Myrmecological News	21	49-57	Vienna, September 2015

Social parasitism and transfer of symbiotic bacteria in ants (Hymenoptera: Formicidae)

Kukka HAAPANIEMI & Pekka PAMILO

Abstract

The lack of phylogenetic congruence between endosymbionts and their hosts suggests that horizontal transmission of endosymbionts has occurred between species. However, the mechanisms of lateral transfer are largely unclear. Since successful transmission of infection most probably occurs when genetic distance between species is small, social parasitism between closely related species has been suggested to offer an important mechanism for interspecies transfer of *Wolbachia*. We compared the *Wolbachia*, *Spiroplasma* and *Entomoplasma* infections of the social parasite *Formica sanguinea* LATREILLE, 1798 and its *Serviformica* hosts to find out if horizontal transmission has occurred. We found that *F. sanguinea* and *Serviformica* mostly harboured infections with different *Wolbachia* strains and had significantly different *Spiroplasma* and *Entomoplasma* infection prevalences. Our results thus indicate that social parasitism between the slave-making ant *F. sanguinea* and its *Serviformic*a hosts does not create substantial opportunities for symbiont transmission. The prevalence data of different *Wolbachia* strains suggest that infections in different *Formica* species are partly strain specific.

Key words: *Wolbachia*, *Spiroplasma*, *Entomoplasma*, social parasitism, horizontal transmission.

Myrmecol. News 21: 49-57 (online 19 January 2015)
ISSN 1994-4136 (print), ISSN 1997-3500 (online)

Received 28 June 2014; revision received 30 October 2014; accepted 30 October 2014
Subject Editor: Daniel J.C. Kronauer

Kukka Haapaniemi (contact author), Department of Biology, University of Oulu, 90014 Oulu, Finland.
E-mail: kukka.haapaniemi@oulu.fi

Pekka Pamilo, Department of Biosciences, University of Helsinki, 00014 Helsinki, Finland.
E-mail: pekka.pamilo@helsinki.fi

Introduction

Ants are common hosts of *Wolbachia* bacteria of the class Alphaproteobacteria, and recent discoveries have shown that they are often also inhabited by other endosymbiotic bacteria such as *Spiroplasma* of the class Mollicutes (RUSSELL & al. 2012, JOHANSSON & al. 2013, KAUTZ & al. 2013). It has been estimated that about 31% of arthropod species are infected by *Wolbachia* and 4% by *Spiroplasma* (RUSSELL & al. 2012) but the estimates are sensitive to taxonomic sampling. Infections vary taxonomically, and some ant genera are particularly enriched with these symbionts (RUSSELL 2012, RUSSELL & al. 2012). In spite of findings of closely related *Wolbachia* strains in closely related ant species (e.g., VILJAKAINEN & al. 2008, FROST & al. 2010), the predominant view is that vertical (maternal) transmission, and thus shared ancestry, is not the sole mode of transmission. It has been observed that the same or similar *Wolbachia* strains often infect distantly related hosts, and closely related hosts can harbour diverse *Wolbachia* strains. These findings clearly show that in addition to maternal transmission *Wolbachia* undergoes horizontal transfer between species (e.g., WERREN & al. 1995, VAVRE & al. 1999, HUIGENS & al. 2004, BALDO & al. 2008, RAYCHOUDHURY & al. 2009). Similarly, the diversity of *Spiroplasma* clades in *Drosophila* species implies that horizontal transmission has played a role in symbiont distribution (HASELKORN & al. 2009).

Empirical and theoretical evidence indicate that horizontal transfer is most readily established among close relatives (ENGELSTÄDTER & HURST 2006, TINSLEY & MAJERUS 2007, RUSSELL & al. 2009), although occasional horizontal transfers across larger taxonomic distances are also important in shaping the *Wolbachia* pandemic (ZUG & al. 2012). Intimate (e.g., symbiotic) contact could create possibilities for transfer of endosymbiotic and other insect-associated bacteria between species. Social parasitism, i.e., a situation in which ants, bees, or wasps parasitize colonies of closely related species, offers a potential system in which endosymbiotic bacteria can spread horizontally between species. This has been inferred in *Solenopsis* fire ants where *Wolbachia* has apparently been transferred horizontally between the inquiline social parasite *Solenopsis daguerrei* and its hosts (DEDEINE & al. 2005). However, the precise mechanisms and vectors of such a transfer are not known and it has been commonly suggested that various parasites (e.g., mites) can act as vectors for *Wolbachia* (see e.g., JAENIKE & al. 2007, RUSSELL 2012).

The widespread occurrence of bacterial endosymbionts in insects relies not only on their efficient transmission but also on the effects the bacteria have on their hosts. Endosymbionts often affect the biology of the host in a way which facilitates spreading and persistence of these bacteria in the host population (MORAN & al. 2008). This can be

done by improving host fitness, for example by protecting the hosts against natural enemies such as viruses, other pathogens and parasitoids (HEDGES & al. 2008, TEIXEIRA & al. 2008, JAENIKE & al. 2010, XIE & al. 2010, ŁUKASIK & al. 2013). Because endosymbionts are commonly maternally transmitted, they can also increase their own fitness by manipulating host reproduction in such a way that there is overproduction of females (e.g., RUSSELL 2012). Both *Wolbachia* (WERREN & al. 2008) and *Spiroplasma* (VENTURA & al. 2012) are known to act as reproductive parasites in some insects, but in most species the nature of the interaction with the host is unknown. In ants, *Wolbachia* infections have been linked to cytoplasmic incompatibility (unsuccessful reproduction due to incompatibility of sperm from *Wolbachia*-infected males with eggs from females that are not infected with the same *Wolbachia* type) and male killing (VAN BORM & al. 2001, WERREN & al. 2008), but direct evidence is lacking (see RUSSELL 2012). However, potential reproductive manipulation by endosymbionts adds interesting elements to the sex allocation conflict between the queens and workers in ants and other social Hymenoptera (KELLER & al. 2001, WENSELEERS & al. 2002, DEBOUT & al. 2010).

Formica sanguinea LATREILLE, 1798 is a social parasite of many ants from the subgenus *Serviformica* (MORI & al. 2000). It uses two strategies to exploit its *Serviformica* hosts. The newly mated *F. sanguinea* queens usurp *Serviformica* nests and kill the resident *Serviformica* queens, thereby avoiding the costs of independent nest founding. *Formica sanguinea* workers from established colonies are also capable of raiding worker pupae from nearby *Serviformica* nests. After eclosion these slaves behave as regular members of the society, performing normal worker tasks and taking care of the slave-maker brood. Since *F. sanguinea* is a facultative social parasite, the workers are capable of nursing broods also independently and slaves are not found in all colonies.

