Title Page

***Wolbachia* in scale insects: a distinct pattern of infection frequencies and potential transfer routes via ant associates**

Ehsan Sanaei^{1*}, Yen-Po Lin², Lyn G Cook¹, Jan Engelstädter¹

¹School of Biological Sciences, The University of Queensland, Brisbane, 4072, Australia

²Department of Plant Medicine, College of Agriculture, National Chiayi University, Chiayi City, 60004, Taiwan

*Corresponding author email: e.sanaei@uq.net.au Phone: +61 7 3365 7959 Fax: +61 7 336 51655

Running Title: *Wolbachia* infection in scale insects

14 **Originality-Significance Statement:**

15 By creating metadata of *Wolbachia* infection in arthropods and applying a fitting an advanced
16 mathematical model on the estimated infection frequency in scale insects, a unique pattern of
17 infection prevalence was detected. In addition, ant-scale insect trophallaxis interaction was
18 suggested as a plausible route of *Wolbachia* transfer

19 **Summary**

20 *Wolbachia* is one of the most successful endosymbiotic bacteria of arthropods. Known as the
21 “master of manipulation”, *Wolbachia* can induce a wide range of phenotypes in its host that
22 can have far-reaching ecological and evolutionary consequences and may be exploited for
23 disease and pest control. However, our knowledge of *Wolbachia*’s distribution and infection
24 rate is unevenly distributed across arthropod groups such as scale insects. We fitted a
25 distribution of within-species prevalence of *Wolbachia* to our data and compared it to
26 distributions fitted to an up-to-date dataset compiled from surveys across all arthropods. The
27 estimated distribution parameters indicate a *Wolbachia* infection frequency of 43.6% (at a
28 10% prevalence threshold) in scale insects. Prevalence of *Wolbachia* in scale insects follows
29 a distribution similar to exponential decline (most species are predicted to have low
30 prevalence infections), in contrast to the U-shaped distribution estimated for other taxa (most
31 species have a very low or very high prevalence). We observed no significant associations
32 between *Wolbachia* infection and scale insect traits. Finally, we screened for *Wolbachia* in
33 scale insect’s ecological associates. We found a positive correlation between
34 *Wolbachia* infection in scale insects and their ant associates, pointing to a possible route of
35 horizontal transfer of *Wolbachia*.

36 **Introduction**

37 *Wolbachia* is one of the most abundant and diverse maternally transmitted endosymbionts on
 38 earth. The majority of *Wolbachia* strains belong to supergroups A and B, which are well
 39 recognised as reproductive parasites in arthropods (Werren *et al.*, 2008). Reproductive
 40 manipulations by *Wolbachia* include Cytoplasmic Incompatibility (CI), Male Killing (MK),
 41 feminization and parthenogenesis induction, and they often provide a relative reproductive
 42 advantage for infected females and thereby foster the maintenance of *Wolbachia* within its
 43 host population (Hoffmann *et al.*, 1990; Bourtzis and O'Neill, 1998; Landmann, 2019). The
 44 effect of *Wolbachia* on its arthropod host also includes a wide range of other induced
 45 phenotypes, from altering the susceptibility to pathogens (Teixeira *et al.*, 2008; Sicard *et al.*,
 46 2014; Gong *et al.*, 2020) and modifying the host's behaviour (Min and Benzer, 1997;
 47 Beltran-Bech and Richard, 2014) to nutrient provisioning (Brownlie *et al.*, 2009). These
 48 diverse phenotypes may impact the host's population and evolutionary dynamics (e.g.,
 49 reduction in mitochondrial genetic diversity, sex ratio distortions, and possibly triggering
 50 speciation) (Tagami *et al.*, 2001; Jiggins, 2003; Engelstädter and Hurst, 2009; Zug and
 51 Hammerstein, 2014; Hoffmann *et al.*, 2015). Given this, as well as promising applications of
 52 *Wolbachia* to control the vector born disease (Ross *et al.*, 2019) or pest species (Liu and Guo,
 53 2019), *Wolbachia* have been extensively studied during the last three decades. Nevertheless,
 54 our knowledge of the distribution and diversity of *Wolbachia* is still in its infancy, and
 55 current data on infection by *Wolbachia* are not evenly distributed across the different groups
 56 of arthropods. While some prominent insect groups (e.g. Diptera (Cooper *et al.*, 2019; Sicard
 57 *et al.*, 2019) and Lepidoptera (Ilinsky and Kosterin, 2017)) have been well studied, there is a
 58 knowledge gap in many other groups, including scale insects (Detcharoen *et al.*, 2019).

59 With more than 8200 described species (García Morales *et al.*, 2016), scale insects
60 (Coccoomorpha, Hemiptera) are a diverse and globally distributed group of sap-sucking
61 insects (Gullan and Cook, 2007). They may feed on a single plant species (specialist), single
62 plant family (monophagous) or multiple plant families (polyphagous) (Lin *et al.*, 2015). Scale
63 insects can be observed in high density, causing nutrient deprivation, and preventing the
64 normal growth of the host's branches (Gullan and Kosztarab, 1997). Given these significant
65 damages to various plant species, some scale insects are considered as serious crop pests.
66 Scale insects are also very diverse in terms of reproduction mode. Gonochoristic sexual
67 reproduction, facultative and obligate parthenogenesis and even hermaphroditism (which is
68 extremely rare in insects) are all observed in different scale insect families and species. There
69 is often considerable intra-species genetic diversity in mitochondrial and nuclear genes, and
70 different karyotype arrangements, even in sibling species (Gullan and Kosztarab, 1997;
71 Cook, 2000; Gavrilov, 2007).

72 As plant materials are rich in carbon but poor in amino acids (Kondo *et al.*, 2008), most
73 members of the suborder Sternorrhyncha (aphids, whiteflies, psyllids and scale insects) are
74 expected or observed to have specialized symbionts with nutrient provision ability (von
75 Dohlen *et al.*, 2001). Indeed, most phytophagous hemipterans possess a symbiotic organ that
76 is formed from specific cells and that harbours various bacteria (Kikuchi *et al.*, 2005;
77 Kuechler *et al.*, 2012), indicating a close association between hemipterans (including scale
78 insects) and their bacterial community (Normand, 1949; Baumann, 2005; Gruwell *et al.*,
79 2007; Matsuura *et al.*, 2009; Sudakaran *et al.*, 2017). In the case of scale insects, both
80 specialized bacteria (usually referred to as primary endosymbionts, e.g. in mealybugs and
81 diaspidids) and secondary endosymbionts (with mostly unknown relationships with their
82 hosts) have been reported (e.g., *Chlamydia* in *Eriococcus spurius* (Everett *et al.*, 2005). In
83 general, the flavobacterial symbionts and Enterobacteriaceae in several scale insect families

(Rosenblueth *et al.*, 2012), *Burkholderia* (Proteobacteria) in *Gossyparia spuria* and *Acanthococcus aceris* (Eriococcidae) (Michalik *et al.*, 2016) and *Cardinium* infection in armoured scale insects (Provencher *et al.*, 2005; Gruwell *et al.*, 2009) are examples of the reported bacterial symbionts in scale insects.

In spite of some available knowledge in scale insect's microbiome, there are only few studies addressing *Wolbachia* infection in scale insects (Rosenblueth *et al.*, 2018). For example, by studying the host's microbiome with antimicrobial components, *Wolbachia* infection was confirmed in *Dactylopius* sp. (Pankewitz *et al.*, 2007) two species of *Drosicha* (Matsuura *et al.*, 2009), *Orthezia urticae*, *Matsucoccus pini* and *Steingelia gorodetskia* (Michalik *et al.*, 2019), and a direct study of *Wolbachia* phage in *Kerria lacca* revealed infection by *Wolbachia* (Kaushik *et al.*, 2019). Other small pieces of information come from very broad *Wolbachia* surveys across all arthropods that serendipitously included a few scale insects, usually with a negative infection status being reported (e.g., single samples of the pseudococcids *Ferrisia virgata* and *Planococcus citri* (Jeyaprakash and Hoy, 2000), *Brevennia rehi* (Behera *et al.*, 2001) and *Maconellicoccus* sp. (Prakash and Puttaraju, 2007); 12 samples of *Pseudococcus longispinus* (Pseudococcidae) and *Coccus* sp. (Coccidae); and 16 samples of *Saissetia oleae* (Coccidae) (Duron *et al.*, 2008)). These limited data depict a low incidence of *Wolbachia* in scale insects compared to other hemipterans, but to date, there has been no study dedicated to assessing this.

