Wolbachia endosymbionts in haplodiploid and diploid scolytine beetles (Coleoptera: Curculionidae: Scolytinae)

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Summary

Haplodiploidy is a sex determination system in which fertilized diploid eggs develop into females and unfertilized haploid eggs develop into males. The evolutionary explanations for this phenomenon include the possibility that haplodiploidy can be reinforced by infection with endosymbiotic bacteria, such as Wolbachia. The subfamily Scolytinae contains species with haplodiploid and diploid sex determination systems. Thus, we studied the association with Wolbachia in 12 diploid and 11 haplodiploid scolytine beetles by analyzing wsp and multilocus sequence typing (MLST) of five loci in this endosymbiont. Wolbachia genotypes were compared with mitochondrial (COI) and nuclear (EF) genotypes in the scolytines. Eight of the 23 scolytine species were infected with Wolbachia, with haplodiploids at significantly higher rates than diploid species. Cloning and sequencing detected multiple infections with up to six Wolbachia strains in individual species. Phylogenetic analyses of wsp and five MLST genes revealed different Wolbachia strains in scolytines. Comparisons between the beetle and Wolbachia phylogenies revealed that closely related beetles were infected with genetically different Wolbachia strains. These results suggest the horizontal transmission of multiple Wolbachia strains between scolytines. We discuss these results in terms of the evolution of different sex determination systems in scolytine beetles.

Introduction

Sex is mostly determined by sex chromosomes, but an alternative system in ants, wasps and bees is haplodiploidy (Cook, 1993). True haplodiploidy is defined as females developing as diploids from fertilized eggs and males as haploids from unfertilized ones. A second type of haplodiploidy is paternal genome elimination (PGE) (Haig, 1993) in which males develop from fertilized eggs but the paternal chromosomes are eliminated during the early ontogenetic stages. PGE is frequently treated as functional haplodiploidy (Brun et al., 1995a). In both types of haplodiploidy, only maternal genome sets are transmitted to offspring.

The evolutionary mechanisms that explain haplodiploidy are still unclear. Some ecological and adaptive hypotheses have been proposed (Engelstädter, 2008), as follows: (i) females that can produce haploid males have twice the genetic fitness advantage compared with females that cannot (Brown, 1963; Smith, 2000); (ii) recessive deleterious mutations will be purged effectively when selection occurs in haploid males (Goldstein, 1994) and (iii) sex ratio control by fertilization is more advantageous under local mate competition (Hamilton, 1967). Recent theoretical studies have shown that maternally transmitted endosymbiotic bacteria may facilitate haplodiploid evolution (Normark, 2004; Engelstädter and Hurst, 2006; Ubeda and Normark, 2006).

Endosymbiotic bacteria, such as Wolbachia, are found in a wide range of insects (Werren et al., 2008; Zug and Hammerstein, 2012). They are asymmetrically (maternally not paternally) transmitted, so they selfishly manipulate host reproduction in order to spread effectively and maintain their infection in host populations (Werren et al., 2008) via cytoplasmic incompatibility (CI;
Hoffmann and Turelli, 1997) and sex ratio distortion (male killing, feminization of genetic males and thelytokous parthenogenesis; e.g. Engelstädter and Hurst, 2009). For example, male-killing bacteria disable the paternal genome sets in male eggs. Under sib mating, the female offspring produced by infected females can use more resources than the offspring of uninfected mothers because the death of sibling males increases the availability of resources (Hurst and Jiggins, 2000). Females that can produce haploid males will be strongly favoured in a population with few males, and this ability will spread rapidly in the population. If endosymbiotic bacteria drive the evolution of a haplodiploid sex determination system, haplodiploidy should originate after the bacterial infection. Hence, the phylogenetic relationships between insects and endosymbionts are expected to co-diverge even if some bacterial losses occur during co-diversification (Frost et al., 2010).

