morrow et al. 2017, Vieira et al. 2017), however, could elucidate relationships to any other symbiont of this bark beetle. This could provide potential culprit organisms to explain the previously observed reproductive incompatibilities in *P. chalcographus*. Further experimentation, e.g., extensive crossing studies or antibiotic treatment, could then be conducted to understand the mechanisms causing incompatibility, thus shedding further light on the evolution and biology of *P. chalcographus* in Europe.

Although mating experiments in *P. chalcographus* are laborious (Avtzis et al. 2008) they would add important knowledge to the beetle's reproductive biology. For example, findings by Führer (1976, 1977) did not show complete incompatibilities among crosses or a clear geographic pattern of altered reproduction. Further, Avtzis et al. (2008) did not detect a distinct genetic basis for reproductive incompatibilities. Therefore, we suggest confirming and re-assessing previous crossing studies, combining them with fine-scale genetic analyses, and relating endosymbiont infections to a reproductive

phenotype to elucidate this chapter of the beetle's biology and to add significant knowledge to the evolution of this weevil subfamily.

Peleg and Norris (1972a,b) reported a scolytine-endosymbiont relationship with potential impacts on the reproductive outcome of the host for the first time. Subsequently, an increasing number of scolytine-endosymbiont associations were described (e.g., Stauffer et al. 1997, Vega et al. 2002, Zchori-Fein et al. 2006, Arthofer et al. 2009, Kawasaki et al. 2010, 2016). Among reproductive manipulators, *Wolbachia* is the best studied symbiont of bark beetles. This bacterium, e.g., might be involved in the sex determination of some species (Vega et al. 2002, Kawasaki et al. 2010, 2016). Kawasaki et al. (2016) screened various bark beetles and found that haplodiploid species are more often infected with this endosymbiont than diploid species, with infection frequencies ranging from 0 to 100%. However, no general infection pattern and no co-evolutionary relationships between beetle and endosymbiont were found (Kawasaki et al. 2016). In addition to their influence in sex determination, endosymbionts like *Wolbachia* and *Rickettsia* can affect the egg production of the scolytine *Coccotrypes dactyliperda* F. (Coleoptera: Curculionidae) (Zchori-Fein et al. 2006). Finally, many endosymbiont infections that alter the reproduction of bark beetle hosts might still be undiscovered, and future research will shed light on the background and evolutionary impacts of this symbiosis.

In conclusion, our findings confirm that *Wolbachia* is not likely a reproductive manipulator of *P. chalcographus*. Moreover, our results suggest that it is improbable that *Cardinium*, *Rickettsia*, and *Spiroplasma* have implications on the evolution of this beetle. If *P. chalcographus* expressed an endosymbiont-driven incompatibility on a European scale, reflected in a central and northeastern lineage (Führer 1976, 1977), we would expect differences in infection patterns in these regions. For example, southern and central European populations of the tephritid fruit fly *R. cerasi* were reported to show unidirectional reproductive incompatibilities (Boller et al. 1976). *Wolbachia* was detected to cause these findings by expressing CI (Riegler and Stauffer 2002) and frequencies of the CI-inducing *Wolbachia* strain change from 100 to 0% within a few kilometers, reflecting this incompatibility pattern (Boller et al. 1976, Riegler and Stauffer 2002, Schuler et al. 2016). In contrast, the infection pattern found here, as well as by other authors (Arthofer et al. 2009, 2010), does not support an endosymbiont-driven incompatibility in *P. chalcographus*.

Alternatively, we propose that other processes, e.g., cycles of glacial and interglacial periods (Hewitt 1996), have likely shaped the genetic structure and evolutionary history of *P. chalcographus* (Avtzis et al. 2008, Bertheau et al. 2013, Schebeck et al. unpublished).

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## References Cited

**Arthofer, W., D. N. Avtzis, M. Riegler, and C. Stauffer. 2010.** Mitochondrial phylogenies in the light of pseudogenes and *Wolbachia*: re-assessment of a bark beetle dataset. Zookeys. 56: 269–280.

**Arthofer, W., M. Riegler, D. N. Avtzis, and C. Stauffer. 2009.** Evidence for low-titre infections in insect symbiosis: *Wolbachia* in the bark beetle *Pityogenes chalcographus* (Coleoptera, Scolytinae). Environ. Microbiol. 11: 1923–1933.

**Avtzis, D. N. 2006.** Race differentiation of *Pityogenes chalcographus* (Coleoptera, Scolytidae): an ecological and phylogeographic approach. PhD dissertation. BOKU University, Vienna, Austria.