In this work, we tested whether social parasitism promotes horizontal transmission of symbiotic bacteria. We estimated the prevalence and sequence diversity of two endosymbionts (*Wolbachia* and *Spiroplasma*) and a gut-associated symbiont (*Entomoplasma*) in the slave-making ant *Formica sanguinea* and its *Serviformica* slaves. If social parasitism facilitates horizontal transmission of symbiotic bacteria, host and parasite species should harbor identical or similar bacterial strains. Moreover, permanent infections are expected mainly when the transfer of infection occurs from host to parasite species. Transfer in the opposite direction may not lead to permanent infections as easily because all slave ants in *F. sanguinea* colonies are sterile workers which do not transfer acquired infections onwards. Thus, we predicted that if social parasitism facilitates horizontal transfer, endosymbionts which are common in the host species are also found in *F. sanguinea*. We also used the prevalence of different bacterial species and strains to evaluate strain-specific susceptibility of their ant hosts.

Materials and methods

Formica sanguinea workers, accompanying *Serviformica* slaves from the same nests (when present), and workers from actual *Serviformica* nests were collected from various populations in Finland. Altogether we analysed 223 ants from 121 nests from 13 different locations (Appendix S1, as digital supplementary material to this article, at the journal's web pages). We analysed one *F. sanguinea* worker per nest (except six nests from which two workers were analysed, N = 105) and one slave ant (N = 58) per nest. Similarly, from actual *Serviformica* nests, one worker ant was analysed (N = 22). In addition, one sexual female (N = 21) and one male (N = 17) were analysed from *F. sanguinea* nests from which sexual offspring were sampled. Two populations (Kiiminki and Tyrnävä) were sampled in two separate years. The *Serviformica* samples were identified morphologically using the key of COLLINGWOOD (1979) and most of them belonged to either *Formica fusca* LINNAEUS, 1758 or *Formica lemani* BONDROIT, 1917. Samples identified as *Formica gagatoides* RUZSKY, 1904, and *Formica picea* NYLANDER, 1846 were collected from one and five nests, respectively. For most analyses we pooled the different *Serviformica* hosts as the species involved were mainly *F. fusca* and *F. lemani* which are morphologically difficult to separate, especially from a small number of individuals (SEPPÄ & al. 2011).

Ants were preserved in 70% ethanol until DNA extraction with DNeasy Blood & Tissue Kit (Qiagen). The quality of DNA was tested by using the same ants in a microsatellite study (HAAPANIEMI & PAMILO 2012) or by amplifying mtDNA with insect-specific PCR primers CB1 (5'-TATGTACTTCCCTGAGGTCAAATATC-3') and CB2 (5'-AATTACACCACCTAATTTATTAGGAAT-3') (JERMIIN & CROZIER 1994). Moreover, four *Formica sanguinea* worker samples were screened for the presence of *Wolbachia* and *Spiroplasma* in different body parts (head, thorax + gaster and legs) separately. For screening Entomoplasmatales (*Spiroplasma* and *Entomoplasma*), universal eubacterial 16S rRNA gene forward primer 27F (named 16SA1 in FUKATSU & al. 2001; 5'-AGAGTTTGATCMT GGCTCAG-3') and *Spiroplasma* specific reverse primer TKSSsp (5'-TAGCCGTGGCTTTCTGGTAA-3') were used (FUKATSU & al. 2001). The amplified sequences were classified as *Entomoplasma* or *Spiroplasma* based on the similarity with sequences in GenBank. *Wolbachia* was screened by *wsp* 81F (5'-TGGTCCAATAAGTGATGAA GAAAC-3') and *wsp* 691R (5'-AAAAATTAAACGCTA CTCCA-3') primers specific to *Wolbachia* surface protein gene *wsp* (BRAIG & al. 1998). All PCRs included negative controls.

PCR mixtures (20 µl) for *Wolbachia* consisted of 1 × reaction buffer (10mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100), 300 µM of each dNTP, 1 µM of each primer, 0.8 U Dynazyme II DNA polymerase (Finnzymes) and approximately 50 ng ant DNA. The amplification profile was as follows: an initial denaturation step of 3 min at 94°C, followed by 35 cycles 30 s at 94°C, 30 s at 55°C, 1 min at 72°C and a final extension step of 5 min at 72°C. PCR mixtures (20 µl) for *Spiroplasma* and *Entomoplasma* consisted of 1 × PCR reaction buffer, 250 µM of each dNTP, 500 nM of each primer, 0.8 U of Dynazyme II DNA polymerase (Finnzymes) and approximately 50 ng of ant DNA. The amplification was performed as follows: 2 min at 94°C, 35 cycles 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension 10 min at 72°C. 5 µl of PCR products were run on agarose gel to ensure the correct size of the amplified fragment. Remaining PCR products were purified with the MinElute (96 UF) PCR

Purification Kit (Qiagen) and cloned using the TOPO TA Cloning Kit (Invitrogen) with half reactions. Five clones per individual were screened to ensure the detection of possible multiple infections (ten clones in cases when only one nest was sampled from the population). Bacterial colonies grown overnight were suspended in 50 µl of sterile water, incubated at 94°C for 10 minutes and screened with PCR using M13 forward and reverse primers flanking the insertion site in the cloning vector (pCR 2.1-TOPO). 3 µl of PCR products were run on agarose gel and a new PCR for sequencing was performed for the positive clones.

Both strands were sequenced from purified PCR products. Sequencing was conducted with the Big Dye Terminator v3.0 Cycle sequencing Kit (Applied Biosystems) according to the instructions of the manufacturer but using 1 / 8 reactions. Sequences were edited in the program Sequencher v. 4.7 (Gene Codes Corporation). Sequence alignments (ClustalW) and molecular evolutionary analyses were conducted using MEGA version 5 (TAMURA & al. 2011). Sequences with > 99% similarity were considered to belong to the same haplogroup and are here called bacterial strains. One representative sequence from each strain was selected and identified using blastn (megablast) searches in GenBank. Sequences representing all the identified strains were deposited to GenBank (accession numbers KJ778174 - KJ778192).

Phylogenetic affiliation of the *Entomoplasma* and *Spiroplasma* strains to those detected from other host organisms were assessed as follows. The sequences obtained here were used as queries in blast searches. Sequences for the phylogenetic analyses were selected from 250 best hits, selecting sequences clustering with those obtained from *Formica* in preliminary phylogenetic analyses. We eliminated redundant sequences (i.e., many similar or identical sequences from the same host) and sequences where the source (i.e., host organism) was not given. The selected *Entomoplasma* and *Spiroplasma* sequences were aligned separately, and neighbour-joining trees were constructed by using the proportion of nucleotide differences as a distance. Bootstrap support of the obtained grouping was calculated by resampling 1000 times.