The most efficient mode for the transmission of endosymbionts, such as Wolbachia, is vertical transmission from the mother to offspring via eggs. Therefore, these parasites manipulate their host's reproduction to enhance their transmission to the next generation, e.g., through the induction of CI or by killing males (Engelstädter and Hurst, 2009). The rate of vertical Wolbachia transmission is an important factor in the maintenance of endosymbionts in the host population (Kawasaki et al., 2014). In addition, incongruence between the phylogeny of Wolbachia and its host shows that these bacteria can switch species boundaries and move horizontally between species (Baldo et al., 2008; Watanabe et al., 2012). The close ecological relationship between different species, such as shared habitats or shared parasitoids, can facilitate the horizontal transmission of Wolbachia (Heath et al., 1999; Stahlhut et al., 2010; Schuler et al., 2013; 2016; Ahmed et al., 2015).

The subfamily Scolytinae (Coleoptera: Curculionidae), also known as bark and ambrosia (fungi-growing) beetles, include diploid species and haplodiploid species (Kirkendall, 1983; 1993) as well as PGE species (Brun et al., 1995b). Scolytinae is a large taxonomic group with approximately 6000 species worldwide (Alonso-Zarazaga and Lyal, 2009), and one-fifth are likely to exhibit haplodiploidy (Normark et al., 1999). The evolution of arthropotous haplodiploidy occurred at least once from diploidy in scolytine beetles (Normark et al., 1999). The causal mechanism has not been elucidated, but endosymbiotic bacteria might have played an important role in the evolution of haplodiploidy (Normark et al., 1999; Normark, 2004). However, little is known about the interactions between scolytine beetles and their endosymbionts.

Endosymbiotic bacteria were first detected in scolytine beetles by Peleg and Norris (1972a,b) in the ambrosia beetle, Xyleborus ferrugineus, which reproduces parthenogenetically in association with Gram-positive endosymbiotic bacteria. No further studies of the endosymbiont-scolytine beetle relationship were reported until Stauffer and colleagues (1997), and Vega and colleagues (2002) described Wolbachia infections in two scolytine bark beetles, i.e., Ips typographus and Hypohenemus hampei respectively. This endosymbiont has also been described in Coccotrypes dactyliperda (Zchori-Fein et al., 2006) and Pityogenes chalcographus (Arthofer et al., 2009). These four bark beetles differ in terms of their biology, wherein H. hampei exhibits PGE, C. dactyliperda has a haplodiploid sex determination system, and I. typographus and P. chalcographus are diploid. Haplodiploidy is closely associated with ambrosia beetles, but Xylosandrus germanus is the only known species to be infected with Wolbachia (Kawasaki et al., 2010).

The objective of this study was to investigate whether haplodiploid scolytines are more frequently associated with Wolbachia than diploid species. Therefore, we screened for Wolbachia in 424 individuals that belonged to 12 diploid and 11 haplodiploid species of scolytine beetles. Moreover, we characterized wsp and performed multilocus sequence typing (MLST) in Wolbachia using a subset of species, which were also characterized by sequencing the mitochondrial COI and nuclear EF1α genes. Our results provide new insights into the occurrence of Wolbachia in haplodiploid and diploid scolytine beetles by highlighting the divergent patterns between Wolbachia and host beetles.

Results

PCR screening for Wolbachia infection in different scolytine beetles

In total, 424 individuals from 23 scolytine beetle species (Table 1) were screened for Wolbachia with wsp primers. Wolbachia was found in eight species: Taphrocyclus bicolor, Euwallacea interjectus, Euwallacea validus, Xyleborus schaufussi, Xyleborus seiyorensis, Xyleborus dispar, Xylosandrus crassiusculus and X. germanus (Fig. 1). Seven of 11 haplodiploid species had Wolbachia infections, whereas 1 of 12 diploid beetles was infected with this endosymbiont. The infection rate at the species level differed significantly between haplodiploid and diploid beetles (GLM, P = 0.011; Fig. 1). The Wolbachia infection rates ranged from 80% in T. bicolor to 100% in E. interjectus, X. seiyorensis, X. dispar and X. germanus (Fig. 1). The infection rates did not differ significantly among eight Wolbachia-infected species (proportion test for multiple comparisons, P = 0.069).
<table>
<thead>
<tr>
<th>Subtribe</th>
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<th>Feed</th>
<th>Sex determination</th>
<th>Mating system</th>
<th>Sex ratio</th>
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7 subtribes; 13 genera; 23 species; 8 bark; 15 12 diploidy; 11 haplodiploidy; 11 female-biased sex ratio