To fill this knowledge gap, we conducted the first broad survey of *Wolbachia* in scale insects, screening *Wolbachia* infection in 689 scale insect samples belonging to 151 species. A well-known problem with such undertakings is cross-study comparisons. *Wolbachia* surveys usually report the fraction of species for which at least one of the tested specimens tested positive for *Wolbachia* infection (e.g., 23% in Australian parasitoid wasps (Klopfstein *et al.*,

2018), 63% in European bees (Gerth *et al.*, 2015), or 23% in insects collected from Indo-Malay rice fields (Wiwatanaratnabutr and Zhang, 2016)). However, due to the natural heterogeneity of *Wolbachia* infection in host populations, combined with biased and incomplete sampling, this raw fraction of species found to be infected cannot readily be compared between surveys. For example, a difference between 20% vs. 40% infection frequency reported in two surveys may be due to the fact that more specimens per species were screened in the latter, or to a higher within-species prevalence of *Wolbachia* in the latter, rather than indicating differences in the actual fraction of infected species (Jiggins *et al.*, 2001). To account for this problem, we adopted the beta-binominal distribution approach pioneered by Hilgenboecker *et al.* (2008) (see also (Zug and Hammerstein, 2014) and (Weinert *et al.*, 2015)) to estimate the infection frequency in scale insects. Based on an up-to-date database of *Wolbachia* infection across arthropods that we compiled from published surveys and meta-analyses, we also estimated the infection frequencies in groups such as aphids and compare them to scale insects. We then tested for plausible associations between *Wolbachia* infection and several ecological and biological features of scale insects, such as host breadth, reproduction mode, the ability to induce galls, host plant family, distribution range, and the number of reported parasitoids species.

Wolbachia strains have a great ability to undertake host shifting among various host species via horizontal transmission (Scholz *et al.*, 2020). Ecological interactions, including prey-predator, host-parasitoid, sharing common food resources or any direct physical interaction, can be a pathway of horizontal transfer of *Wolbachia* (Hunter *et al.*, 2003; Kittayapong *et al.*, 2003; Le Clec'h *et al.*, 2013; Brown and Lloyd, 2015; Li *et al.*, 2017). Similar to many insect species, scale insects have close interactions with many arthropod groups in their community, including predators and parasitoids. In addition, many scale insect species have a mutual relation with ants through trophallaxis interactions (Hölldobler *et al.*, 1990; Buckley and

Gullan, 1991, 1). As such, ants consume scale insect's honeydew and in return provide protection to scale insects (Gullan *et al.*, 1993). Here, we screened for *Wolbachia* infection with the several ecological associate groups (especially ants and parasitoid wasps) to interrogate the frequency of joint infection between scale insects and their associates. Based on that, we hypothesise the plausible common route of *Wolbachia* horizontal transmission in scale insects.

Results

Results of scale insect screen

Of the 151 species of scale insects that we screened for *Wolbachia*, 47 species (30.5%) had at least one specimen that tested positive for *Wolbachia*. We screened 689 specimens, amongst which 138 (20.1%) tested positive (File S2). After fitting a beta-binomial model to our data combined with previously published results, we estimate that 86.3% of scale insect species are infected at a prevalence threshold of 0.1%, and 43.6% are infected with a *Wolbachia* prevalence of 10% or more.

Comparison of Wolbachia incidence between scale insect and other arthropod groups

Table 1 and Figure 1 show our estimates for *Wolbachia* incidence and prevalence distributions in different arthropod groups. A likelihood ratio test indicates that hemipterans are characterised by a distribution of *Wolbachia* prevalence that is significantly different from that estimated in insects overall ($P = 0.0006$). In particular, at all three prevalence thresholds, hemipterans are estimated to have a higher infection frequency than arthropods in general. Similarly, the distribution of *Wolbachia* prevalence in scale insects is significantly different from the one in other hemipterans ($P = 7 \times 10^{-10}$) even though the predicted fraction of infected species is similar.

The plotted prevalence distribution of *Wolbachia* indicates that the pattern of infection in scale insect species is quite distinct from other groups (Figure 2). While species of all non-scale insect hemipteran families were estimated to have a U-shaped distribution (indicating that most species either have a very high or a very low infection rate), the distribution of infection in scale insect species represents an exponential decline. This distribution signposting most of scale insect species are predicted to have a low to intermediate number of infected individuals.

Variation in infection probability between groups of scale insects

By adopting our estimated scale insect parameters ($\alpha = 0.535$, $\beta = 1.454$), we estimated the infection probability for each species (File S2). The mean of infection probability was then calculated for each scale insect family (Table S2A). By filtering out the families with less than 40 specimens and 20 species, the probability of infection and its standard error is shown in Figure 3. At 10% infection prevalence threshold, coccids, eriococcids, pseudococcids have mean species infection probabilities of 0.39, 0.46 and 0.48 respectively (Figure 3A, Table S2A), which is close to the estimated mean for the whole superfamily Coccoidea (43.6%) (Table 1).

The mean infection probability of well sampled scale insect species grouped according to the major host plant family from which it was collected (those plant families with more than 25 specimens and 5 scale insect species) is plotted in Figure 3B (the complete list is provided in Table S2B). Most of the scale insects were collected from plants belonging to family Myrtaceae (113 species and 265 specimens). Therefore, it is not surprising that the infection probability of those samples (around 44% at 10% infection prevalence, see Figure 3B, Table S3A) is identical to that estimated for Coccoidea as a whole (Figure 1, Table 1). From host

families with a sufficient number of specimens, Fabaceae had the highest infection probability (64% at a $c=10\%$ infection prevalence threshold).

Association tests

Fisher's exact tests failed to find a significant association between *Wolbachia* infection and various biological and ecological traits of scale insects. The mean P value of 10,000 replicates test was not significant for any character ($P > 0.05$) (Table 2).

Infection in pairs of scale insects and their associates

150 pairs of scale insects and their associates were categorized into seven groups (based on associate taxonomy) and screened for *Wolbachia* (Table S4). Based on the infection status of the scale insect and its direct associate in each pair, contingency tables were generated for three groups: wasps, ants and sum of all associates (Table 3). Fisher's exact tests performed on each contingency table indicate there is significant non-random association between the *Wolbachia* infection of scale insects and all associates (P -value = 0.0008), and scale insects and ants (P -value = 0.0013). However, no significant association was observed between scale insects and wasp's infection (P -value = 0.6169). The estimated Pearson Phi showed that there is a positive association between scale insect and all associates ($\phi = 0.28$) and scale insect and ants ($\phi = 0.52$).

Discussion

An overview on Wolbachia infection in scale insects

By conducting the first broad survey of *Wolbachia* in scale insects and compiling an up to date comprehensive database of *Wolbachia* infection in arthropods, we obtained a comparative view of the distribution of *Wolbachia* in scale insects and other hemipterans. Our results indicate that although the fraction of infected scale species is not different from

other groups of Hemiptera, the pattern of infection prevalence is distinct; in contrast to the U-shaped beta-distributions of *Wolbachia* prevalence estimated for other arthropods, the pattern in scale insects is similar to one of exponential decline (Figure 2). This means that most scale insect species are predicted to be infected with *Wolbachia* at a low to intermediate prevalence, with very few species in which *Wolbachia* is at or near fixation.

One plausible hypothesis to explain the predominance of such low prevalence infections is that *Wolbachia* may not be able to induce CI in scale insects, given that CI is predicted to always drive the infection to greater than 50% prevalence. This could be a result of the peculiar reproductive system of scale insects, which may have the potential to reduce or prevent the expression of the CI phenotype. In female scale insects, “vestibule cells” located at the neck of the ovariole receive the sperm from the spermatheca (or in some species, cells and chambers near the ovariole), and keep them for hours to days before transferring them to the oocyte (Koteja, 1990; Robison, 1990). While the function of scale insect vestibule cells is mostly unknown, they may biochemically assess the quality of sperms (Normark, 2009) and block or limit those that have been modified by *Wolbachia*. In support of this notion, studies in other insect groups have reported an impact of *Wolbachia* on sperm aside from CI modification. For example, *Wolbachia* infection may lower sperm competitive ability (Champion de Crespigny and Wedell, 2006; Lewis *et al.*, 2011) and might chemically mark the modified sperm cells (Riparbelli *et al.*, 2007) in a way that they can be distinguished from the normal sperm cells by the female reproductive system. It will be interesting to investigate whether the vestibule cells in scale insects do indeed have a role in blocking *Wolbachia*-modified sperm. Moreover, we need to identify the phenotypes induced by *Wolbachia* in scale insects (which to date are completely unknown), and ascertain whether CI is indeed rare or even absent in this group.

Whilst many surveys for *Wolbachia* report the fraction of species where at least one specimen tests positive, here we used a beta-binominal distribution approach to estimate the probability of infection. This enables us to provide an updated estimation of infection probability in arthropods and estimated that 46.7% and 74.6% of arthropod species are infected with *Wolbachia* at thresholds of 10% and 0.1%, respectively. It is important to note that our methodology is slightly different from that of Weinert *et al.* (2015) and thus the mentioned estimation is different ((Weinert *et al.* (2015) estimated 52% infection probability at 0.1% threshold). We also extensively improved our knowledge on *Wolbachia* infection rate in different scale insect families. Coccids (with more than 1100 described species, (García Morales *et al.*, 2016)), eriococcids (comprising the highest portion of gall inducers in scale insects (Cook and Gullan, 2004)) and pseudococcids (mealybugs) are three large families of scale insects with respectively 38%, 45% and 47% infection probability at >10% prevalence (Table S2A, Figure 3A). By grouping scale insects based on their host-plant families, we also found that scale insects that feed on Fabaceae plants have a higher infection probability (64%) than other main plant families (35% in Casuarinaceae, 44% in Myrtaceae, and 18% in Proteaceae) (Table S2B, Figure 3B). Such higher infection probability may relate to the higher species diversity and ecological interactions in Fabaceae plants (Wojciechowski, 2005; Villaseñor *et al.*, 2007).

