***Wolbachia* infections in Australian ichneumonid parasitoid wasps (Hymenoptera: Ichneumonidae): evidence for adherence to the global equilibrium hypothesis**

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Wolbachia are intracellular bacteria that infect a wide range of arthropod and nematode hosts, whose reproduction they manipulate to increase rates of vertical transmission. Horizontal transmission between unrelated species also occurs; pathways and mechanisms are still under debate, but parasitoid wasps have been suggested as potential vectors. Here, we perform the largest screening of *Wolbachia* in any parasitoid group by sampling 228 species of Ichneumonidae from Australia. The observed infection frequency of 23% is very close to the proposed global equilibrium frequency of ~20%. Rates of gain and loss are very similar in comparisons between different parasitoid lifestyles, such as ecto- vs. endoparasitoids. Genotyping *Wolbachia* showed identical strains in several unrelated ichneumonid species and other insect groups from around the world. A permutation test comparing *Wolbachia* strains in parasitoids and their host orders did not yield conclusive results, nor did a test for a latitudinal gradient. We argue that future studies should carefully consider the time scale of the processes under investigation. The speed at which *Wolbachia* move horizontally is probably so high that potential patterns are quickly erased and cannot be detected when sampling at the species level, nor with the slowly evolving multilocus sequence typing set of bacterial markers.

ADDITIONAL KEYWORDS: Bayesian inference – phylogenetic comparative method – multilocus sequence typing.

INTRODUCTION

Members of the *Wolbachia pipientis* group are endosymbiotic bacteria that infect arthropods and nematodes (Casiraghi *et al.*, 2005). They are especially prevalent in the reproductive organs of their hosts, although they can also be found in somatic tissues (Dobson *et al.*, 1999). As intracellular bacteria, they are transmitted vertically from mother to offspring via the egg. In order to increase vertical transmission, many

Wolbachia strains have been found to manipulate their hosts' reproduction in various ways, from killing male embryos to feminization of genetic males or parthenogenesis induction (PI) (Werren, Baldo & Clark, 2008). The most commonly found reproductive manipulation, cytoplasmic incompatibility (CI), acts in more complex ways, causing an incompatibility between the sperm of infected males and the eggs of uninfected females. Besides vertical transmission, horizontal transmission has to occur at considerable rates, given that the phylogenies of *Wolbachia* and their hosts show very little sign of congruence (e.g. Werren, Zhang & Guo, 1995b; Ahmed, Breinholt & Kawahara, 2016), and the same

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bacterial strain can sometimes be found in hosts from different orders or even classes (Heath *et al.*, 1999; Ahmed *et al.*, 2016).

Wolbachia are probably the most common endosymbiotic bacteria in the world, infecting ~40–66% of arthropod species (Hilgenboeker *et al.*, 2008; Zug & Hammerstein, 2012; Weinert *et al.*, 2015). The exact number of species infected at any given time is difficult to estimate, as infections typically show either very high or very low frequencies in a population (Hilgenboeker *et al.*, 2008). This pattern is in agreement with population dynamic models that predict a lower and an upper stable equilibrium of CI-inducing *Wolbachia* frequencies in a host population (Turelli, 1994; Hancock, Sinkins & Godfray, 2011). However, additional factors are certainly at play when it comes to the long-term dynamics of *Wolbachia* and their hosts; beneficial effects of the endosymbionts have been demonstrated repeatedly in nematodes, where they are considered mostly mutualistic (Taylor & Hoerauf, 1999), but have also been shown in some arthropods (Panteleev *et al.*, 2007), which might provide an additional explanation for their ubiquity.