Results

Wolbachia

The samples of both *Formica sanguinea* and *Serviformica* were commonly infected with *Wolbachia*, but with different strains of bacteria (GenBank: KJ778186-KJ778192). We screened *Wolbachia* from *F. sanguinea* in 99 nests, and workers from 50 nests were infected (Tab. 1). The infection prevalence in worker ants varied significantly (from 3% to 100%) among the four populations in which at least 12 nests were screened (Appendix S2, $\chi^2 = 41.5$, df = 3, P < 0.001). Infection frequencies were similar in males (12 out of 17) and sexual females (15 / 21) of F. sanguinea (Tab. 1).

As multiple clones were screened, we obtained altogether 388 *wsp* sequences from *Formica sanguinea*, and 386 were of the *Wolbachia* strain *w*Fex1 (Fig. 1a) previously identified from other *Formica* species (REUTER & KELLER 2003, VILJAKAINEN & al. 2008). The remaining two sequences came from different nests in Kiiminki, and one of them represented strain *w*Fex2 also detected in other *Formica* ants (REUTER & KELLER 2003, VILJAKAINEN & al. 2008). The other one showed sequence similarity to the

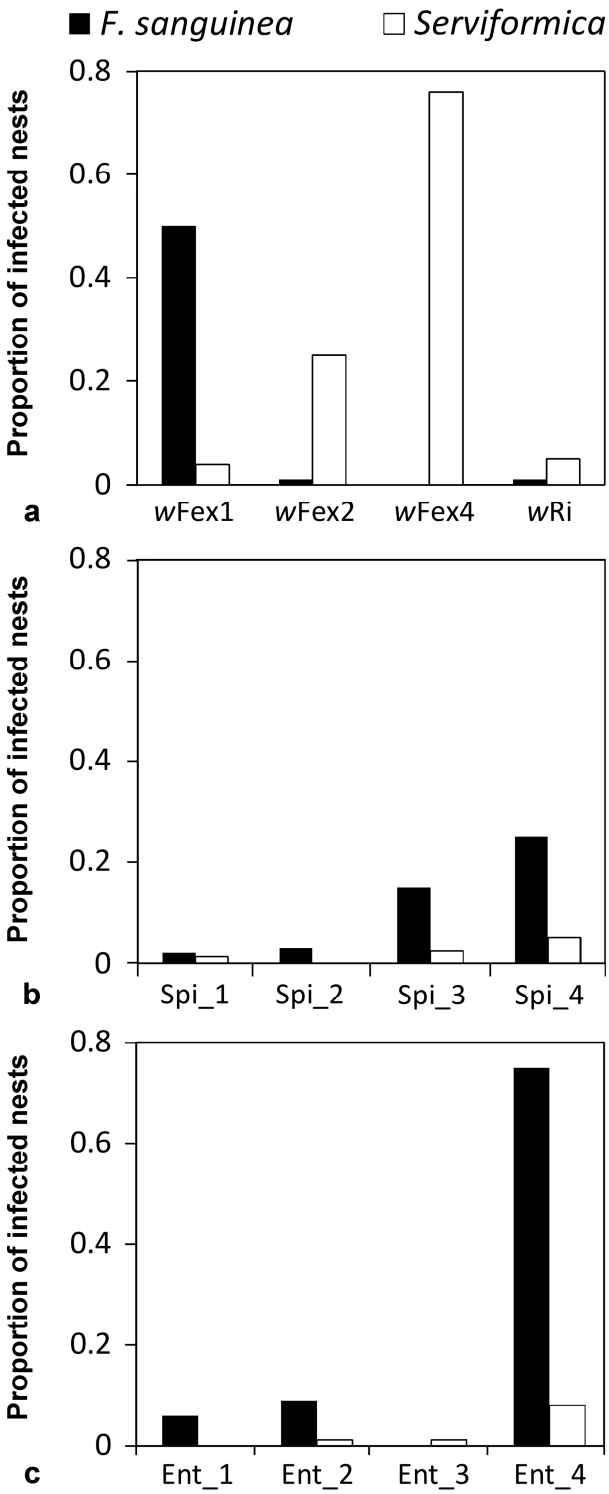

Fig. 1: Proportion of nests of *Formica sanguinea* and of the *Serviformica* species infected by different types of (a) *Wolbachia* (*w*Fex1 - *w*Ri), (b) *Spiroplasma* (Spi_1 - Spi_4), and (c) *Entomoplasma* (Ent_1 - Ent_4). Four distinct haplotype groups were identified from each bacterium.

wsp gene in the whole genome sequence of *Wolbachia w*Ri from *Drosophila simulans* (CP001391) and to sequences found earlier in the spider *Cybaeus eutypus* and wasps of the genera *Trichomalopsis* and *Spalangia*. The same variant similar to *w*Ri was also detected in four out of five *F. picea* (*Serviformica*) samples collected from the same re-

Tab. 1: Number of nests studied (n) and the percentage of ants observed to carry *Wolbachia*, *Spiroplasma*, and *Entomoplasma* bacteria.

	n	*Wolbachia* [%]	*Spiroplasma* [%]	*Entomoplasma* [%]	*Wolbachia* + *Spiroplasma* [%]
Formica sanguinea					
workers	99	50.5	79.8	19.2	47.5
males	17	70.6	64.7	41.2	52.9
gynes	21	71.4	61.9	47.6	38.1
Serviformica	80	96.3	10.0	7.5	8.8

gion (Kiiminki, all these samples from *F. sanguinea* nests) but not in any other *Serviformica* sample. The strain *w*Fex1 which was abundant in *F. sanguinea* was found as a rare variant in only three *Serviformica* nests (Fig. 1a, one independent nest and two samples from *F. sanguinea* nests). Overall, the infection prevalence in *Serviformica* was high and 77 out of 80 colonies (96%; including both independent colonies and samples from *F. sanguinea* nests) carried *Wolbachia*. Most of them carried sequences identified as *w*Fex2 (22% of all sequences) or *w*Fex4 (72%) (Fig. 1a). Double infections with both *w*Fex2 and *w*Fex4 were common (11% of samples) and detected in seven different geographical locations.

The four *Wolbachia* strains (*w*Fex1, *w*Fex2, *w*Fex4 and the one similar to *w*Ri) differed from each other by 50 - 111 nucleotides (8.5 - 19%), the total alignment being 588 nucleotides. Within the strains, nucleotide variation was small, the largest pairwise difference being ten nucleotides in *w*Fex1. The rare variants were mostly singletons (81 - 100% depending on the strain) and only three variants were shared by more than three sequences. It is thus possible that many variants represent PCR errors. The single sequence of *w*Fex2 in *Formica sanguinea* differed by five to seven nucleotides (0.9 - 1.2%) from the sequences found in *Serviformica*, while the pairwise differences among the 18 sequences from *Serviformica* ranged from zero to four nucleotides. Otherwise, it is evident that there are no major subdivisions within the strains.