No evidence of significant associations between Wolbachia infection and scale insect traits

Host breadth, distributional range and the number of reported parasitoid species are variables that could correlate with ecological interactions and consequently the probability of *Wolbachia* infection. Compared to monophagous scale insects, polyphagous scale insect species may be exposed to more diverse ecological interactions. Similarly, species with a broad distribution and those that are visited with more parasitoid species might have a higher chance to be infected as they experience more ecological interactions. Moreover, in many

insects with the ability to induce galls, it is expected that the gall may provide a level of protection against parasitoids and predators and consequently decrease the extent of direct physical interactions between the insect and environment (Cornell, 1983; Stone and Schönrogge, 2003; Miller *et al.*, 2009). This might be true for some scale insects that are capable of inducing galls (e.g. eriococcids) and hence protect the female and newborn nymphs from the physical interactions with other insects (Gullan and Kosztarab, 1997; Cook and Gullan, 2004). However, in this study, we did not find any significant association to support any of the mentioned scenarios. This is in line with mixed results among other studies testing for correlations between *Wolbachia* infection and host traits. For example, a positive correlation between *Wolbachia* prevalence and ecological traits of bees (including host-parasite interactions, nesting, and phenology) has been reported (Gerth *et al.*, 2015), but other studies have found no such correlations (e.g. (Shoemaker *et al.*, 2002; Klopstein *et al.*, 2018; Kajtoch *et al.*, 2019).

Another well-known host feature that can directly or indirectly be affected by *Wolbachia* is the reproductive mode. Parthenogenesis induction is one of the best known host phenotypes effected by *Wolbachia* (Weeks and Breeuwer, 2001; Huigens, 2003). This is well recorded in hymenopterans, weevils, thrips and mites (Vandekerckhove *et al.*, 1999; Son *et al.*, 2008; Rodriguero *et al.*, 2010; Ma and Schwander, 2017; Elias-Costa *et al.*, 2019), but in many other cases, *Wolbachia* infection is not related to the reproductive mode or female-biased sex ratio (e.g. in the case of the isopod *Jaera albifrons* (Ribardièrre *et al.*, 2018), gall wasps (Schuler *et al.*, 2018) and *Altica* flea beetles (Wei *et al.*, 2020)). In scale insects, there are several parthenogenetic species. It is demonstrated that a parthenogenetic lineage in armoured scale insect (*Aspidiotus nerii*) has a positive association with *Cardinium* infection and thus *Cardinium* might be the causative agent of parthenogenesis reproduction mode (Provencher *et al.*, 2005). However, while the current study is unable to directly test the role

of *Wolbachia* in parthenogenesis reproduction in scale insects, we could not find any significant association between the infection and reproduction mode. If *Wolbachia* induces parthenogenesis, then it is expected to have near 100% infection frequency in a parthenogenetic lineage (Zhu *et al.*, 2007; Russell and Stouthamer, 2011). None of the 19 scale insect species with obligate parthenogenesis for which we sampled more than one specimen had 100% *Wolbachia* infection frequency (the highest infection frequency is 50% in *Saissetia miranda*) (Table S2), which is additional evidence against *Wolbachia*-mediated parthenogenesis in scale insects. If *Wolbachia* is not the causative agent of parthenogenesis in scale insects, and since the other classic *Wolbachia* manipulation phenotypes (CI, MK and feminisation) all rely on sexual reproduction, one might expect to see a negative association between *Wolbachia* and parthenogenesis. However, this was also not observed, presumably because other phenotypic effects of *Wolbachia* are responsible for its maintenance in parthenogenetic species (Zhu *et al.*, 2007). The commonness of parthenogenesis reproduction mode in scale insect species may also partly explain the low *Wolbachia* prevalence in scale insects overall.

It is important to note that our estimated infection probability in scale insects (like the majority of hemipterans) was estimated from female samples. While there is a possibility that the infection rate varies between males and females (and that consequently the outcome of the association tests could depend on which sex is used), further study is needed to investigate whether this is the case in scale insects. In addition, we did not incorporate host or *Wolbachia* phylogeny in our association tests. Ideally, comparative methods such as those proposed in Hadfield *et al.* (2014) should be applied, but due to lack of genetic data and hence phylogenetic trees for either party we were unable to do so. However, given that phylogenetic history is usually envisioned as producing associations rather than annihilating

them, we do not expect our negative result of an absence of associations with all traits we considered to be affected by the choice of our cruder method.

Plausible routes of Wolbachia horizontal transfers in scale insects

Finally, we sought to identify plausible routes of *Wolbachia* horizontal transmission in scale insects. Our results, which are based on infection in pairs of scale insects and their associates, indicate that there is a positive association between scale insect and ant infection, but not between scale insects and wasps (Table 3). Although host-parasitoid interactions are suggested as a plausible route of *Wolbachia* horizontal transmission (Sanaei *et al.*, 2020), it is unknown how common this route is compared to alternatives (Cook and Butcher, 1999; Heath *et al.*, 1999; Vavre *et al.*, 1999; Huigens *et al.*, 2000; Raychoudhury *et al.*, 2009; Kageyama *et al.*, 2010; Morrow *et al.*, 2014; Ahmed *et al.*, 2015). Indeed, host-parasitoid interactions usually impose fitness costs on the host and hence reduce the probability of *Wolbachia* horizontal transmission from the parasitoid to the host. On the other hand, ecological interactions between ants and hemipterans can be more stable and there are often bidirectional fitness benefits (Depa *et al.*, 2020). From inter-colony and inter-species social interactions (Frost *et al.*, 2010; Andersen *et al.*, 2012) to various direct and indirect ecological interactions with other insects, ants are considered as one of the major groups facilitating horizontal transfer of *Wolbachia* and other microbes, mainly via trophobiosis (mediated by foods) (Pringle and Moreau, 2017; Tolley *et al.*, 2019). Ants can have mutualistic relations with hemipteran species that produce honeydew, including scale insects, aphids and whiteflies (Buckley, 1987; Delabie, 2001; Wilson and Hölldobler, 2005). Indeed, many scale insects can be found with generalist or specialist ant species that protect them from parasites and predators while producing honeydew for them (Way, 1954; Bartlett, 1961; Bach, 1991; Itioka and Inoue, 1996; Gullan and Kosztarab, 1997; Heckroth *et al.*, 1998; Marco, and Kent, 2008). In some cases, such relations are obligatory and by removing the

ant, the fitness of scale insects is dramatically decreased (Abbott and Green, 2007). Moreover, hemipteran microbiota may also affect the composition of honeydew (as is reported in aphids (Sabri *et al.*, 2013)), and may indirectly affect ant-scale insect interactions. However, to confirm the horizontal transmission between ants and scale insects, *Wolbachia* strain determination is necessary.

Our study provides a first overview of *Wolbachia* infection in scale insects and their associates. Studies uncovering the phenotypes induced by *Wolbachia*, its interaction with the host microbiome, its distribution within species, and the phylogenetic relationships between strains are needed for a more complete picture of *Wolbachia*'s role in scale insects.

Experimental Procedures

Sampling and DNA extraction

A total of 689 individuals belonging to 151 species, 52 genera, and nine families of scale insects were screened for *Wolbachia*. Except for six males of *Cryptes baccatus*, all samples were adult females and might have included developing offspring in ovoviviparous species. The majority of these samples were collected in Australia, with other samples representing all continents except Antarctica (Table S1, Figure S1:2). All collections were made under the relevant Australian state/territory authorities. For each sample, the geographic coordination and the host plant species were recorded (File S1). Samples belonging to the same species that are reported from the same location were always collected from different trees (File S1). Due to the focus of scale insect taxonomy in Australia, our database mostly includes scale insects from the families Coccidae, Pseudococcidae, and Eriococcidae (38, 89, and 19 species, respectively), with an additional nine species of Diaspididae, five species of Monophlebidae, and a single species each from the Asterolecaniidae, Cerococcidae.

Kerriidae and Phenacoleachiidae. Some of the scale insects' samples were assigned only to the genus level as they have not yet been described, or a major taxonomic revision is required for that group.

During field work, direct arthropod associates of scale insects, including ants, wasps, flies, beetles, thrips, and mites, were also collected and added to the database. The ecological association of these arthropods with scale insects are confirmed either by direct observation of physical interactions (e.g., oviposition of parasitoids, ants trophallaxis, predation by beetles) or by rearing the collected scale insects in the lab and later preserving larvae or adults of parasitoids (e.g., wasps) emerging from them into the ethanol. For most of the associates, we were not able to determine identification to species.