**Avtzis, D. N., W. Arthofer, and C. Stauffer. 2008.** Sympatric occurrence of diverged mtDNA lineages of *Pityogenes chalcographus* (Coleoptera, Scolytinae) in Europe. Biol. J. Linnean Soc. 94: 331–340.

**Bertheau, C., H. Schuler, W. Arthofer, D. N. Avtzis, F. Mayer, S. Krumböck, Y. Moodley, and C. Stauffer. 2013.** Divergent evolutionary histories of two sympatric spruce bark beetle species. Mol. Ecol. 22: 3318–3332.

**Boller, E. F., K. Russ, V. Vallo, and G. L. Bush. 1976.** Incompatible races of European cherry fruit fly, *Rhagoletis cerasi* (Diptera: Tephritidae), their origin and potential use in biological control. Entomol. Exp. Appl. 20: 237–247.

**Bordenstein, S. R., and S. R. Bordenstein. 2011.** Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. PLoS One. 6: e29106.

**Bracewell, R. R., B. J. Bentz, B. T. Sullivan, and J. M. Good. 2017.** Rapid neo-sex chromosome evolution and incipient speciation in a major forest pest. Nat. Commun. 8: 1593.

**Braig, H. R., W. Zhou, S. L. Dobson, and S. L. O'Neill. 1998.** Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. J. Bacteriol. 180: 2373–2378.

**Chiel, E., E. Zchori-Fein, M. Inbar, Y. Gottlieb, T. Adachi-Hagimori, S. E. Kelly, M. K. Asplen, and M. S. Hunter. 2009.** Almost there: transmission routes of bacterial symbionts between trophic levels. PLoS One. 4: e4767.

**Duron, O., D. Bouchon, S. Boutin, L. Bellamy, L. Zhou, J. Engelstädter, and G. D. Hurst. 2008.** The diversity of reproductive parasites among arthropods: *Wolbachia* do not walk alone. BMC Biol. 6: 27.

**Engelstädter, J., and G. D. D. Hurst. 2009.** The ecology and evolution of microbes that manipulate host reproduction. Annu. Rev. Ecol. Evol. Syst. 40: 127–149.

**Führer, E. 1976.** Fortpflanzungsphysiologische Unverträglichkeit beim Kupferstecher (*Pityogenes chalcographus* L.) – Ein neuer Ansatz zur Borkenkäferbekämpfung? Forstarchiv. 6: 114–117.

**Führer, E. 1977.** Studien über intraspezifische Inkompatibilität bei *Pityogenes chalcographus* L. (Col., Scolytidae). J. Appl. Entomol. 83: 286–297.

**Grégoire, J.-C., K. F. Raffa, and B. S. Lindgren. 2015.** Economics and politics of bark beetles, pp. 585–613. *In* F. E. Vega and R. W. Hofstetter (eds.), Bark beetles: biology and ecology of native and invasive species. Academic Press, New York, NY.

**Hagimori, T., Y. Abe, S. Date, and K. Miura. 2006.** The first finding of a *Rickettsia* bacterium associated with parthenogenesis induction among insects. Curr. Microbiol. 52: 97–101.

**Hewitt, G. M. 1996.** Some genetic consequences of ice ages, and their role in divergence and speciation. Biol. J. Linnean Soc. 58: 247–276.

**Himler, A. G., T. Adachi-Hagimori, J. E. Bergen, A. Kozuch, S. E. Kelly, B. E. Tabashnik, E. Chiel, V. E. Duckworth, T. J. Dennehy, E. Zchori-Fein, et al. 2011.** Rapid spread of a bacterial symbiont in an invasive whitefly is driven by fitness benefits and female bias. Science. 332: 254–256.

**Hoffmann, A. A., and M. Turelli. 1997.** Cytoplasmatic incompatibility in insects, pp. 42–80. *In* S. L. O'Neill, A. A. Hoffmann, and J. H. Werren (eds.), Influential passengers: inherited microorganisms and arthropod reproduction. Oxford University Press, Oxford, United Kingdom.

**Hofstetter, R. W., J. Dinkins-Bookwalter, T. S. Davis, and K. D. Klepzig. 2015.** Symbiotic associations of bark beetles, pp. 209–245. *In* F. E. Vega and R. W. Hofstetter (eds.), Bark beetles: biology and ecology of native and invasive species. Academic Press, New York, NY.

**Hunter, M. S., S. J. Perlman, and S. E. Kelly. 2003.** A bacterial symbiont in the *Bacteroidetes* induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc. Biol. Sci. 270: 2185–2190.