Spiroplasma and *Entomoplasma*

The 16S rRNA primers amplified sequences which were identified as either *Spiroplasma* (KJ778180 - KJ778185) or *Entomoplasma* (KJ778174 - KJ778179) on the basis of similarity to sequences in GenBank. *Spiroplasma* is considered endosymbiotic and *Entomoplasma* is an insect-associated gut bacterium (FUNARO & al. 2011). Both bacteria were common in *Formica sanguinea*, 80% of nests being infected by *Spiroplasma* and 19% by *Entomoplasma* on the basis of worker data. *Entomoplasma* was amplified slightly but not significantly more frequently from sexual individuals than from workers, and the fraction of infected nests was 27% if these were included. The respective frequencies in the combined *Serviformica* material were 10% (eight nests) in *Spiroplasma* and 8% (six nests) in *Entomoplasma*, about half of these from the few *F. picea* samples from Kiiminki. Entomoplasmatales-specific primers successfully amplified *Spiroplasma* sequences from head, legs and thorax + gaster of ants. In some cases these same primers also amplified sequences which on the basis of blast searches belonged to non-target bacteria (e.g., *Lactobacillus*). Specifically, these non-target sequences were amplified from *Serviformica* samples, showing that when *Spiroplasma / Entomoplasma* infections were absent, the primer specificity was not absolute.

The sequence difference between *Spiroplasma* and *Entomoplasma* was 9.4 - 14.4% (47 - 72 nucleotides out of 495 in total; Appendix S3). Within both *Spiroplasma* and *Entomoplasma* we could distinguish four major variants or phylotype groups by using the 99% similarity criteria (RUSSELL & al. 2012). The difference between the major variants within *Spiroplasma* (Spi_1 - Spi_4) were 1.4 - 6.8% and within *Entomoplasma* (Ent_1 - Ent_4) 1.4 - 5.5% (Appendix S3). The most common variants (Ent_4 and Spi_4) were the same in both *Formica sanguinea* and *Serviformica* (Fig. 1b, c). Within the variants, most nucleotide differences were singletons. As both *Spiroplasma* and *Entomoplasma* were rare in *Serviformica* (except in *F. picea*), and were represented mainly by the most common variants, it was not easy to infer possible transfers between the species. The only indication was that the *Serviformica* ant carrying the variant Spi_2 of *Spiroplasma* came from a *F. sanguinea* nest which also had this same rare variant. The prevalence of both *Entomoplasma* and *Spiroplasma* in worker ants showed significant heterogeneity among the four *F. sanguinea* population samples ($\chi^2 = 9.9$ and 10.2, respectively, df = 3, $P < 0.01$). The prevalence was significantly smaller in *Serviformica* than in *F. sanguinea* ($\chi^2 = 4.8$, df = 1, $P < 0.05$ for *Entomoplasma* and $\chi^2 = 87.6$, df = 1, $P < 0.001$ for *Spiroplasma*). As with *Wolbachia*, both males and females of *F. sanguinea* were similarly infected also by *Spiroplasma* (11 out of 17 males, 13 out of 21 females) and *Entomoplasma* (7 / 17 in males, 10 / 21 in females; Tab. 1).

The infections by *Wolbachia* and *Spiroplasma* were positively associated in *Formica sanguinea* ($\chi^2 = 11.3$, df = 1, $P < 0.001$), i.e., there was a significant excess of individuals with either both or neither of the two endosymbionts. *Entomoplasma* showed no significant coexistence with either *Wolbachia* or *Spiroplasma*. It should be noted that we have probably underestimated the possible coexistence of *Entomoplasma* and *Spiroplasma* because they were amplified with the same primers and may have outcompeted each other during amplification.

All the *Entomoplasma* sequences clustered in the neighbour-joining tree with sequences obtained from some Neotropical ant hosts with a 55% bootstrap support (Fig. 2), while *Entomoplasma* from army ants (ant6-ant9 in Fig. 2) formed a separate cluster. The *Spiroplasma* sequences did not show any clear clusters regarding the host taxa and the bootstrap values were low (Fig. 2).