All collected samples were preserved individually in ethanol prior to DNA extraction. DNA extraction was done using either the Bioline DNA extraction kit (Bioline Corporation, London, UK) or the CTAB (Cetyl Trimethyl Ammonium Bromide) method (Semple *et al.*, 2015) from whole insects. The quality of extracted DNAs was tested with Arthropoda 18S ribosomal RNA primers (2880, Br) (von Dohlen and Moran, 1995). A positive PCR band was taken as evidence for successful DNA extraction, and samples with negative bands were discarded from the study.

Wolbachia infection determination

In order to determine the *Wolbachia* infection status by PCR, we used primers for the following *Wolbachia* genes: 16S rRNA, the five *Wolbachia* Multilocus Sequence Typing scheme (MLST) genes (fbpA, coxA, ftsZ, gatB, hcpA), and *wsp*. A detailed information on design of *Wolbachia* specific 16S rRNA primers, all other MLST primers used in this study, and the PCR configuration is provided in File S3 (including Table S2). We assigned positive infection status to a sample if it had a positive 16S rRNA band and a positive band for at least

one of the other genes. We chose this approach because single primers sometimes fail to amplify their corresponding gene fragments in some strains (Simões *et al.*, 2011). As a positive control, we used extracted DNA of *Drosophila melanogaster* with a confirmed *Wolbachia* infection by wMel strain (Monsanto-Hearne and Johnson, 2018) for all PCRs.

Estimating and comparing Wolbachia incidence between scale insects and other arthropods

To compare *Wolbachia* incidence in scale insects with other arthropods, we first compiled an up-to-date, comprehensive list of arthropod species that have been tested for *Wolbachia* infection status (File S1). The starting point for this dataset were the datasets provided by Weinert (2015) and Charlesworth *et al.* (2019). We then added data from an additional 114 publications resulting in the most comprehensive *Wolbachia* incidence dataset compiled to date (to October 10th 2020), including 152,694 tested specimens from 6640 arthropod species. We used only samples that were tested individually for *Wolbachia* and filtered out species where samples had been pooled for the PCR.

We followed an approach put forward by Hilgenboecker *et al.* (2008), where a beta-binomial distribution was fitted to our data to estimate the distribution of *Wolbachia* prevalence within species. Specifically, we obtained maximum likelihood estimates for the parameters α and β defining a beta distribution for infection by numerically optimising the joint log-likelihood function of the beta-binomial model for a given data set, with initial values taken from the maximum likelihood estimates obtained by Hilgenboecker *et al.* (2008) ($\alpha=0.12$, $\beta=0.36$). The probability x_c that a randomly chosen species is infected with *Wolbachia* with a prevalence of at least c was then calculated by integrating over the probability density function of the beta distribution. We used three different thresholds levels c (0.1, 0.01 and 0.001) to cover a wide range of scenarios of potential interest. Finally, we obtained 1,000

bootstrap values for α , β and x_c , based on which we calculated confidence intervals for these parameters using the *basic method* (Davison and Hinkley, 1997).

We obtained maximum likelihood estimates and confidence intervals for α , β and x_c for the following groups: all arthropods, all insects, all hemipterans and all hemipteran families for which sufficient data exist. Apart from the scale insects, these families included the Aphididae, Cicadellidae, Delphacidae, Lygaeidae, Miridae and Pentatomidae, all of which are families with at least 10 genera, 25 species and 100 specimens in the *Wolbachia* survey dataset. Scale insect families were pooled into the Coccoidea superfamily (total 764 samples) for this analysis. To test for differences in infection frequencies between two groups, we conducted likelihood ratio tests on the estimated maximum likelihood ratios of two models: one where we estimated parameters for a larger group (e.g., all Hemiptera), and one where we estimated different parameters for two subgroups (e.g., scale insects and all other Hemiptera). Specifically, we calculated the test statistic $-2(\log L_T - \log L_1 - \log L_2)$, where L_T , L_1 and L_2 are the maximum likelihoods of the larger group and the two subgroups, respectively, and then compared this value to a chi-square distribution with two degrees of freedom.

Biological and ecological data for scale insects

In order to test for associations between *Wolbachia* infection and scale insect biological and ecological traits, we collated data from two of the authors' (LGC and YPL) personal scale insect databases and the information available online in the ScaleNet database (García Morales *et al.*, 2016). The following data were compiled for each species (File S2):

1. Host breadth: By counting the number of reported host plant species and families of each scale insect species, those feeding on a single host species were assigned as “specialist”, those feeding on more than one host plant species within a single family

were assigned as “monophagous” and those species that feed on two or more host families were assigned as “polyphagous”.

2. Geographic distribution: the categories “limited”, “broad” and “cosmopolitan” were used to refer to scale insect species with a distribution range of fewer than 2 million km², between 2 and 6 million km², and beyond 6 million km² area, respectively. Therefore, Australian endemic species are categorized as having either a “limited” or “broad” distribution, and most of globally invasive pest species are categorized as “cosmopolitan”.

3. Reproduction mode: scale insects are classified into categories "sexual", "facultative parthenogenesis", "obligate parthenogenesis" and "hermaphrodite". However, populations of some species may be observed with different reproduction modes in different lineages or geographic areas. Specifically, *Aspidiotus nerii* and *Ferrisia virgata* can be either obligate parthenogens or sexual, and *Icerya aegyptiaca* can reproduce either by facultative parthenogenesis and sexually. Therefore, the reproduction mode of these was assigned as “mixed”. Further, out of the 151 species of these study, 24 species were assigned reproduction mode “unknown”, and therefore excluded from the analysis.

4. Parasitised: For some well-studied scale insect species, the information about foes mostly consisted of lists of parasitoid species, summarized in ScaleNet (García Morales *et al.*, 2016). Scale insect species with more than 10 recorded parasitoids were assigned as “highly parasitised”, and those with fewer (but not zero/unknown) were assigned as “low parasitised”. However, this kind of information was only available for 27 species of our study. Therefore, the analysis was run only for these species.

5. Gall: “Gall maker” versus “Non-gall maker” are the two categories for this trait.

443 Association tests between *Wolbachia* infection and scale insect characters

444 By adopting the specific estimated α , β parameters for scale insects, we estimated the
 445 infection probability for each species with three different frequency thresholds c . We adopted
 446 the following equation in which infection frequency (p) within a given species, c is the
 447 minimum prevalence above which we want to classify a species as being infected, H is the
 448 hypothesis that the species is infected (i.e. that $p \geq c$), and D is the *Wolbachia* infection data
 449 we collected in the current study for this species comprised of the number of sampled
 450 specimens (n) and the number of specimens ($k \leq n$) that tested positive for *Wolbachia*. We
 451 can then obtain the probability that the species is infected as

$$452 \quad P(H|D) = \frac{P(D \cap H)}{P(D)} \quad (1)$$

453 Here, $P(D \cap H)$ is the probability that the data are obtained and the species is infected, given
 454 by

$$455 \quad P(D \cap H) = \int_c^1 f_{\text{beta}(\alpha, \beta)}(p) f_{\text{binom}(p, n)}(k) dp \quad (2)$$

456 where

$$457 \quad f_{\text{beta}(\alpha, \beta)}(p) = \frac{p^{\alpha-1}(1-p)^{\beta-1}}{B(\alpha, \beta)} \quad \text{and} \quad f_{\text{binom}(p, n)}(k) = \binom{n}{k} p^k (1-p)^{n-k} \quad (3)$$

458 are the probability density functions for the beta and binomial distribution, respectively, and
 459 $B(\alpha, \beta)$ is the beta function. Similarly, the probability $P(D)$ of observing the data is

$$460 \quad P(D) = \int_0^1 f_{\text{beta}(\alpha, \beta)}(p) f_{\text{binom}(p, n)}(k) dp = \binom{n}{k} \frac{B(k + \alpha, n - k + \beta)}{B(\alpha, \beta)}, \quad (4)$$

461 which is the probability density of the beta-binomial distribution. After inserting the
 462 expressions for $P(D \cap H)$ and $P(D)$ into Eq.1 and simplifying we obtain

$$P(H|D) = 1 - \frac{\tilde{B}(c, k + \alpha, n - k + \beta)\Gamma(n + \alpha + \beta)}{\Gamma(k + \alpha)\Gamma(n - k + \beta)}, \quad (5)$$

where $\tilde{B}$ is the incomplete beta function and Γ is the gamma function. From the probability of infection for each species, the mean of the probability of infection can be calculated for each scale insect family, and also across host plant families that scale insect species were collected from.

In order to test for associations between *Wolbachia* infection status and the scale insects' ecological and biological traits, species represented by single specimens were first filtered out. For the "parasitised" ecological trait, species with an "unknown" value were also filtered out. For this test, we first generated random infection statuses for each species by drawing from a Bernoulli distribution with their estimated infected probabilities. Then we ran Fisher's exact test (Fisher, 1934) between these randomly generated infection statuses and a given species trait. This procedure was repeated 10,000 times, yielding a distribution of *P*-values, which is an indicator of a possible correlation between a trait and infection status.