Studies that attempt to screen a larger number of randomly chosen species and that include a single or very few individuals per species will, in most cases, only register infections which are near to fixation in a population. Such screenings have typically

found *Wolbachia* frequencies of ~20% with a very high consistency across groups and geographical regions (Table 1; Werren, Windsor & Guo, 1995a; West *et al.*, 1998; Kittayapong *et al.*, 2003; Duron *et al.*, 2008; Hilgenboeker *et al.*, 2008; Russell *et al.*, 2012). Several exceptions to this rule have been reported, and not all of these can be explained by their focus on a particular taxonomic group where *Wolbachia* has been recorded before (Table 1; Schilthuizen & Stouthamer, 1998; Kyei-Poku *et al.*, 2006; Gerth *et al.*, 2015; Klopstein, Kropf & Baur, 2016). Rather, certain taxonomic groups seem to be especially prone to *Wolbachia* infection and thus might deserve further study (e.g. Rokas *et al.*, 2002; Ahmed *et al.*, 2013; Gerth *et al.*, 2015). Nevertheless, based on the apparent 20% rule, Werren & Windsor (2000) suggested a 'global equilibrium' of *Wolbachia* infections as a result of a balance between infection and endosymbiont loss over evolutionary time scales.

A number of mechanisms have been suggested for *Wolbachia* transmission between species. First, rare hybridization events followed by vertical transmission in the new host have been demonstrated in a number of cases where the *Wolbachia* also mediates mitochondrial DNA transfer between species (reviewed by Klopstein *et al.*, 2016). But this pathway is limited by the potential for hybridization and cannot explain the occurrence of the same *Wolbachia* strain in hosts of different families or even orders; these truly horizontal

Table 1. Infection frequencies found in previous screening studies of *Wolbachia*

Study	Study group	Region	Sampling*	Total	Infected	Percentage
Werren <i>et al.</i> (1995a)	Insecta (and a few Arachnida)	Neotropic	Random	154	26	17
Schilthuizen & Stouthamer (1998)	Insecta from <i>Diplolepis</i> galls	Paleartic	Targeted	8	5	63
West <i>et al.</i> (1998)	Lepidoptera and Hymenoptera	Paleartic	Random	83	18	22
Werren & Windsor (2000)	Insecta (and other Arthropoda)	Nearctic	Random	145	28	19
Kittayapong <i>et al.</i> (2003)	Insecta in rice fields	Indo-Malaya	Random	209	49	23
Kyei-Poku <i>et al.</i> (2006)	Fly hosts and their wasp parasitoids	Nearctic, Palearctic	Targeted	30	18	60
Duron <i>et al.</i> (2008)	Insecta (and other Arthropoda)	Paleartic	Random	102	18	18
Russel <i>et al.</i> (2012)	Lepidoptera	Worldwide	Random	158	39	25
Russel <i>et al.</i> (2012)	Formicidae	Worldwide	Random	70	20	29
Gerth <i>et al.</i> (2015)	Hymenoptera, <i>Anthophila</i>	Europe	Targeted	170	107	63
Klopstein <i>et al.</i> (2016)	Ichneumonidae, <i>Diplazon</i>	Europe	Targeted	15	7	47
Total				1144	335	29
Total of random/species-level studies				921	198	21

*Sampling: random = species sampled randomly with respect to previous knowledge of *Wolbachia* infections; targeted = in groups (or systems) with prior knowledge of infection.

transmissions seem to be very common over evolutionary time scales, and several possible pathways have been demonstrated, for instance between predator and prey, via entomophagous mites and nematodes, between parasitoids that feed inside the same host egg, or even via plant tissue (Taylor & Hoerauf, 1999; Huigens *et al.*, 2000; Werren *et al.*, 2008; Yun *et al.*, 2010; Brown & Lloyd, 2015; Li *et al.*, 2017). Parasitoid wasps acting as vectors are another tantalizing possibility, given their intimate relationship with their hosts during their entire larval development and the broad host ranges of some species (Cook & Butcher, 1999). In the laboratory, horizontal transmission from an infected host (*Drosophila simulans*) to an uninfected lineage of parasitoids (*Leptopilina boulardi*) was successfully established in the offspring of 0.7–3.0% of females (Cook & Butcher, 1999; Heath *et al.*, 1999). In silverleaf whiteflies, it has been shown that their aphelinid parasitoids can carry the bacteria on mouthparts and ovipositor to uninfected individuals (Ahmed *et al.*, 2015b). Evidence from wild populations is mostly indirect, in the form of nearly identical *Wolbachia* sequences from hosts and their parasitoids (Werren *et al.*, 1995b; Vavre *et al.*, 1999a; Batista *et al.*, 2010). If host–parasitoid transmission is confirmed to be a major factor in the spread of *Wolbachia*, one might expect parasitoids to show higher infection rates than other insect groups, owing to increased exposure (Cook & Butcher, 1999).