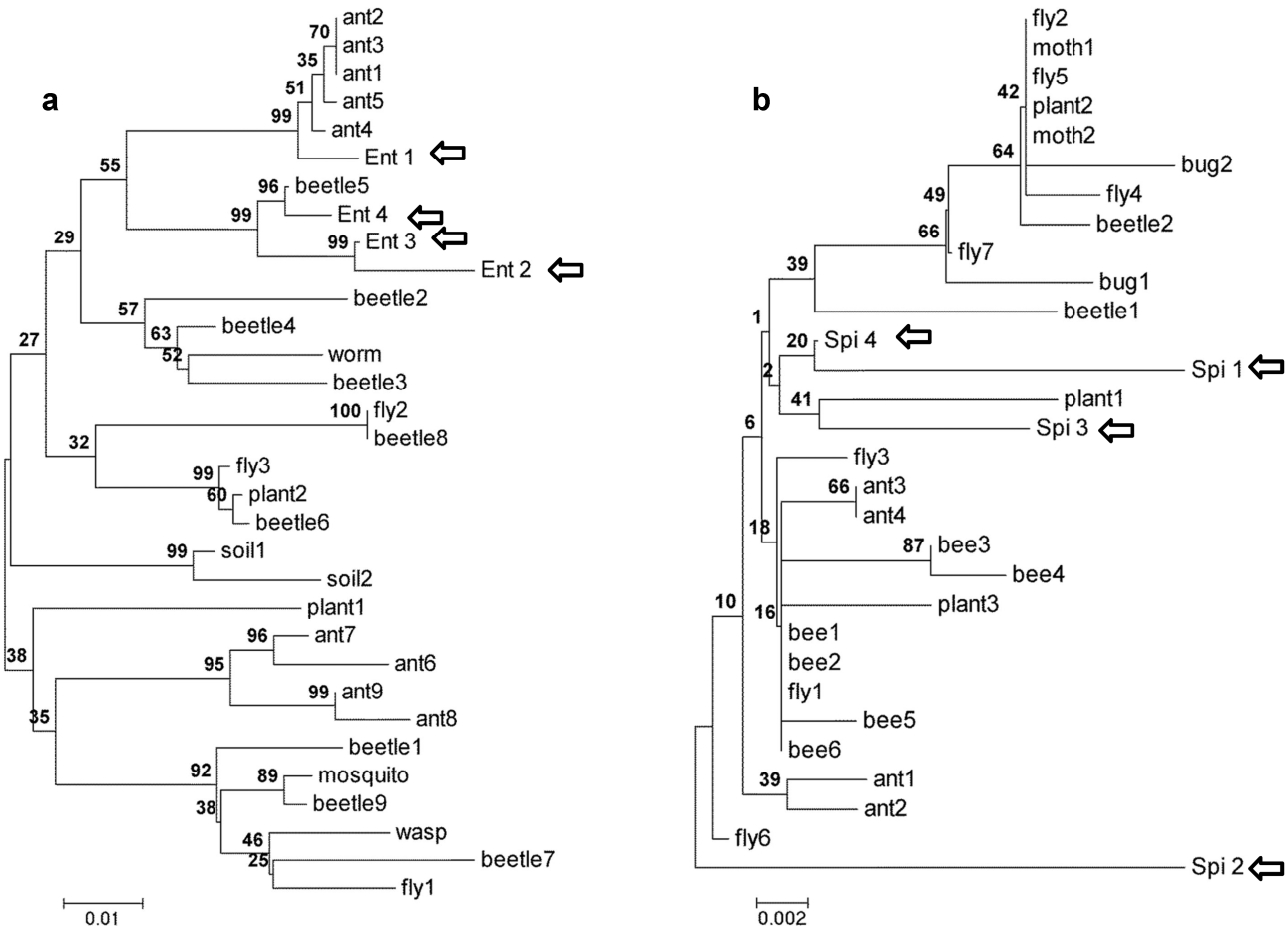

Fig. 2: Neighbour-joining trees of the (a) *Entomoplasma* and (b) *Spiroplasma*. The sequences from the present study are indicated by arrows and denoted as Ent_1 - Ent_4 and Spi_1 - Spi_4, and the sequences from GenBank are referred to with the common name of the host organism (or other source) and an arbitrary number. The list of the sequences is in Appendix S4. The length of the alignment is 319 bp for *Entomoplasma* and 345 bp for *Spiroplasma* and the distance is the proportion of nucleotide differences (p-distance). Rooting is made by midpoint, and the numbers associated to the branching points give the bootstrap support values. Note the different distance scales.

Discussion

Our main aim was to test whether nest sharing in social parasitism promotes interspecific horizontal transfer of symbiotic bacteria, which would be seen as sharing of genetically similar bacterial strains. Furthermore, comparisons with results from other *Formica* ants allow inferring possible host specificity of the endosymbionts.

DEDEINE & al. (2005) found in fire ants that the socially parasitic inquiline *Solenopsis daguerrei* SANTSCHI, 1930 shared one of its nine *Wolbachia* variants with the host species *S. invicta* BUREN, 1972 and another one with *S. richteri* FOREL, 1909. This led to the conclusion that social parasitism can represent a significant mechanism of interspecies transfer of endosymbionts. A phylogenetic analysis of *Wolbachia* from different *Solenopsis* species lends further support to the conclusion that horizontal transfer has taken place through the social parasite (MARTINS & al. 2012). *Formica sanguinea* and its *Serviformica* hosts mostly harbour infections by different bacterial strains (*Wolbachia*) in spite of close contacts within colonies. This result is clear (Fig. 1a) and applies thus to both of the main host species *F. fusca* and *F. lemani* which we pooled here for the analyses.

Even though the horizontal transfer of endosymbionts between *Formica sanguinea* and its *Serviformica* hosts seems to be rare, we detected one case where recent transfer may have taken place. The *Wolbachia* strain similar to *w*Ri was found in several nests of *F.* (*Serviformica*) *picea* and *F. sanguinea* in the same location, suggesting exchange of bacteria between these species. The same *F. picea* samples carried *Entomoplasma* and *Spiroplasma* infections, suggesting that the prevalence of these bacteria can vary among the *Serviformica* species. Rarity of *Entomoplasma* and *Spiroplasma* in other *Serviformica* species agreed with the results of SIRVIÖ & PAMILO (2010) who could not amplify them in any of the numerous *F.* (*Serviformica*) *cinerea* MAYR, 1853 samples. As the *F. picea* samples came from one locality, it is not possible to conclude whether this species generally differs from other *Serviformica* ants by carrying a different set of bacteria or whether there have been recent horizontal transfers in the population under study here. In addition, there were three cases where a *Wolbachia* variant common in *F. sanguinea* (*w*Fex1) was detected in *Serviformica*, and one case in which the variant *w*Fex2, common in *Serviformica*, was found in *F. sanguinea*. It is impossible to conclude whether this occasional sharing derives from social parasitism, be-

cause the same strains are shared by many *Formica* ants (REUTER & KELLER 2003, VILJAKAINEN & al. 2008) and the bacteria can apparently also spread in the absence of social parasitism However, if social parasitism enhances horizontal transfer, we hypothesized that the transfer from *F. sanguinea* to *Serviformica* is expected to be a dead end because the slave workers do not produce female offspring. This means that sharing of *w*Fex2 provides the only possible example of horizontal transfer associated with cohabitation of the same nests by the slaves and slave-makers. We should point out that a small possibility might also exist for transferring endosymbionts from *F. sanguinea* to its slave species during the short periods when raiding their nests. In whichever direction the infection might be transferred, the drastic difference in the relative frequency of *Wolbachia* strains between *F. sanguinea* and *Serviformica* suggests that the role of social parasitism in horizontal transfer can be weak or that the bacterial strains are host specific.

Currently not much is known about the process of horizontal transmission for *Spiroplasma* in arthropod species but horizontal transfers have had an important impact on the taxonomic distribution of *Spiroplasma* in *Drosophila* species (HASELKORN & al. 2009). Phylogenetic analysis of *Spiroplasma*-related ant symbionts suggests repeated acquisition of infections (KAUTZ & al. 2013), and our results are concordant with this (Fig. 2). The phylogenetic distribution of *Entomoplasma* haplotypes gives a somewhat different picture. The branch lengths of the *Entomoplasma* tree are longer and some well supported clades appear to be host specific (Fig. 2) However, combined phylogenetic analysis and molecular clock dating suggest that *Entomoplasma* may also have been horizontally transferred between army ant subfamilies during their long-term evolution (FUNARO & al. 2011). Thus, horizontal transfer seems to play a role in the dynamics of *Spiroplasma* and *Entomoplasma* bacteria, but the mechanisms and time frames of such transfers are not well understood.

Our results likely underestimated the prevalence of *Entomoplasma* and *Spiroplasma* infections, as these bacteria were amplified with the same primers, facing competitive amplification. The present results show that *Spiroplasma* has a high prevalence (80%) in *Formica sanguinea* but a low prevalence in *Serviformica* (9%), with *F. picea* as a possible exception. *Spiroplasma* infections appear to be systemic, since they were successfully detected in different ant tissues. Previously reported *Spiroplasma* infection frequencies in arthropods overall are low (3.8%; RUSSELL & al. 2012) and infection was not identified in the transcriptome of *F. exsecta* NYLANDER, 1846 (JOHANSSON & al. 2013). However, in the genus *Polyrhachis* (tribe Camponotini) 20.7% of studied species were infected (RUSSELL & al. 2012). In their study including 95 ant species, KAUTZ & al. (2013) found 28.4% of ant species were infected with Entomoplasmatales bacteria (including both *Spiroplasma* and *Entomoplasma*), and ISHAK & al. (2011) reported a high abundance of *Spiroplasma* in several colonies of *Solenopsis geminata* (FABRICIUS, 1804). Further studies are needed to explain the seemingly sporadic existence of *Spiroplasma* infections in some ant species.