Scale insects and their associates

An associate pair is formed from a scale insect and a direct associate specimen. As such we have 150 associate pairs categorized into nine groups, mostly wasp (n=81) and ant (n=42) associate groups. We generated a contingency table for all groups, scale insect-wasp and scale insect-ant groups where the rows are the numbers of infected vs. uninfected scale insects and the columns are the numbers of infected and uninfected associates. We then ran Fisher's exact test to check whether there is a correlation between infection status of the scale insects with their associates. Finally, we estimated the phi coefficient (mean square contingency coefficient) (Harald, 1946) for each contingency table, to determine whether the correlation is positive or negative.

486 *Code*

487 All data analysis, statistics and plotting was done in R v3.6.0 (R Core Team, 2020). We used
488 the following R packages: tidyverse (Wickham *et al.*, 2019), rmisc (Hope, 2013), grid
489 (Murrell, 2020), mosaic (Pruim *et al.*, 2020), and ggpubr (Kassambara, 2020). Scripts are
490 provided as File S4.

491 **References**

- 492 Abbott, K.L. and Green, P.T. (2007) Collapse of an ant–scale mutualism in a rainforest on
493 Christmas Island. *Oikos* **116**: 1238–1246.
- 494 Ahmed, M.Z., Li, S.-J., Xue, X., Yin, X.-J., Ren, S.-X., Jiggins, F.M., et al. (2015) The
495 intracellular bacterium *Wolbachia* uses parasitoid wasps as phoretic vectors for
496 efficient horizontal transmission. *PLoS Pathog* **11**: e1004672.
- 497 Andersen, S.B., Boye, M., Nash, D.R., and Boomsma, J.J. (2012) Dynamic *Wolbachia*
498 prevalence in *Acromyrmex* leaf-cutting ants: potential for a nutritional symbiosis. *J*
499 *Evol Biol* **25**: 1340–1350.
- 500 Bach, C.E. (1991) Direct and indirect interactions between ants (*Pheidole megacephala*),
501 scales (*Coccus viridis*) and plants *Pluchea indica*. *Oecologia* **87**: 233–239.
- 502 Bartlett, B.R. (1961) The Influence of Ants Upon Parasites, Predators, and Scale Insects. *Ann*
503 *Entomol Soc Am* **54**: 543–551.
- 504 Baumann, P. (2005) Biology of Bacteriocyte-Associated Endosymbionts of Plant Sap-
505 Sucking Insects. *Annu Rev Microbiol* **59**: 155–189.
- 506 Behera, L., Sahu, S.C., Rajamani, S., and Mohan, M. (2001) Molecular evidence for
507 *Wolbachia* in rice insects. *Curr Sci* **81**: 1299–1300.
- 508 Beltran-Bech, S. and Richard, F.-J. (2014) Impact of infection on mate choice. *Anim Behav*
509 **90**: 159–170.

510 Bourtzis, K. and O'Neill, S. (1998) "Wolbachia" Infections and Arthropod Reproduction.
511 *BioScience* **48**: 287–293.

512 Brown, A.N. and Lloyd, V.K. (2015) Evidence for horizontal transfer of Wolbachia by a
513 *Drosophila* mite. *Exp Appl Acarol* **66**: 301–311.

514 Brownlie, J.C., Cass, B.N., Riegler, M., Witsenburg, J.J., Iturbe-Ormaetxe, I., McGraw, E.A.,
515 and O'Neill, S.L. (2009) Evidence for Metabolic Provisioning by a Common
516 Invertebrate Endosymbiont, *Wolbachia pipientis*, during Periods of Nutritional Stress.
517 *PLOS Pathog* **5**: e1000368.

518 Buckley, R. (1987) Ant-Plant-Homopteran Interactions. *Adv Ecol Res* **16**: 53–85.

519 Buckley, R. and Gullan, P. (1991) More Aggressive Ant Species (Hymenoptera: Formicidae)
520 Provide Better Protection for Soft Scales and Mealybugs (Homoptera: Coccidae,
521 Pseudococcidae). *Biotropica* **23**: 282–286.

522 Champion de Crespigny, F.E. and Wedell, N. (2006) Wolbachia infection reduces sperm
523 competitive ability in an insect. *Proc R Soc B Biol Sci* **273**: 1455–1458.

524 Charlesworth, J., Weinert, L.A., Araujo, E.V., and Welch, J.J. (2019) Wolbachia, Cardinium
525 and climate: an analysis of global data. *Biol Lett* **15**: 20190273.

526 Cook, J.M. and Butcher, R.D.J. (1999) The transmission and effects of Wolbachia bacteria in
527 parasitoids. *Res Popul Ecol* **41**: 15–28.

528 Cook, L.G. (2000) Extraordinary and extensive karyotypic variation: A 48-fold range in
529 chromosome number in the gall-inducing scale insect *Apiomorpha* (Hemiptera:
530 Coccoidea: Eriococcidae). *Genome* **43**: 255–263.

531 Cook, L.G. and Gullan, P.J. (2004) The gall-inducing habit has evolved multiple times
532 among the eriococcid scale insects (Sternorrhyncha: Coccoidea: Eriococcidae). *Biol J*
533 *Linn Soc* **83**: 441–452.

534 Cooper, B.S., Vanderpool, D., Conner, W.R., Matute, D.R., and Turelli, M. (2019)
535 Wolbachia Acquisition by *Drosophila yakuba*-Clade Hosts and Transfer of
536 Incompatibility Loci Between Distantly Related Wolbachia. *Genetics* **212**: 1399–
537 1419.

538 Cornell, H.V. (1983) The Secondary Chemistry and Complex Morphology of Galls Formed
539 by the Cynipinae (Hymenoptera): Why and How? *Am Midl Nat* **110**: 225–234.

540 Davison, A.C. and Hinkley, D.V. (1997) Bootstrap Methods and Their Application,
541 Cambridge University Press.

542 Delabie, J.H.C. (2001) Trophobiosis Between Formicidae and Hemiptera (Sternorrhyncha
543 and Auchenorrhyncha): an Overview. *Neotrop Entomol* **30**: 501–516.

544 Depa, Ł., Kaszyca-Taszkowska, N., Taszakowski, A., and Kanturski, M. (2020) Ant-
545 induced evolutionary patterns in aphids. *Biol Rev* **n/a**:

546 Detcharoen, M., Arthofer, W., Schlick-Steiner, B.C., and Steiner, F.M. (2019) Wolbachia
547 megadiversity: 99% of these microorganismic manipulators unknown. *FEMS*
548 *Microbiol Ecol* **95**: fuz151.

549 von Dohlen, C.D., Kohler, S., Alsop, S.T., and McManus, W.R. (2001) Mealybug β -
550 proteobacterial endosymbionts contain γ -proteobacterial symbionts. *Nature* **412**: 433–
551 436.

552 von Dohlen, C.D. and Moran, N.A. (1995) Molecular phylogeny of the homoptera: a
553 paraphyletic taxon. *J Mol Evol* **41**: 211–223.

554 Duron, O., Bouchon, D., Boutin, S., Bellamy, L., Zhou, L., Engelstädter, J., and Hurst, G.D.
555 (2008) The diversity of reproductive parasites among arthropods: Wolbachia do not
556 walk alone. *BMC Biol* **6**: 27.

557 Elias-Costa, A.J., Confalonieri, V.A., Lanteri, A.A., and Rodriguero, M.S. (2019) Game of
558 clones: Is Wolbachia inducing speciation in a weevil with a mixed reproductive
559 mode? *Mol Phylogenet Evol* **133**: 42–53.

560 Engelstädter, J. and Hurst, G.D.D. (2009) What Use Are Male Hosts? The Dynamics of
561 Maternally Inherited Bacteria Showing Sexual Transmission or Male Killing. *Am Nat*
562 **173**: E159–E170.

563 Everett, K.D.E., Thao, M., Horn, M., Dyszynski, G.E., and Baumann, P. (2005) Novel
564 chlamydiae in whiteflies and scale insects: endosymbionts ‘Candidatus Fritschea
565 bemisiae’ strain Falk and ‘Candidatus Fritschea eriococci’ strain Elm. *Int J Syst Evol*
566 *Microbiol* **55**: 1581–1587.

567 Fisher, R.A. (1934) Statistical methods for research workers, 5th ed. Edinburgh and London:
568 Oliver and Boyd.

569 Frost, C.L., Fernandez-Marin, H., Smith, J.E., and Hughes, W.O.H. (2010) Multiple gains
570 and losses of Wolbachia symbionts across a tribe of fungus-growing ants. *Mol Ecol*
571 **19**: 4077–4085.

572 García Morales, M., Denno, B.D., Miller, D.R., Miller, G.L., Ben-Dov, Y., and Hardy, N.B.
573 (2016) ScaleNet: a literature-based model of scale insect biology and systematics.
574 *Database* **2016**..

575 Gavrilov, I.A. (2007) A catalog of chromosome numbers and genetic systems of scale insects
576 (Homoptera: Coccinea) of the world. **37**: 46.

577 Gerth, M., Saeed, A., White, J.A., and Bleidorn, C. (2015) Extensive screen for bacterial
578 endosymbionts reveals taxon-specific distribution patterns among bees
579 (Hymenoptera, Anthophila). *FEMS Microbiol Ecol* **91**: 1–12.