Although much information on the biology of *Wolbachia* comes from parasitic wasps, this concerns only species that have already been previously reared in the laboratory, especially the genetic model system *Nasonia* (Werren *et al.*, 2010) and groups used for biological control, such as *Trichogramma* (Vavre, Girin & Boulétreau, 1999b) or *Encarsia* (White *et al.*, 2009). To our knowledge, no parasitoid group has to date been subjected to a broader screening effort, and it thus remains unclear whether *Wolbachia* are more common in groups with this lifestyle. Furthermore, different parasitoid strategies, such as ecto- vs. endoparasitism (i.e. development inside or outside of the host) or idiobiosis vs. koinobiosis (i.e. the host is inactivated and its development arrested, or the host is allowed to continue to develop), entail a different level of intensity in the interaction with their hosts and thus might result in different *Wolbachia* transmission rates. Finally, data from a large number of species might allow for estimation of the prevalence of host–parasitoid transmissions of *Wolbachia*.

The largest family of parasitoid wasps is the Ichneumonidae, with > 24 000 species currently described and at least several times as many still awaiting discovery (Townes, 1969; Yu, Van Achterberg & Horstmann, 2012). As parasitoids of holometabolan

insects and spiders, they might be expected to represent powerful vectors for *Wolbachia*. Ichneumonids attack only egg, larval or pupal host stages and never allow their hosts to develop into an adult, unless parasitism is unsuccessful. Transmission of *Wolbachia* might thus be biased towards host-to-parasitoid transmissions, but data on this are currently missing. To date, *Wolbachia* infections have been detected in ~15 species of ichneumonids from five of the 40 extant subfamilies (Werren *et al.*, 1995a; Batista *et al.*, 2010; Duploux *et al.*, 2015; Lu *et al.*, 2015; Klopstein *et al.*, 2016), but most groups have never been tested.

In the present study, we screened 348 females of 228 species from 18 subfamilies of Australian ichneumonids for *Wolbachia* infection. To our knowledge, this is the largest screening ever undertaken in a parasitoid group and the largest for the Australian continent. We studied infection frequencies in parasitoids with different lifestyles and tested for an effect by the wasp phylogeny. Sequencing six genes for the *Wolbachia* infections and comparing them with known strains in the online *Wolbachia* strain database (<http://pubmlst.org/wolbachia>; Baldo *et al.*, 2006b), we looked for potential matches with isolates from presumed host groups. A latitudinal gradient of infection frequencies has recently been found in Australian tephritid fruit flies and in a review of Lepidopteran infections (Ahmed *et al.*, 2015a; Morrow *et al.*, 2015). Here, we investigated whether such a pattern was also present in Australian ichneumonid wasps.