About one fifth of *Formica sanguinea* individuals carried *Entomoplasma*, and *Serviformica* showed again much lower infection frequencies. In army ants *Entomoplasma*

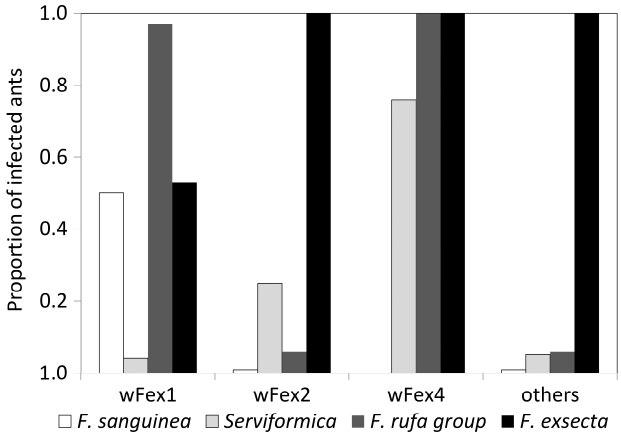

Fig. 3: The proportion of worker individuals in *Formica* species infected by different *Wolbachia* strains (several strains are pooled in the class "others"). The data for *F. sanguinea* (n = 99) and *Serviformica* (n = 80) are derived from this study, for the *F. rufa* group (n = 31) from VILJAKAINEN & al. (2008), and for *F. exsecta* (n = 34) from REUTER & KELLER (2003).

infections are generally absent from eggs and larvae, and thus these bacteria are suggested to act as gut associates (FUNARO & al. 2011). *Entomoplasma* bacteria are shown to be slightly enriched among predatory ant genera, where they are suggested to have a non-essential, but specialized relationship with their hosts (FUNARO & al. 2011, ANDERSON & al. 2012). N-isotope signatures, which reflect the trophic positions of the organisms, showed no differences among three *Formica* subgenera, including *F. sanguinea* and *Serviformica* (FIEDLER & al. 2007). It needs to be determined whether this result indicates that the species are trophically similar or that there are dietary differences but the isotope signatures are shaped by the gut bacteria in the more predatory *F. sanguinea*.

The average *Wolbachia* infection frequency in *Formica sanguinea* workers (51%) was clearly lower than in *Serviformica* (96%) in this study (but only 19% in *F.* (*Serviformica*) *cinerea*; see SIRVIÖ & PAMILO 2010) and was lower than has been reported in the *Formica rufa* group (100%; VILJAKAINEN & al. 2008) and *Formica exsecta* (100%; REUTER & KELLER 2003), where generally all individuals have multiple *Wolbachia* infections (Fig. 3). The infection frequency was similar to that in workers of *Formica truncorum* FABRICIUS, 1804 (45%; WENSELEERS & al. 2002) and *Acromyrmex echinatior* (FOREL, 1899) (45%; VAN BORM & al. 2001) in which adult workers are suggested to lose their infection over time. Our samples of sexual individuals were too small to draw firm conclusions, but the proportion of infected individuals was similar in newly eclosed gynes and males, and in adult workers in the same nests.

The meta-analysis by RUSSELL & al. (2012) gives weak support to the hypothesis initially proposed by WENSELEERS & al. (1998) that ants with independent colony founding are less often infected by *Wolbachia*. The species of *Serviformica* commonly establish new nests independently, whereas the nest-founding queens of *Formica exsecta*, *F. sanguinea* and the *F. rufa* group species temporarily parasitize *Serviformica* nests (or join existing con-

specific nests). There is thus no evident association between nest founding and *Wolbachia* in *Formica* ants, except regarding the specific strain *w*Fex1 which was very rare in *Serviformica* but common in the other *Formica* species.

A broad systematic survey by KAUTZ & al. (2013) suggests that there is a tendency that related *Wolbachia* to some extent infect related host ants from the same geographic region, even though *Wolbachia* infections in insects are generally not very host specific. Similarly, RUSSELL & al. (2009) demonstrated in their work including both ants and lycaenid butterflies that related *Wolbachia* commonly infect related hosts. The most common *Wolbachia* strains (as identified on the basis of the *wsp* sequence) were *w*Fex1 in *F. sanguinea* and *w*Fex2 and *w*Fex4 in *Serviformica*. These strains have been earlier found in other *Formica* ants, in *F. exsecta* (subgenus *Coptoformica*; REUTER & KELLER 2003) and in the *F. rufa* group complex (six species of the subgenus *Formica* s.str.; VILJAKAINEN & al. 2008), supporting qualitatively the previous findings that related hosts are infected by similar endosymbiont strains (Fig. 3). However, colonies and individuals of several *Formica* species are commonly infected by several *Wolbachia* strains: *F. exsecta* by four or five strains and *F. rufa* group ants by both *w*Fex1 and *w*Fex4. This suggests that there are differences in how readily different bacterial strains are able to infect a specific host species either due to specificity of the bacteria or selectivity of the ant hosts. Thus, *F. sanguinea* lacked the strain *w*Fex4 which is common in the other *Formica* ants, and the strain *w*Fex1 was very rare in *Serviformica* but common in all the other species (Fig. 3). As the same methods have readily shown multiple infections (REUTER & KELLER 2003, VILJAKAINEN & al. 2008), it is unlikely that the observed differences would be amplification artefacts. Further support to the conclusion that infections by *Wolbachia* in different *Formica* species are strain specific is added by the study of SIRVIÖ & PAMILO (2010) who found that even though 19% of *F.* (*Serviformica*) *cinerea* colonies were estimated to carry *Wolbachia*, the *wsp* sequences could not be amplified. This indicates that *F. cinerea* carries a specific *Wolbachia*, at least with regards to the *wsp* gene.

The fact that *Formica sanguinea* lacks several *Wolbachia* strains which are commonly found in other *Formica* ants suggests interspecific differences in susceptibility, but the observed variation in the prevalence of *w*Fex1 suggests also intraspecific differences among the *F. sanguinea* populations. The strain *w*Fex1 is commonly found in the *F. rufa* group ants throughout their geographical distribution (VILJAKAINEN & al. 2008), so the geographical distribution of the strain is not a limiting factor for infections. It seems also unlikely that the variation in the prevalence of *Wolbachia* in *F. sanguinea* would result from interaction between different endosymbionts. We found no association between *Entomoplasma* and *Wolbachia*, and the association between *Spiroplasma* and *Wolbachia* was positive. In other words, the presence of *Spiroplasma* also predicted the presence of *Wolbachia*. This association at least partly reflects population differences as, for example, we found no endosymbionts in the northernmost location of Pudasjärvi. Otherwise the prevalence showed no evident geographical gradient, unlike the situation in the *Solenopsis* ants (MARTINS & al. 2012).