580 Gong, J.-T., Li, Y., Li, T.-P., Liang, Y., Hu, L., Zhang, D., et al. (2020) Stable Introduction
581 of Plant-Virus-Inhibiting Wolbachia into Planthoppers for Rice Protection. *Curr Biol*.

582 Gruwell, M.E., Morse, G.E., and Normark, B.B. (2007) Phylogenetic congruence of armored
583 scale insects (Hemiptera: Diaspididae) and their primary endosymbionts from the
584 phylum Bacteroidetes. *Mol Phylogenet Evol* **44**: 267–280.

585 Gruwell, M.E., Wu, J., and Normark, B.B. (2009) Diversity and Phylogeny of Cardinium
586 (Bacteroidetes) in Armored Scale Insects (Hemiptera: Diaspididae). *Ann Entomol Soc*
587 *Am* **102**: 1050–1061.

588 Gullan, P.J., Buckley, R.C., and Ward, P.S. (1993) Ant-Tended Scale Insects (Hemiptera:
589 Coccidae: Myzolecanium) within Lowland Rain Forest Trees in Papua New Guinea. *J*
590 *Trop Ecol* **9**: 81–91.

591 Gullan, P.J. and Cook, L.G. (2007) Phylogeny and higher classification of the scale insects
592 (Hemiptera: Sternorrhyncha: Coccoidea). *Zootaxa* 413–425.

593 Gullan, P.J. and Kosztarab, M. (1997) Adaptations in Scale Insects. *Annu Rev Entomol* **42**:
594 23–50.

595 Hadfield, J.D., Krasnov, B.R., Poulin, R., and Nakagawa, S. (2014) A Tale of Two
596 Phylogenies: Comparative Analyses of Ecological Interactions. *Am Nat* **183**: 174–
597 187.

598 Harald, C. (1946) Mathematical Methods of Statistics, Princeton. US: University Press.

599 Heath, B.D., Butcher, R.D.J., Whitfield, W.G.F., and Hubbard, S.F. (1999) Horizontal
600 transfer of Wolbachia between phylogenetically distant insect species by a naturally
601 occurring mechanism. *Curr Biol* **9**: 313–316.

602 Heckroth, H.-P., Fiala, B., Gullan, P.J., Idris, A.H., and Maschwitz, U. (1998) The soft scale
603 (Coccidae) associates of Malaysian ant-plants. *J Trop Ecol* **14**: 427–443.

604 Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., and Werren, J.H. (2008)
605 How many species are infected with Wolbachia? – a statistical analysis of current
606 data. *FEMS Microbiol Lett* **281**: 215–220.

607 Hoffmann, A.A., Ross, P.A., and Rašić, G. (2015) Wolbachia strains for disease control:
608 ecological and evolutionary considerations. *Evol Appl* **8**: 751–768.

609 Hoffmann, A.A., Turelli, M., and Harshman, L.G. (1990) Factors affecting the distribution of
610 cytoplasmic incompatibility in *Drosophila simulans*. *Genetics* **126**: 933–948.

611 Hölldobler, B., Holldobler, F.P. of B.B., Wilson, E.O., and Wilson, H.C. in E. and
612 U.R.P.E.E.O. (1990) *The Ants*, Harvard University Press.

613 Hope, R.M. (2013) Package ‘Rmisc’, contains many functions useful for data analysis and
614 utility operations.

615 Huigens, M.E. (2003) On the evolution of Wolbachia-induced parthenogenesis in
616 *Trichogramma* wasps.

617 Huigens, M.E., Luck, R.F., Klaassen, R.H.G., Maas, M., Timmermans, M., and Stouthamer,
618 R. (2000) Infectious parthenogenesis. *Nature* **405**: 178–179.

619 Hunter, M.S., Perlman, S.J., and Kelly, S.E. (2003) A bacterial symbiont in the Bacteroidetes
620 induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*.
621 *Proc R Soc Lond B Biol Sci* **270**: 2185–2190.

622 Ilinsky, Y. and Kosterin, O.E. (2017) Molecular diversity of Wolbachia in Lepidoptera:
623 Prevalent allelic content and high recombination of MLST genes. *Mol Phylogenet*
624 *Evol* **109**: 164–179.

625 Itioka, T. and Inoue, T. (1996) The Consequences of Ant-Attendance to the Biological
626 Control of the Red Wax Scale Insect *Ceroplastes rubens* by *Anicetus beneficus*. *J*
627 *Appl Ecol* **33**: 609–618.

628 Jeyaprakash, A. and Hoy, M.A. (2000) Long PCR improves Wolbachia DNA amplification:
629 wsp sequences found in 76% of sixty-three arthropod species. *Insect Mol Biol* **9**: 393–
630 405.

631 Jiggins, F.M. (2003) Male-Killing Wolbachia and Mitochondrial DNA: Selective Sweeps,
632 Hybrid Introgression and Parasite Population Dynamics. *Genetics* **164**: 5–12.

633 Jiggins, F.M., Schulenburg, J.H.G. von der, Hurst, G.D.D., and Majerus, M.E.N. (2001)
634 Recombination confounds interpretations of Wolbachia evolution. *Proc R Soc Lond B*
635 *Biol Sci* **268**: 1423–1427.

636 Kageyama, D., Narita, S., Imamura, T., and Miyanoshita, A. (2010) Detection and
637 identification of Wolbachia endosymbionts from laboratory stocks of stored-product
638 insect pests and their parasitoids. *J Stored Prod Res* **46**: 13–19.

639 Kajtoch, Ł., Kolasa, M., Kubisz, D., Gutowski, J.M., Ścibior, R., Mazur, M.A., and
640 Holecová, M. (2019) Using host species traits to understand the Wolbachia infection
641 distribution across terrestrial beetles. *Sci Rep* **9**: 1–15.

642 Kassambara, A. (2020) ggpubr: “ggplot2” Based Publication Ready Plots.

643 Kaushik, S., Sharma, K.K., Ramani, R., and Lakhanpaul, S. (2019) Detection of Wolbachia
644 Phage (WO) in Indian Lac Insect [*Kerria lacca* (Kerr.)] and Its Implications. *Indian J*
645 *Microbiol* **59**: 237–240.

646 Kikuchi, Y., Meng, X.-Y., and Fukatsu, T. (2005) Gut Symbiotic Bacteria of the Genus
647 Burkholderia in the Broad-Headed Bugs *Riptortus clavatus* and *Leptocoris chinensis*
648 (Heteroptera: Alydidae). *Appl Environ Microbiol* **71**: 4035–4043.

649 Kittayapong, P., Jamnongluk, W., Thipaksorn, A., Milne, J.R., and Sindhusake, C. (2003)
650 Wolbachia infection complexity among insects in the tropical rice-field community.
651 *Mol Ecol* **12**: 1049–1060.

652 Klopstein, S., van Der Schyff, G., Tierney, S., and Austin, A.D. (2018) Wolbachia infections
653 in Australian ichneumonid parasitoid wasps (Hymenoptera: Ichneumonidae):
654 evidence for adherence to the global equilibrium hypothesis. *Biol J Linn Soc* **123**:
655 518–534.

656 Kondo, T., Gullan, P.J., and Williams, D.J. (2008) Coccidology. The study of scale insects
657 (Hemiptera: Sternorrhyncha: Coccoidea). *Corpoica Cienc Tecnol Agropecu* **9**: 55–61.

658 Koteja, J. (1990) Embryonic development, oviparity and viviparity. In *Armored scale insects:*
659 *their biology, natural enemies, and control, volume A*. Amsterdam: Elsevier, pp.
660 233–242.

661 Kuechler, S.M., Renz, P., Dettner, K., and Kehl, S. (2012) Diversity of Symbiotic Organs and
662 Bacterial Endosymbionts of Lygaeoid Bugs of the Families Blissidae and Lygaeidae
663 (Hemiptera: Heteroptera: Lygaeoidea). *Appl Environ Microbiol* **78**: 2648–2659.

664 Landmann, F. (2019) The Wolbachia Endosymbionts. In *Bacteria and Interacellularity*.
665 ASM PRESS, Washington, DC: Wiley, pp. 139–214.

666 Le Clec'h, W., Chevalier, F.D., Genty, L., Bertaux, J., Bouchon, D., and Sicard, M. (2013)
667 Cannibalism and predation as paths for horizontal passage of Wolbachia between
668 terrestrial isopods. *PloS One* **8**: e60232.

669 Lewis, Z., Champion de Crespigny, F.E., Sait, S.M., Tregenza, T., and Wedell, N. (2011)
670 Wolbachia infection lowers fertile sperm transfer in a moth. *Biol Lett* **7**: 187–189.

671 Li, S.-J., Ahmed, M.Z., Lv, N., Shi, P.-Q., Wang, X.-M., Huang, J.-L., and Qiu, B.-L. (2017)
672 Plantmediated horizontal transmission of Wolbachia between whiteflies. *ISME J* **11**:
673 1019–1028.

674 Lin, Y., Cook, D.H., Gullan, P.J., and Cook, L.G. (2015) Does host-plant diversity explain
675 species richness in insects? A test using Coccidae (Hemiptera). *Ecol Entomol* **40**:
676 299–306.