MATERIAL AND METHODS

TAXON SAMPLING, DNA EXTRACTION AND *COI* SEQUENCING

We analysed ichneumonid wasps collected in Malaise traps and by sweep netting over the course of the last 10 years at numerous Australian locations, with a focus on South Australia. Most specimens were from the Waite Insect and Nematode Collection (WINC) at the University of Adelaide, where vouchers are deposited. A number of specimens were collected in the Kimberley region of Western Australia by O. R. Edwards and R. K. Didham; these are deposited at the Australian National Insect Collection (ANIC), Canberra (Supporting information, [File S1](#)). Specimens were sorted to subfamily, and genera were identified according to Gauld (1984a). Within each genus, we sorted specimens to morpho-species and extracted one to four specimens from each. DNA extraction was performed using the Gentra Puregene extraction kit (Gentra Systems, Minneapolis, MN, USA). For specimens with a total length of < 10 mm, whole-body extractions were performed, with the specimen left intact during the process. For larger

specimens, we extracted the metasoma only. Sufficiency of DNA quality for further analyses was assessed by performing two PCR attempts with different primers to amplify the mitochondrial cytochrome oxidase subunit 1 (*COI*) marker for each wasp. We used two primer pairs targeting different portions of the gene, first the 5' half (barcoding region; Folmer *et al.*, 1994; LCO 5'-GGTCAACAAATCATAAAGATATTGG-3' and HCO 5'-TAAACTTCAGGGTGACCAAAAAATCA-3') and second, a section starting 200 bp downstream from it (Simon *et al.*, 1994; C1-J-1718 5'-GGAGGATTTGGAAATTGATTAGTTCC-3' and C1-N-2329 5'-ACTGTAAATATATGATGAGCTCA-3'). The PCR assays were performed in a total volume of 25 µL using Immolase Polymerase (Bioline) and a premixed buffer with dNTPs and potassium (MRT buffer; Bioline). The relative volumes of the PCR components were 16.4 µL water, 5 µL MRT buffer, 1 µL of each 5 mM primer, 0.1 µL of the enzyme, and 1.5 µL genomic extract. The PCR protocols used 5 min initial denaturation, followed by 37 cycles of 30 s denaturation, 30 annealing at 50 °C, and 45 s extension, followed by 3 min of final extension. PCR products were sent to the Australian Genome Research Facility Ltd (AGRF) for purification and Sanger sequencing in both forward and reverse directions. All *COI* sequences were transmitted to GenBank under accession numbers KY446943–KY447295 (Supporting information, File S1).

The *COI* sequences obtained were translated into amino acid sequences with MEGA 6.1 (Tamura *et al.*, 2013) and aligned using the MUSCLE algorithm with default settings. Two gap positions of length 3 and 6 bp, respectively, were detected. A BLAST search was performed to ensure that the wasp *COI* segment was successfully targeted by the primers. In *Yezoceryx* (Acaenitinae), the primers either did not bind well or had an internal binding site (LCO, see Folmer *et al.*, 1994), so only a short fragment could be obtained. As this specimen was the only representative of the subfamily, we used a sequence of the same genus from GenBank, as for the single specimen of *Leptobatopsis* (Banchinae). For all the remaining specimens, we used our own *COI* sequences for further analysis.

DEFINING OPERATIONAL TAXONOMIC UNITS

Only a few groups of Australian Ichneumonidae have been revised at the species level (Gauld, 1977, 1984a, 1984b; Klopstein, 2016a, 2016b), and we have to expect that a majority of the taxa included in this study have not yet been formally described. In a pragmatic approach towards the delimitation of putative species, we combined our morpho-species approach (focusing on characters usually informative at the species level for ichneumonid wasps; e.g. Gauld, 1984b; Klopstein, 2014),

with molecular species delimitation based on barcoding sequences. However, the population biology of the mitochondrial *COI* can be influenced by endosymbiotic bacteria, such as *Wolbachia*, and is therefore not always reliable for species delimitation, as has been shown in multiple cases (e.g. Hurst & Jiggins, 2005; Klopstein *et al.*, 2016). It should always be combined with evidence from additional markers or morphology. From the obtained *COI* sequences, we calculated uncorrected pairwise distances. The distribution of these distances was bimodal, with most comparisons either < 1.5 or > 3.5%. This is the range in which most insect studies have found what is known as the 'barcoding gap', i.e. the difference between intraspecific and interspecific variation (Smith *et al.*, 2008; Klopstein, 2014). However, 26 pairwise comparisons lay between those values and thus may or may not represent discrete biological species. We took a conservative approach to defining operational taxonomic units (OTUs) by using the upper end of the observed gap as a threshold, thus probably ending up with an underestimation of the real number of species (which should be borne in mind when assessing intra-OTU variability of *Wolbachia* infections; see Results section).