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8 bark/D: Diploidy; HD: Haplodiploidy; R: Random mating; S: Sib mating; FB: Female-biased sex ratio
The accession numbers are summarized in Table S1. (LC041011–LC041014). Details of the host beetles and strain from Wolbachia had four different strains designated as products. Seven plasmids from two of different strains by cloning and sequencing the PCR multiple had double peaks, which indicated the presence of multiple sequences, the chromatograms of (AB588930).

Fig. 1. Infection rates of Wolbachia in 12 diploid and 11 haplodiploid scolytine beetle species. Gray bars show the infection rates per species. Numbers at the right of the graph indicate the number of beetles examined.

**Phylogenetic characterization of Wolbachia strains based on wsp and MLST**

We characterized 11 wsp sequences from 8 scolytine beetle species. Six species (T. bicolor, E. interjectus, E. validus, X. schaufussi, X. seiryorenisis and X. crassiusculus) had clear single-peak chromatograms, which suggested the presence of only a single Wolbachia strain. The strains were designated as wTbi from T. bicolor (GenBank Accession No. LC041010), wEi from E. interjectus (AB588925), wEv from E. validus (AB588926), wXsc from X. schaufussi (AB588928), wXse from X. seiryorenisis (AB588929) and wXc from X. crassiusculus (AB588930). Wolbachia strains that infect X. germanus have already been named as wXge1 to wXge5 (Kawasaki et al., 2010), but an additional novel Wolbachia strain from X. germanus was designated as wXge6 (LC041015). Unlike the previously described sequences, the chromatograms of wsp from X. dispar had double peaks, which indicated the presence of multiple Wolbachia strains. Thus, we segregated the alleles of different strains by cloning and sequencing the PCR products. Seven plasmids from two X. dispar individuals had four different strains designated as wXdi1 to wXdi4 (LC041011–LC041014). Details of the host beetles and the accession numbers are summarized in Table S1.

The phylogenetic analyses of wsp showed that the Wolbachia strains infecting haplodiploid scolytine beetles belonged to the A supergroup (Fig. 2), whereas the wTbi that infected the diploid T. bicolor belonged to the B supergroup (Fig. 2). Three major clades were reconstructed based on wsp. The first clade included wEi, wXse, wXge1 and wXge6, and the second comprised wXc and wXge4. The third clade contained wEv and wXsc. The other strains (wXge2, wXge3, wXge5, wXdi1–4 and wTbi) were located at distinct nodes.

Five MLST genes from six different species were concatenated and aligned with strains from the Wolbachia MLST database. The MLST phylogenetic relationships (Fig. S1) detected two major clades, one of which comprised wEi, wXse and wXge1. Similar to the wsp gene, these strains were identical based on the ftsZ, coxA and gatB loci, but they exhibited polymorphisms in the hcaA and fbpA genes (Fig. S2). By contrast, wEv and wXsc were identical based on wsp but also for all five MLST loci. The wXc, wXge2, wXge3 and wXge5 strains exhibited polymorphisms at almost all of the MLST loci.

**Discordance between Wolbachia and genetic diversity of the hosts**

In this comparison, we only used ambrosia beetles captured in Japan to exclude the effects of geographic and ecological traits. The molecular phylogenetic relationships of the beetles were reconstructed using the putative amino acid sequences of the combined EF1a, COI and COI nucleotide sequences (Fig. 3A). The different clades indicated monophyly for each subtribe (Table 1).