677 Liu, X.-D. and Guo, H.-F. (2019) Importance of endosymbionts Wolbachia and Rickettsia in
678 insect resistance development. *Curr Opin Insect Sci* **33**: 84–90.

679 Ma, W.-J. and Schwander, T. (2017) Patterns and mechanisms in instances of endosymbiont-
680 induced parthenogenesis. *J Evol Biol* **30**: 868–888.

681 Marco, S.B. and Kent, M.D. (2008) Host-handling behaviours in parasitoids of the black
682 scale: a case for ant-mediated evolution. *J Anim Ecol* **70**: 237–247.

683 Matsuura, Y., Koga, R., Nikoh, N., Meng, X.-Y., Hanada, S., and Fukatsu, T. (2009) Huge
684 Symbiotic Organs in Giant Scale Insects of the Genus *Drosicha* (Coccoidea:
685 Monophlebidae) Harbor Flavobacterial and Enterobacterial Endosymbionts. *Zoolog
686 Sci* **26**: 448–456.

687 Michalik, K., Szklarzewicz, T., Kalandyk-Kołodziejczyk, M., Jankowska, W., and Michalik,
688 A. (2016) Bacteria belonging to the genus *Burkholderia* are obligatory symbionts of
689 the eriococcids *Acanthococcus aceris* Signoret, 1875 and *Gossyparia spuria* (Modeer,
690 1778) (Insecta, Hemiptera, Coccoidea). *Arthropod Struct Dev* **45**: 265–272.

691 Michalik, K., Szklarzewicz, T., Kalandyk-Kołodziejczyk, M., and Michalik, A. (2019)
692 Bacterial associates of *Orthezia urticae*, *Matsucoccus pini*, and *Steingelia gorodetskia*
693 - scale insects of archaeocoid families Ortheziidae, Matsucoccidae, and Steingeliidae
694 (Hemiptera, Cocomorpha). *Protoplasma* **256**: 1205–1215.

695 Miller, D.G., Ivey, C.T., and Shedd, J.D. (2009) Support for the microenvironment
696 hypothesis for adaptive value of gall induction in the California gall wasp, *Andricus
697 quercuscalifornicus*. *Entomol Exp Appl* **132**: 126–133.

698 Min, K.-T. and Benzer, S. (1997) *Wolbachia*, normally a symbiont of *Drosophila*, can be
699 virulent, causing degeneration and early death. *Proc Natl Acad Sci* **94**: 10792–10796.

700 Monsanto-Hearne, V. and Johnson, K.N. (2018) *Wolbachia*-mediated protection of
701 *Drosophila melanogaster* against systemic infection with its natural viral pathogen
702 *Drosophila C virus* does not involve changes in levels of highly abundant miRNAs. *J
703 Gen Virol* **99**: 827–831.

704 Morrow, J.L., Frommer, M., Shearman, D.C.A., and Riegler, M. (2014) Tropical tephritid
705 fruit fly community with high incidence of shared Wolbachia strains as platform for
706 horizontal transmission of endosymbionts. *Environ Microbiol* **16**: 3622–3637.

707 Murrell, P. (2020) grid package | R Documentation.

708 Normand, H. (1949) Contribution au catalogue de coléoptères de Tunisie. Fascicule 4. *Bull*
709 *Société Sci Nat Tunis* **2**: 65–104.

710 Normark, B.B. (2009) 13 - Unusual gametic and genetic systems. In *Sperm Biology*.
711 Birkhead, T.R., Hosken, D.J., and Pitnick, S. (eds). London: Academic Press, pp.
712 507–538.

713 Pankewitz, F., Zöllmer, A., Hilker, M., and Gräser, Y. (2007) Presence of Wolbachia in
714 Insect Eggs Containing Antimicrobially Active Anthraquinones. *Microb Ecol* **54**:
715 713–721.

716 Prakash, B.M. and Puttaraju, H.P. (2007) Frequency of infection with A and B supergroup
717 Wolbachia in insects and pests associated with mulberry and silkworm. *J Biosci* **32**:
718 671–676.

719 Pringle, E.G. and Moreau, C.S. (2017) Community analysis of microbial sharing and
720 specialization in a Costa Rican ant–plant–hemipteran symbiosis. *Proc R Soc B* **284**:
721 20162770.

722 Provencher, L.M., Morse, G.E., Weeks, A.R., and Normark, B.B. (2005) Parthenogenesis in
723 the *Aspidiotus nerii* Complex (Hemiptera: Diaspididae): A Single Origin of a
724 Worldwide, Polyphagous Lineage Associated with Cardinium Bacteria. *Ann Entomol*
725 *Soc Am* **98**: 629–635.

726 Pruim, R., Kaplan, D.T., and Horton, N.J. (2020) mosaic: Project MOSAIC Statistics and
727 Mathematics Teaching Utilities.

728 Raychoudhury, R., Baldo, L., Oliveira, D.C., and Werren, J.H. (2009) Modes of acquisition
729 of Wolbachia: horizontal transfer, hybrid introgression, and codivergence in the
730 Nasonia species complex. *Evolution* **63**: 165–183.

731 Ribardire, A., Centanni, J., Dano, A., Coudret, J., Daguin-Thibaut, C., Houbin, C., et al.
732 (2018) Female-biased sex ratios unrelated to Wolbachia infection in European species
733 of the Jaera albifrons complex (marine isopods). *J Exp Mar Biol Ecol* **509**: 91–98.

734 Riparbelli, M.G., Giordano, R., and Callaini, G. (2007) Effects of Wolbachia on sperm
735 maturation and architecture in Drosophila simulans Riverside. *Mech Dev* **124**: 699–
736 714.

737 Robison, G.W.J. (1990) Sperm ultrastructure, behaviour, and evolution. In *Armored scale*
738 *insects: their biology, natural enemies, and control, volume A*. Amsterdam: Elsevier,
739 pp. 205–220.

740 Rodrguero, M.S., Confalonieri, V.A., Guedes, J.V.C., and Lanteri, A.A. (2010) Wolbachia
741 infection in the tribe Naupactini (Coleoptera, Curculionidae): association between
742 thelytokous parthenogenesis and infection status. *Insect Mol Biol* **19**: 631–640.

743 Rosenblueth, M., Martnez-Romero, J., Ramrez-Puebla, S.T., de Len, A.V.-P., Rosas-Prez,
744 T., Bustamante-Brito, R., et al. (2018) Endosymbiotic microorganisms of scale
745 insects. **21**: 53–69.

746 Rosenblueth, M., Sayavedra, L., Smano-Snchez, H., Roth, A., and Martnez-Romero, E.
747 (2012) Evolutionary relationships of flavobacterial and enterobacterial endosymbionts
748 with their scale insect hosts (Hemiptera: Coccoidea). *J Evol Biol* **25**: 2357–2368.

749 Ross, P.A., Turelli, M., and Hoffmann, A.A. (2019) Evolutionary Ecology of Wolbachia
750 Releases for Disease Control. *Annu Rev Genet* **53**: 93–116.

751 Russell, J.E. and Stouthamer, R. (2011) The genetics and evolution of obligate reproductive
752 parasitism in *Trichogramma pretiosum* infected with parthenogenesis-inducing
753 *Wolbachia*. *Heredity* **106**: 58–67.

754 Sabri, A., Vandermoten, S., Leroy, P.D., Haubruge, E., Hance, T., Thonart, P., et al. (2013)
755 Proteomic Investigation of Aphid Honeydew Reveals an Unexpected Diversity of
756 Proteins. *PLOS ONE* **8**: e74656.

757 Sanaei, E., Charlat, S., and Engelstädter, J. (2020) *Wolbachia* host shifts: routes,
758 mechanisms, constraints and evolutionary consequences. *Biol Rev* **n/a**:

759 Scholz, M., Albanese, D., Tuohy, K., Donati, C., Segata, N., and Rota-Stabelli, O. (2020)
760 Large scale genome reconstructions illuminate *Wolbachia* evolution. *Nat Commun*
761 **11**: 5235.

762 Schuler, H., Egan, S.P., Hood, G.R., Busbee, R.W., Driscoe, A.L., and Ott, J.R. (2018)
763 Diversity and distribution of *Wolbachia* in relation to geography, host plant affiliation
764 and life cycle of a heterogonic gall wasp. *BMC Evol Biol* **18**: 37.

765 Semple, T.L., Gullan, P.J., Hodgson, C.J., Hardy, N.B., and Cook, L.G. (2015) Systematic
766 review of the Australian ‘bush coconut’ genus *Cystococcus* (Hemiptera:
767 Eriococcidae) uncovers a new species from Queensland. *Invertebr Syst* **29**: 287–312.

768 Shoemaker, D.D., Machado, C.A., Molbo, D., Werren, J.H., Windsor, D.M., and Herre, E.A.
769 (2002) The distribution of *Wolbachia* in fig wasps: correlations with host phylogeny,
770 ecology and population structure. *Proc R Soc Lond B Biol Sci* **269**: 2257–2267.