Our results showed that the slave-making *Formica sanguinea* and its *Serviformica* hosts are infected by different bacterial strains (*Wolbachia*) or carry symbionts with very different prevalence (*Spiroplasma*, *Entomoplasma*). This can be due to a lack of suitable transfer vectors in spite of regular physical and behavioural contacts between adult ants and also between slave workers and *F. sanguinea* broods. Comparative data also show some host specificity of different *Wolbachia* strains within the genus *Formica*. It is thus also possible that the observed difference between *F. sanguinea* and *Serviformica* in their community of symbiotic bacteria does not derive from the lack of transferring vectors but from specificity of the bacteria or selectivity of the ant hosts (see also RUSSELL & al. 2009). It is not easy to distinguish between these alternatives but it is clear that successful horizontal transfer between these species is uncommon as they form the only species pairs among the studied *Formica* ants which do not have a single *Wolbachia* strain with high prevalence in both (Fig. 3). Proper testing of the suggested host specificity of the endosymbionts and their possible lateral transfer, or lack of it, would require thorough screening of various endosymbiotic species with multi locus sequence typing of the strains.

Acknowledgements

We thank Riitta Jokela for valuable help with the laboratory work and the reviewers for constructive and helpful comments. The work has been financially supported by the Academy of Finland. Personal grants to K.H. were admitted from Oskar Öflund's Foundation and from Societas pro Fauna et Flora Fennica.

References

ANDERSON, K.E., RUSSELL, J.A., MOREAU, C.S., KAUTZ, S., SULLAM, K.E., HU, Y.I., BASINGER, U., MOTT, B.M., BUCK, N. & WHEELER, D.E. 2012: Similar microbial communities are shared among related and trophically similar ant species. – Molecular Ecology 21: 2282-2296.

BALDO, L., AYOUB, N.A., HAYASHI, C.Y., RUSSELL, J.A., STAHLHUT, J.K. & WERREN, J.H. 2008: Insight into the routes of *Wolbachia* invasion: high levels of horizontal transfer in the spider genus *Agelenopsis* revealed by *Wolbachia* strain and mitochondrial DNA diversity. – Molecular Ecology 17: 557-569.

BRAIG, H.R., ZHOU, W., DOBSON, S.L. & O'NEILL, S.L. 1998: Cloning and characterization of a gene encoding the major surface protein of the bacterial endosymbiont *Wolbachia pipientis*. – Journal of Bacteriology 180: 2373-2378.

COLLINGWOOD, C.A. 1979: The Formicidae (Hymenoptera) of Fennoscandia and Denmark. – Fauna Entomologica Scandinavica 8: 1-174.

DEBOUT, G.D.G., FREDERICKSON, M.E., ARON, S. & YU, D.W. 2010: Unexplained split sex ratios in the neotropical plant-ant, *Allomerus octoarticulatus* var. *demerarae* (Myrmicinae): a test of hypotheses. – Evolution 64: 126-141.

DEDEINE, F., AHRENS, M., CALCATERRA, L. & SHOEMAKER, D.D. 2005: Social parasitism in fire ants (*Solenopsis* spp.): a potential mechanism for interspecies transfer of *Wolbachia*. – Molecular Ecology 14: 1543-1548.

ENGELSTÄDTER, J. & HURST, G.D.D. 2006: The dynamics of parasite incidence across host species. – Evolutionary Ecology 20: 603-616.

FIEDLER, K., KUHLMANN, F., SCHLICK-STEINER, B.C., STEINER, F.M. & GEBAUER, G. 2007: Stable N-isotope signatures of central European ants – assessing positions in a trophic gradient. – Insectes Sociaux 54: 393-402.

FROST, C.L., FERNANDEZ-MARIN, H., SMITH, J.E. & HUGHES, W. O.H. 2010: Multiple gains and losses of *Wolbachia* symbionts across a tribe of fungus growing ants. – Molecular Ecology 19: 4077-4085.

FUKATSU, T., TSUCHIDA, T., NIKOH, N. & KOGA, R. 2001: *Spiroplasma* symbiont of the pea aphid, *Acyrthosiphon pisum* (Insecta: Homoptera). – Applied and Environmental Microbiology 67: 1284-1291.

FUNARO, C.F., KRONAUER, D.J.C., MOREAU, C.S., GOLDMAN-HUERTAS, B., PIERCE, N.E. & RUSSELL, J.A. 2011: Army ants harbor a host-specific clade of *Entomoplasmatales* bacteria. – Applied and Environmental Microbiology 77: 346-350.

HAAPANIEMI, K. & PAMILO, P. 2012: Reproductive conflicts in polyandrous and polygynous ant *Formica sanguinea*. – Molecular Ecology 21: 421-430.

HASELKORN, T.S., MARKOW, T.A. & MORAN, N.A. 2009: Multiple introductions of the *Spiroplasma* bacterial endosymbiont into *Drosophila*. – Molecular Ecology 18: 1294-1305.

HEDGES, L.M., BROWNLIE, J.C., O'NEILL, S.L. & JOHNSON, K.N. 2008: *Wolbachia* and virus protection in insects. – Science 322: 702.

HUIGENS, M.E., DE ALMEIDA, R.P., BOONS, P.A., LUCK, R.F. & STOUTHAMER, R. 2004: Natural interspecific and intraspecific horizontal transfer of parthenogenesis-inducing *Wolbachia* in *Trichogramma* wasps. – Proceedings of the Royal Society B-Biological Sciences 271: 509-515.

ISHAK, H.D., PLOWES, R., SEN, R., KELLNER, K., MEYER, E., ESTRADA, D.E., DOWD, S.E. & MUELLER, U.G. 2011: Bacterial diversity in *Solenopsis invicta* and *Solenopsis geminata* ant colonies characterized by 16S amplicon 454 pyrosequencing. – Microbial Ecology 61: 821-831.

JAENIKE, J., POLAK, M., FISKIN, A., HELOU, M. & MINHAS, M. 2007: Interspecific transmission of endosymbiotic *Spiroplasma* by mites. – Biology Letters 3: 23-25.

JAENIKE, J., UNCKLESS, R., COCKBURN, S.N., BOELIO, L.M. & PERLMAN, S.J. 2010: Adaptation via symbiosis: recent spread of a *Drosophila* defensive symbiont. – Science 329: 212-215.

JERMIIN, L.S. & CROZIER, R.H. 1994: The cytochrome b region in the mitochondrial DNA of the ant *Tetraponera rufoniger*: Sequence divergence in Hymenoptera may be associated with nucleotide content. – Journal of Molecular Evolution 38: 282-294.