Mapping the distribution of Wolbachia against its host (Fig. 3) indicated that there was a discordant relationship, which suggested frequent horizontal transfer of Wolbachia within the subfamily Scolytinae. For example, E. interjectus and E. validus are closely related sister species, but they were infected with two genetically different Wolbachia strains. By contrast, X. seiryorenisis and E. interjectus are genetically separated haplodiploid beetles, but they were infected with genetically identical Wolbachia strains. Related species, such as X. crassiusculus and X. germanus, differed in terms of their infection status, wherein X. crassiusculus was infected with the wXc strain whereas X. germanus had six different strains. X. crassiusculus and X. germanus also shared two genetically related strains. Five additional Wolbachia strains were found only in X. germanus. Xyleborus schaufussi is infected by Wolbachia, but we could not detect any Wolbachia strains in the closely related species X. seriatus. Therefore, stable trends in the Wolbachia infections of haplodiploid beetles were not observed, thereby suggesting that the horizontal
Fig. 2. Molecular phylogenetic tree obtained for Wolbachia strains infecting haplodiploid scolytine beetles based on the wsp gene using neighbour-joining (NJ) and maximum-likelihood (ML) methods. Each node shows the strain name with the host beetle. Bootstrap values > 50% estimated using 1000 replicates are shown above (NJ) and below (ML) each node. An arrow shows the bootstrap values obtained by NJ and ML between wXge1 and wEi. Wolbachia strains infecting Aedes albopictus and Brugia malayi are markers of the B and D supergroups respectively.

Fig. 3. Phylogenetic trees for (A) scolytine species and (B) Wolbachia strains. A. The phylogeny of the scolytines was estimated using the combined amino acid sequences of EF1α and COI (total length = 470 amino acids). The analysis included 13 scolytine species. Bootstrap values > 50% inferred from 1000 replicates are shown above (neighbour-joining) and below (maximum-likelihood) each node. B. The simplified phylogenetic relationships between Wolbachia strains infecting scolytine species were drawn based on Figs 2 and S1. Relationships between host beetles and infecting Wolbachia strains are indicated by the corresponding coloured lines (Fig S1). The outgroup was wBm in Brugia malayi, which belongs to the D supergroup.
transmission of Wolbachia has occurred frequently, rather than co-speciation between the beetles and Wolbachia.

Discussion
Our comparative study shows that the reproductive bacterial endosymbiont Wolbachia is widespread in haplodiploid and diploid scolytine beetles. However, the infections are significantly biased toward haplodiploid species rather than diploid mating species (Fig. 1). Three possibilities may explain the correlation of the biased infection rate with ecological traits, as follows. First, it is possible that the evolution of haplodiploidy is attributable to Wolbachia infection. According to theoretical studies (Normark, 2004; Engelstädter and Hurst, 2006), reproductive manipulators, such as Wolbachia, can accelerate the evolution of haplodiploidy (including PGE) from diploidy (Engelstädter and Hurst, 2009). Normark and colleagues (1999) stated that endosymbiotic bacteria are factors that can induce haplodiploidy in scolytine beetles. Similar to the present study, Vega and colleagues (2002) also detected Wolbachia in the PGE beetle H. hampei, and they suggested that the endosymbiont may cause the elimination of the paternal genome. These results are supported by a theoretical model, which showed that haplodiploidizing endosymbionts can become beneficial for their female hosts (Normark, 2004). Hence, our results support these previous studies. However, if Wolbachia is the evolutionary key to haplodiploidy in scolytine beetles, Wolbachia infection should have occurred prior to the evolution of haplodiploidy before the subsequent co-divergence between Wolbachia and host beetles. However, we found no indication of the coevolution of Wolbachia and its hosts (Fig. 3).