771 Sicard, M., Bonneau, M., and Weill, M. (2019) *Wolbachia* prevalence, diversity, and ability
772 to induce cytoplasmic incompatibility in mosquitoes. *Curr Opin Insect Sci* **34**: 12–20.

773 Sicard, M., Dittmer, J., Grève, P., Bouchon, D., and Braquart-Varnier, C. (2014) A host as an
774 ecosystem: *Wolbachia* coping with environmental constraints. *Environ Microbiol* **16**:
775 3583–3607.

776 Simões, P.M., Mialdea, G., Reiss, D., Sagot, M. -F., and Charlat, S. (2011) Wolbachia
777 detection: an assessment of standard PCR Protocols. *Mol Ecol Resour* **11**: 567–572.

778 Son, Y., Luckhart, S., Zhang, X., Lieber, M.J., and Lewis, E.E. (2008) Effects and
779 implications of antibiotic treatment on Wolbachia-infected vine weevil (Coleoptera:
780 Curculionidae). *Agric For Entomol* **10**: 147–155.

781 Stone, G.N. and Schönrogge, K. (2003) The adaptive significance of insect gall morphology.
782 *Trends Ecol Evol* **18**: 512–522.

783 Sudakaran, S., Kost, C., and Kaltenpoth, M. (2017) Symbiont Acquisition and Replacement
784 as a Source of Ecological Innovation. *Trends Microbiol* **25**: 375–390.

785 Tagami, Y., Miura, K., and Stouthamer, R. (2001) How Does Infection with Parthenogenesis-
786 Inducing Wolbachia Reduce the Fitness of Trichogramma? *J Invertebr Pathol* **78**:
787 267–271.

788 Teixeira, L., Ferreira, Á., and Ashburner, M. (2008) The Bacterial Symbiont Wolbachia
789 Induces Resistance to RNA Viral Infections in *Drosophila melanogaster*. *PLOS Biol*
790 **6**: e1000002.

791 Tolley, S.J.A., Nonacs, P., and Sapountzis, P. (2019) Wolbachia Horizontal Transmission
792 Events in Ants: What Do We Know and What Can We Learn? *Front Microbiol* **10**:
793 296.

794 Vandekerckhove, T.T.M., Watteyne, S., Willems, A., Swings, J.G., Mertens, J., and Gillis,
795 M. (1999) Phylogenetic analysis of the 16S rDNA of the cytoplasmic bacterium
796 Wolbachia from the novel host *Folsomia candida* (Hexapoda, Collembola) and its
797 implications for wolbachial taxonomy. *FEMS Microbiol Lett* **180**: 279–286.

798 Vavre, F., Fleury, F., Lepetit, D., Fouillet, P., and Boulétreau, M. (1999) Phylogenetic
799 evidence for horizontal transmission of Wolbachia in host-parasitoid associations.
800 *Mol Biol Evol* **16**: 1711–1723.

801 Villaseñor, J.L., Maeda, P., Rosell, J.A., and Ortiz, E. (2007) Plant families as predictors of
802 plant biodiversity in Mexico. *Divers Distrib* **13**: 871–876.

803 Way, M.J. (1954) Studies on the Association of the Ant *Oecophylla*
804 *longinoda* (Latr.) (Formicidae) with the Scale Insect *Saissetia zanzibarensis* Williams (Coccidae). *Bull Entomol Res*
805 **45**: 113–134.

807 Weeks, A.R. and Breeuwer, J. a. J. (2001) Wolbachia–induced parthenogenesis in a genus of
808 phytophagous mites. *Proc R Soc Lond B Biol Sci* **268**: 2245–2251.

809 Wei, J., Segraves, K.A., Xiao, B.-H., Li, W.-Z., Yang, X.-K., and Xue, H.-J. (2020) High
810 prevalence of Wolbachia infection does not explain unidirectional cytoplasmic
811 incompatibility of *Altica* flea beetles. *Ecol Entomol* **45**: 67–78.

812 Weinert, L.A., Araujo-Jnr, E.V., Ahmed, M.Z., and Welch, J.J. (2015) The incidence of
813 bacterial endosymbionts in terrestrial arthropods. *Proc R Soc B Biol Sci* **282**:
814 20150249.

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

817 Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D., François, R., et al. (2019)
818 Welcome to the Tidyverse. *J Open Source Softw* **4**: 1686.

819 Wilson, E.O. and Hölldobler, B. (2005) The rise of the ants: A phylogenetic and ecological
820 explanation. *Proc Natl Acad Sci* **102**: 7411–7414.

821 Wiwatanaratnabutr, I. and Zhang, C. (2016) Wolbachia infections in mosquitoes and their
822 predators inhabiting rice field communities in Thailand and China. *Acta Trop* **159**:
823 153–160.

824 Wojciechowski, M.F. (2005) *Astragalus* (Fabaceae): A molecular phylogenetic perspective.
825 *Brittonia* **57**: 382–396.

Zhu, D.-H., He, Y.-Y., Fan, Y.-S., Ma, M.-Y., and Peng, D.-L. (2007) Negative evidence of parthenogenesis induction by *Wolbachia* in a gallwasp species, *Dryocosmus kuriphilus*. *Entomol Exp Appl* **124**: 279–284.

Zug, R. and Hammerstein, P. (2014) Bad guys turned nice? A critical assessment of *Wolbachia* mutualisms in arthropod hosts. *Biol Rev* **90**: 89–111.

Figure and Table legends:

Table 1: Probability of *Wolbachia* infection in various arthropods groups. Numbers of genera, species, screened specimens (#Specimens), infected specimens (#Infected), and frequency of infected species with ≥ 1 infected specimens (InfFreq), maximum likelihood estimates for the α and β parameters and the probability of infection at three different prevalence thresholds ($x_{0.1}$, $x_{0.01}$, $x_{0.001}$) are provided for each group.

Group	#Genera	#Species	#Specimens	#Infected	#InfFreq	α	β	$x_{0.1}$	$x_{0.01}$	$x_{0.001}$
Arthropods	2726	6482	152525	68990	39.23%	0.159	0.384	0.467	0.633	0.746
Insects	2391	5754	134948	64146	40.16%	0.170	0.388	0.487	0.656	0.767
Order Hemiptera	409	765	20947	9396	45.75%	0.229	0.428	0.566	0.746	0.851
Family Aphididae	44	93	2467	1071	51.61%	0.396	0.337	0.593	0.756	0.852
Family Cicadellidae	24	69	1896	808	60.86%	0.849	0.564	0.720	0.894	0.960
Family Delphacidae	17	28	4415	3480	60.71%	0.685	0.437	0.622	0.801	0.894
Family Lygaeidae	25	34	105	55	50%	1.074	0.307	0.650	0.804	0.889
Family Miridae	19	42	583	242	35.71%	0.706	0.603	0.527	0.734	0.849
Family Pentatomidae	34	50	136	14	16%	0.726	0.521	0.233	0.360	0.464
Superfamily Coccoidea	57	160	764	151	29.37%	0.535	1.454	0.436	0.721	0.863

842

843

844 Table 2: The mean of P value of 10,000 replicated Fisher's Exact Tests from *Wolbachia*
845 infection and five different tested characters of scale insects. None is significant.

Tested Character	Mean <i>P</i> -value
Infection vs Distribution	0.64
Infection vs Host breadth	0.14
Infection vs Existence of gall	0.70
Infection vs Number of parasitoids	0.84
Infection vs Reproduction	0.13

846

847 Table 3: Details of infection status in wasps, ants and all associate groups (ant, wasps,
848 beetles, flies, mites, moths and thrips). N = total number of pairs, UU = number of pairs in
849 which both parts of a pair are uninfected, IU = number of pairs in which the scale insect is
850 infected but not the associate, UI = number of pairs in which the associate part is infected but
851 not the scale insect, II = number of pairs in which both parts of a pair are infected, *P* = *P*-
852 value of Fisher's exact test and its significant level (*P* < 0.05) is indicated by “*”. ϕ =
853 estimated phi.

Group	N	UU	IU	UI	II	<i>P</i>	ϕ
Wasps	81	39	16	12	9	0.6169	0.06
Ants	42	9	0	17	21	0.0013*	0.52
All	150	61	23	30	36	0.0008*	0.28

854

855 Figure 1. The probability of *Wolbachia* infection for arthropods, insects, hemipterans and
856 seven hemipteran groups (including superfamily of scale insects, Coccoidae) at three

857 different infection prevalence thresholds (grey at 10%, orange at 1% and blue at 0.1%). The
858 error bars are the estimated confidence intervals from bootstrap values.

859 Figure 2. The estimated distribution of infection prevalence. A) Maximum likelihood
860 estimates for scale insects (red line), with blue lines showing the bootstrap estimates. B)
861 Maximum likelihood estimates for each hemipteran group including scale insects.

862 Figure 3. The mean infection probability for A) three main scale insects' families and B)
863 four main host plants which scale insect species were collected from at three different
864 prevalence thresholds (grey at 10%, orange at 1% and blue at 0.1%) with error bars (standard
865 errors) calculated from infection probability of each scale insect's species. Dots are estimated
866 infection probability for each species.

867

A

Probability Density

B

A

Infection probability

B