**Jaenike, J., R. Unckless, S. N. Cockburn, L. M. Boelio, and S. J. Perlman. 2010.** Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. Science. 329: 212–215.

**Jiggins, F. M., G. D. Hurst, C. D. Jiggins, J. H. v d Schulenburg, and M. E. Majerus. 2000**. The butterfly *Danaus chrysippus* is infected by a male-killing *Spiroplasma* bacterium. Parasitology. 120: 439–446.

**Kawasaki, Y., M. Ito, K. Miura, and H. Kajimura. 2010**. Superinfection of five *Wolbachia* in the alnus ambrosia beetle, *Xylosandrus germanus* (Blandford) (Coleoptera: Curuculionidae). Bull. Entomol. Res. 100: 231–239.

**Kawasaki, Y., H. Schuler, C. Stauffer, F. Lakatos, and H. Kajimura. 2016**. *Wolbachia* endosymbionts in haplodiploid and diploid scolytine beetles (Coleoptera: Curculionidae: Scolytinae). Environ. Microbiol. Rep. 8: 680–688.

**Larkin, M. A., G. Blackshields, N. P. Brown, R. Chenna, P. A. McGettigan, H. McWilliam, F. Valentin, I. M. Wallace, A. Wilm, R. Lopez, et al. 2007**. Clustal W and Clustal X version 2.0. Bioinformatics. 23: 2947–2948.

**Lawson, E. T., T. A. Mousseau, R. Klaper, M. D. Hunter, and J. H. Werren. 2001**. *Rickettsia* associated with male-killing in a buprestid beetle. Heredity. 86: 497–505.

**Martinez, J., I. Tolosana, S. Ok, S. Smith, K. Snoeck, J. P. Day, and F. M. Jiggins. 2017**. Symbiont strain is the main determinant of variation in *Wolbachia*-mediated protection against viruses across *Drosophila* species. Mol. Ecol. 26: 4072–4084.

**Moran, N. A., J. P. McCutcheon, and A. Nakabachi. 2008**. Genomics and evolution of heritable bacterial symbionts. Annu. Rev. Genet. 42: 165–190.

**Morrow, J. L., A. A. G. Hall, and M. Riegler. 2017**. Symbionts in waiting: the dynamics of incipient endosymbiont complementation and replacement in minimal bacterial communities of psyllids. Microbiome. 5: 58.

**Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter. 2003**. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. USA 100: 1803–1807.

**Peleg, B., and D. M. Norris. 1972a**. Bacterial symbiote activation of insect parthenogenetic reproduction. Nat. New Biol. 236: 111–112.

**Peleg, B., and D. M. Norris. 1972b**. Symbiotic interrelationships between microbes and ambrosia beetles: VII. Bacterial symbionts associated with *Xyleborus ferrugineus*. J. Invertebr. Pathol. 20: 59–65.

**Postner, M. 1974**. Scolytidae (= Ipidae), Borkenkäfer, pp. 334–482. *In* W. Schwenke (ed.), Die Forstschädlinge Europas, 2. Band. Verlag Paul Parey, Hamburg, Berlin, Germany.

**Raffa, K. F., J.-C. Grégoire, and B. S. Lindgren. 2015**. Natural history and ecology of bark beetles, pp. 1–40. *In* F. E. Vega and R. W. Hofstetter (eds.), Bark beetles: biology and ecology of native and invasive species. Academic Press, New York, NY.

**Riegler, M. 1999**. Untersuchungen zu *Wolbachia* (α-Proteobacteria) in *Ips typographus* L. (Coleoptera, Scolytidae) und anderen Arten der Rhynchophora. Master thesis. BOKU University, Vienna, Austria.

**Riegler, M., and C. Stauffer. 2002**. *Wolbachia* infections and superinfections in cytoplasmically incompatible populations of the European cherry fruit fly *Rhagoletis cerasi* (Diptera, Tephritidae). Mol. Ecol. 11: 2425–2434.

**Sakurai, M., R. Koga, T. Tsuchida, X. Y. Meng, and T. Fukatsu. 2005**. *Rickettsia* symbiont in the pea aphid *Acyrthosiphon pisum*: novel cellular tropism, effect on host fitness, and interaction with the essential symbiont *Buchnera*. Appl. Environ. Microbiol. 71: 4069–4075.

**Schuler, H., K. Köppler, S. Daxböck-Horvath, B. Rasool, S. Krumböck, D. Schwarz, T. S. Hoffmeister, B. C. Schlick-Steiner, F. M. Steiner, A. Telschow, et al. 2016**. The hitchhiker's guide to Europe: the infection dynamics of an ongoing *Wolbachia* invasion and mitochondrial selective sweep in *Rhagoletis cerasi*. Mol. Ecol. 25: 1595–1609.