All seven Wolbachia-infected haplodiploid species had Wolbachia strains that belonged to the A supergroup, but the only diploid species infected with this endosymbiont had a Wolbachia strain from the B supergroup (Fig. 2). We can explain this observation by the independent horizontal transmission of different Wolbachia strains. We found two different clades in the A supergroup in which wXsc and wEv were genetically identical based on wsp and the MLST genes, whereas wXge1, wEi and wXse were separated by just a few SNPs but highly diverged from wXsc and wEv (Figs S1 and S2). This suggests that haplodiploid beetles are infected by two different genetic lineages of Wolbachia. However, our comparison with the genetic background of their hosts showed that closely related strains were present in distantly related species of haplodiploid beetles (Fig. 3). This suggests that Wolbachia probably has horizontal routes, rather than coevolving with its host. In addition, if the evolution of haplodiploidy in scolytine beetles was driven by Wolbachia, this endosymbiont should have become fixed in these species. However, our data do not support this assumption because we found that all E. interjectus, X. seiryorensis, X. dispar and X. germanus individuals were infected with Wolbachia, whereas the endosymbionts were not ubiquitous in E. validus, X. schaufussi and X. crassiusculus, and four species did not have infections with Wolbachia. It is not clear whether these species were never infected by Wolbachia or if a historic infection was lost (Morrow et al., 2015). Moreover, we cannot exclude the possibility that certain Wolbachia strains were present only at low titers, so they were not detectable using our conventional sequencing approach. In general, we cannot exclude the possibility that Wolbachia played a role during haplodiploid evolution in scolytine species, but our data do not support this hypothesis. It is also possible that other endosymbionts, such as Cardinium, Rickettsia or Spiroplasma (e.g. Duron et al., 2008), could have played roles in the haplodiploid evolution of scolytine beetles.

The second possibility is that haplodiploidy is favourable, or that diploidy is unfavourable, for Wolbachia infection and maintenance. In particular, differences in the sex ratio with these two reproduction systems (Kirkendall, 1993) should have an important effect on Wolbachia infection (Wenseleers et al., 1998). Most of the forms of reproductive manipulation (male killing, feminization and parthenogenesis) caused by Wolbachia are known to bias the sex ratio toward females (Werren et al., 2008), and haplodiploid scolytine species are known to have a female-biased sex ratio (Mizuno and Kajimura, 2002; Peer and Taborsky, 2004; Biedermann, 2010). If the distortion of the sex ratio is not caused by Wolbachia, then this endosymbiont does not necessarily manipulate host reproduction, and their relationship might no longer be considered parasitic. Therefore, a female-biased sex ratio may make Wolbachia advantageous. As a result, Wolbachia can infect haplodiploid scolytine species at higher rates than diploid species. However, it is also possible that we underestimated the occurrence of Wolbachia in diploid scolytine species. First, Wolbachia may be present in populations at frequencies ranging from 100% (e.g. Schuler et al., 2013) to 1.3% (Sun et al., 2007). The low number of individual diploid scolytines may have influenced our results. For example, Stauffer and colleagues (1997) found that Wolbachia infected all of the I. typographus individuals tested in their study, whereas we could not detect this endosymbiont in the present study. Our results agree with a recent study, which did not detect Wolbachia in populations of I. typographus from Western Carpathia, Slovakia (Michalková et al., 2012). Arthofer and colleagues (2009) and Vega and colleagues (2002) also detected Wolbachia in scolytine beetles using more
sensitive PCR techniques, such as nested PCR. Therefore, it is also possible that Wolbachia is present in scolytine beetles at lower densities, and, thus, we may have overlooked these bacteria in our survey.

The incongruence of the Wolbachia phylogeny with that of its hosts suggests that haplodiploid scolytine beetles have acquired different Wolbachia strains horizontally from independent sources. Previous studies have shown that the ecological overlap of different species by sharing the same host provides the opportunity for Wolbachia exchange between different species (Stahlhut et al., 2010; Schuler et al., 2013; 2016). Different factors, such as shared parasitoids (Heath et al., 1999; Vavre et al., 1999; Ahmed et al., 2015), cannibalism (Le Clec’h et al., 2013) and the host plant itself (Sintupachee et al., 2006), can facilitate the switch between different species boundaries. All of the haplodiploid species tested in this study are also known as ambrosia beetles. Ambrosia beetles live in galleries within logs for almost their entire life cycle. During larval growth within the galleries, the adults block the entrances of the galleries in order to prevent attack by natural enemies, such as parasitoids and predators. Therefore, low parasitism rates with the wasps that attack ambrosia beetles have been reported (Kenis et al., 2004).