JOHANSSON, H., DHAYGUDE, K., LINDSTRÖM, S., HELANTERÄ, H., SUNDSTRÖM, L. & TRONTTI, K. 2013: A metatranscriptomic approach to the identification of microbiota associated with the ant *Formica exsecta*. – Public Library of Science One 8: e79777.

KAUTZ, S., RUBIN, B.E.R. & MOREAU, C.S. 2013: Bacterial infections across the ants: frequency and prevalence of *Wolbachia*, *Spiroplasma* and *Asaia*. – Psyche 2013: 936341.

KELLER, L., LIAUTARD, C., REUTER, M. & BROWN, W.D. 2001: Sex ratio and *Wolbachia* infection in the ant *Formica exsecta*. – Heredity 87: 227-233.

ŁUKASIK, P., VAN ASCH, M., GUO, H., FERRARI, J. & GODFRAY, H.C.J. 2013: Unrelated facultative endosymbionts protect aphids against a fungal pathogen. – Ecology Letters 16: 214-218.

MARTINS, C., SOUZA, R.F. & BUENO, O.C. 2012: Presence and distribution of the endosymbiont *Wolbachia* among *Solenopsis* spp. (Hymenoptera: Formicidae) from Brazil and its evolutionary history. – Journal of Invertebrate Pathology 109: 287-296.

MORAN, N.A., MCCUTCHEON, J.P. & NAKABACHI, A. 2008: Genomics and evolution of heritable bacterial symbionts. – Annual Review of Genetics 42: 165-190.

MORI, A., GRASSO, D.A. & LE MOLI, F. 2000: Raiding and foraging behavior of the blood-red ant, *Formica sanguinea* LATREILLE (Hymenopetra: Formicidae). – Journal of Insect Behavior 13: 421-438.

RAYCHOUDHURY, R., BALDO, L., OLIVEIRA, D.C.S.G. & WERREN, J.H. 2009: Modes of acquisition of *Wolbachia*: horizontal transfer, hybrid introgression, and codivergence in the *Nasonia* species complex. – Evolution 63: 165-183.

REUTER, M. & KELLER, L. 2003: High levels of multiple *Wolbachia* infection and recombination in the ant *Formica exsecta*. – Molecular Biology and Evolution 20: 748-753.

RUSSELL, J.A. 2012: The ants (Hymenoptera: Formicidae) are unique and enigmatic hosts of prevalent *Wolbachia* (Alphaproteobacteria) symbionts. – Myrmecological News 16: 7-23.

RUSSELL, J.A., FUNARO, C.F., GIRALDO, Y.M., GOLDMAN-HUERTAS, B., SUH, D., KRONAUER, D.J.C., MOREAU, C.S. & PIERCE, N.E. 2012: A veritable menagerie of heritable bacteria from ants, butterflies, and beyond: broad molecular surveys and a systematic review. – Public Library of Science One 7: e51027.

RUSSELL, J.A., GOLDMAN-HUERTAS, B., MOREAU, C.S., BALDO, L. STAHLHUT J.K., WERREN, J.H. & PIERCE, N.E. 2009: Specialization and geographic isolation among *Wolbachia* symbionts from ants and lycaenid butterflies. – Evolution 63: 624-640.

SEPPÄ, P., HELANTERÄ, H., TRONTTI, K., PUNTTILA, P., CHERNENKO, A., MARTIN, S.J. & SUNDSTRÖM, L. 2011: The many ways to delimit species: hairs, genes and surface chemistry. – Myrmecological News 15: 31-41.

SIRVIÖ, A. & PAMILO, P. 2010: Multiple endosymbionts in populations of the ant *Formica cinerea*. – BioMed Central Evolutionary Biology 10: 335.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011: MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance and maximum parsimony methods. – Molecular Biology and Evolution 28: 2731-2739.

TEIXEIRA, L., FERREIRA, A. & ASHBURNER, M. 2008: The bacterial symbiont *Wolbachia* induces resistance to RNA viral infections in *Drosophila melanogaster*. – Public Library of Science Biology 6: 2753-2763.

TINSLEY, M.C. & MAJERUS, M.E. 2007: Small steps or giant leaps for male killers? Phylogenetic constraints to male killer host shifts. – BioMed Central Evolutionary Biology 7: 238.

VAN BORM, S., WENSELEERS, T., BILLEN, J. & BOOMSMA, J.J. 2001: *Wolbachia* in leafcutter ants: a widespread symbiont that may induce male killing or incompatible matings. – Journal of Evolutionary Biology 14: 805-814.

VAVRE, F., FLEURY, F., LEPETIT, D., FOUILLET, P. & BOULETREAU, M. 1999: Phylogenetic evidence for horizontal transmission of *Wolbachia* in host-parasitoid associations. – Molecular Biology and Evolution 16: 1711-1723.

VENTURA, I.M., MARTINS, A.B., LYRA, M.L., ANDRADE, C.A.C., CARVALHO, K.A. & KLACZKO, L.B. 2012: *Spiroplasma* in *Drosophila melanogaster* populations: Prevalence, male-killing, molecular identification, and no association with *Wolbachia*. – Microbial Ecology 64: 794-801.

VILJAKAINEN, L., REUTER, M. & PAMILO, P. 2008: *Wolbachia* transmission dynamics in *Formica* wood ants. – BioMed Central Evolutionary Biology 8: 55.

WENSELEERS, T., ITO, F.,VAN BORM, S., HUYBRECHTS, R., VOLCKAERT, F. & BILLEN, J. 1998: Widespread occurrence of the micro-organism *Wolbachia* in ants. – Proceedings of the Royal Society B-Biological Sciences 265: 1447-1452.

WENSELEERS, T., SUNDSTRÖM, L. & BILLEN, J. 2002: Deleterious *Wolbachia* in the ant *Formica truncorum*. – Proceedings of the Royal Society B-Biological Sciences 269: 623-629.

WERREN, J.H., BALDO, L. & CLARK, M.E. 2008: *Wolbachia*: master manipulators of invertebrate biology. – Nature Reviews Microbiology 6: 741-751.

WERREN, J.H., ZHANG, W. & GUO, L.R. 1995: Evolution and phylogeny of *Wolbachia* – reproductive parasites of arthropods. – Proceedings of the Royal Society B-Biological Sciences 261: 55-63.

XIE, J., VILCHEZ, I. & MATEOS, M. 2010: *Spiroplasma* bacteria enhance survival of *Drosophila hydei* attacked by the parasitic wasp *Leptopilina heterotoma*. – Public Library of Science One 5: e12149.

ZUG, R., KOEHNCKE, A. & HAMMERSTEIN, P. 2012: Epidemiology in evolutionary time: the case of *Wolbachia* horizontal transmission between arthropod host species. – Journal of Evolutionary Biology 25: 2149-2160.