**Schwerdtfeger, F. 1929**. Ein Beitrag zur Fortpflanzungsbiologie des Borkenkäfers *Pityogenes chalcographus* L. J. Appl. Entomol. 15: 335–427.

**Stauffer, C., M. M. M. van Meer, and M. Riegler. 1997**. The presence of the Protobacteria *Wolbachia* in European *Ips typographus* (Col., Scolytidae) populations and the consequences for genetic data. Mitt. Dtsch. Ges. Allg. Angew. Entomol. 11: 709–711.

**Teixeira, L., A. Ferreira, and M. Ashburner. 2008**. The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. PLoS Biol. 6: e2.

**Toju, H., and T. Fukatsu. 2011**. Diversity and infection prevalence of endosymbionts in natural populations of the chestnut weevil: relevance of local climate and host plants. Mol. Ecol. 20: 853–868.

**Vega, F. E., P. Benavides, J. A. Stuart, and S. L. O'Neill. 2002**. *Wolbachia* infection in the coffee berry borer (Coleoptera: Scolytidae). Ann. Entomol. Soc. Am. 95: 374–378.

**Vieira, A. S., M. O. Ramalho, C. Martins, V. G. Martins, and O. C. Bueno. 2017**. Microbial communities in different tissues of *Atta sexdens rubropilosa* leaf-cutting ants. Curr. Microbiol. 74: 1216–1225.

**Weeks, A. R., and R. Stouthamer. 2004**. Increased fecundity associated with infection by a *Cytophaga*-like intracellular bacterium in the predatory mite, *Metaseiulus occidentalis*. Proc. Biol. Sci. 271 (Suppl 4): S193–S195.

**Weeks, A. R., F. Marec, and J. A. Breeuwer. 2001**. A mite species that consists entirely of haploid females. Science. 292: 2479–2482.

**Weeks, A. R., R. Velten, and R. Stouthamer. 2003**. Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc. Biol. Sci. 270: 1857–1865.

**Wegensteiner, R., B. Wermelinger, and M. Herrmann. 2015**. Natural enemies of bark beetles: predators, parasitoids, pathogens, and nematodes, pp. 247–304. *In* F. E. Vega and R. W. Hofstetter (eds.), Bark beetles: biology and ecology of native and invasive species. Academic Press, New York, NY.

**Weinert, L. A., M. C. Tinsley, M. Temperley, and F. M. Jiggins. 2007**. Are we underestimating the diversity and incidence of insect bacterial symbionts? A case study in ladybird beetles. Biol. Lett. 3: 678–681.

**Weinert, L. A., E. V. Araujo-Jnr, M. Z. Ahmed, and J. J. Welch. 2015**. The incidence of bacterial endosymbionts in terrestrial arthropods. Proc. Biol. Sci. 282: 20150249.

**Werren, J. H., L. Baldo, and M. E. Clark. 2008**. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6: 741–751.

**Xie, J., S. Butler, G. Sanchez, and M. Mateos. 2014**. Male killing *Spiroplasma* protects *Drosophila melanogaster* against two parasitoid wasps. Heredity. 112: 399–408.

**Zchori-Fein, E., and S. J. Perlman. 2004**. Distribution of the bacterial symbiont *Cardinium* in arthropods. Mol. Ecol. 13: 2009–2016.

**Zchori-Fein, E., Y. Gottlieb, S. E. Kelly, J. K. Brown, J. M. Wilson, T. L. Karr, and M. S. Hunter. 2001**. A newly discovered bacterium associated with parthenogenesis and a change in host selection behavior in parasitoid wasps. Proc. Natl. Acad. Sci. USA 98: 12555–12560.

**Zchori-Fein, E., S. J. Perlman, S. E. Kelly, N. Katzir, and M. S. Hunter. 2004**. Characterization of a '*Bacteroidetes*' symbiont in *Encarsia* wasps (Hymenoptera: Aphelinidae): proposal of '*Candidatus* Cardinium hertigii'. Int. J. Syst. Evol. Microbiol. 54: 961–968.

**Zchori-Fein, E., C. Borad, and A. R. Harari. 2006**. Oogenesis in the date stone beetle, *Coccotrypes dactyliperda*, depends on symbiotic bacteria. Physiol. Entomol. 31: 164–169.

**Zhou, W., F. Rousset, and S. O'Neill. 1998**. Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. Biol. Sci. 265: 509–515.