Common habitats and/or resource availability appear to be a more plausible explanation for the horizontal transmission of Wolbachia in haplodiploid scolytine beetles. The ranges of the host trees used by ambrosia beetles depend partially on the species, but ambrosia beetles can usually exploit a wide range of different host trees provided that their symbiotic fungi can grow in the tree. For example, X. germanus and E. validus can co-occur on Japanese cypress (Chamaecyparis obtusa) (Y. Kawasaki, pers. obs.). However, these beetles are infected with different Wolbachia strains. Maples (Acer japonicum) can be attacked by five different ambrosia beetles: X. crassiusculus, X. germanus, X. seiryorensis, Scolytotylus mikado and S. tycoon. While the latter two species were not infected by Wolbachia, but the first three harboured different Wolbachia strains. Therefore, a subsequent study should focus on sampling different beetles from the same trees to determine whether the host might play a role in Wolbachia transfer.

In conclusion, for the first time, we showed that Wolbachia is present in seven haplodiploid and one diploid scolytine species. We found no indication of co-phylogeny between Wolbachia and its hosts, but the occurrence of genetically different Wolbachia strains in genetically similar species suggests that the horizontal acquisition of Wolbachia has occurred frequently in distant phylogenetic clades. Our results do not indicate that Wolbachia drove the evolution of the haplodiploid and diploid sex determination systems in scolytine beetles, but we cannot exclude a potential role of this endosymbiont. For example, the historic gain and loss of Wolbachia could have obscured the original pattern. In addition, future studies should focus on other endosymbionts, such as Cardinium, Rickettsia or Spiroplasma, to understand the evolutionary roles of endosymbionts in the sex determination systems of scolytine beetles.

Acknowledgements

We would like to thank F.E. Vega for critical comments on an earlier version of the manuscript and S.L. O’Neill for valuable comments to the study. We are very grateful to W. Arthofer, R.A. Beaver, P.H.W. Biedermann, A.I. Cognato, J. Huclr and M. Schebeck for sharing their precious knowledge. We are very grateful to A. Ueda, M. Nishimura, T. Hogen, Y. Imaizumi, N. Yamaguchi, N. Takabe and M. Ito for collecting and providing specimens. This study was financially supported by Grants-in-Aid for Scientific Research (18405012, 20405025 and 26292083 to H.K.), the IFO (Institute for Fermentation, Osaka) Foundation (2007) to H.K., the Showa-Houkoukai (Ito Chube’e) Foundation (2008) to H.K., the Austrian Science Fund FWF project (J-3527-B22) to H.S., and the FWF project ID10219 to C.S. The stay of Y.K. in Vienna was supported by the Nagoya University International Academic Exchange Scholarship for Oversea Study Program.

References


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Supporting Information

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Fig. S1. Molecular phylogenetic tree of Wolbachia strains infecting scolytine species based on the concatenated sequences of five MLST genes (2079 base pairs) using the neighbor-joining (NJ) method. The strains in scolytine beetles are shown in bold letters. Bootstrap values > 50% obtained by the NJ method are shown above or below the branches. The analysis included 20 additional Wolbachia strains, most of which were detected in coleopteran species (Table S2).

Fig. S2. Neighbor-joining trees for individual Wolbachia MLST genes with a simplified Wolbachia genome map modified from Baldo and colleagues (2006). Wolbachia strains infecting scolytine species are described by the strain names given in bold letters and other Wolbachia strains by ST (Table S2). Bootstrap values > 50% are shown above the branches.

Table S1. Summary of strain names, hosts, accession numbers and sequence types for Wolbachia strains used in the present study
Table S2. Profiles of the sequence types and allelic numbers used in the present study