PHYLOGENETIC TREE OF THE WASP HOSTS FOR HYPOTHESIS TESTING

For comparative studies, a phylogeny is needed to validate statistical testing (Felsenstein, 1985). Unfortunately, the tree for ichneumonid wasps is far from resolved, and we needed to take phylogenetic uncertainty into account. In an attempt to do so, we obtained a dataset including representatives of all major lineages of ichneumonids from an unpublished study by Andrew Bennett and co-workers (see Acknowledgements). This dataset is based on three genes: 28S, the F2 copy of *elongation factor 1-α* (Klopstein & Ronquist, 2013) and *COI*. To these data, we added our *COI* sequences in order to place our samples on the tree. The resulting matrix with 368 taxa and 1924 sites was analysed both under maximum likelihood (ML; RAxML 8.2; Stamatakis, 2014) and Bayesian inference (MrBayes 3.2.2; Ronquist *et al.*, 2012) after partitioning by gene and first plus second vs. third codon positions for the two protein-coding genes and under the GTR + Γ + I in RAxML and the 'mixed' model in MrBayes (Huelsenbeck, Larget & Alfaro, 2004). Both a search for the ML tree and 1000 bootstrap iterations were conducted in RAxML, whereas MrBayes was run for 100 million generations, with every 5000th generation sampled. Topology convergence on this complex dataset was inherently difficult to attain, with the four independent runs repeatedly getting stuck on different local optima. Examining the sets of trees obtained in the

independent runs, we found that most subfamilies and lower taxonomic levels were recovered as monophyletic most of the time, but relationships among subfamilies and among some isolated taxa with long branches were notoriously unstable. This was not likely to be detrimental for the comparative method, as the unstable parts concern rather short branches close to the root of the ichneumonid tree. We decided to use a conservative burn-in of 50 million generations and sample 1000 equally spaced trees from all four runs to account for phylogenetic uncertainty in the hypothesis testing. For testing purposes, all trees were pruned in R (R Core Team, 2014) using the 'drop.tip' function from the APE package (Paradis, Claude & Strimmer, 2004) to include only the Australian specimens from this study, removing Bennett's species. All trees were uploaded to TreeBase (www.treebase.org, study accession <http://purl.org/phylo/treebase/phyloids/study/TB2:S21501?x-access-code=d70380e91ad6be9b00e172c7e9a4f4f8&format=html>).

WOLBACHIA SCREENING AND MULTILOCUS SEQUENCE TYPING

Wolbachia bacteria can be detected from a host individual by selectively amplifying specific bacterial genes. As amplification success can vary depending on the DNA quality, bacterial strain and primers used, we screened all specimens with two primer pairs, one for the *Wolbachia* surface protein gene (*wsp*) and one for a highly conserved gene involved in cell division (*ftsZ*; Baldo *et al.*, 2006b). Specimens with at least one of the two genes showing a clear band on an agarose gel were then also typed for the five genes included in the multilocus sequence typing (MLST) scheme for *Wolbachia* (Baldo *et al.*, 2006b). The PCR conditions were as for *COI*, with primer sequences and annealing temperatures available on the MLST web page (<http://pubmlst.org/wolbachia/>). *Wolbachia* genes were Sanger sequenced at AGRF and transmitted to the MLST database.

Infection status (single or multiple infections) was determined based on all available genes. If one or more genes showed multiple peaks in a region where sequence quality was sufficiently high, we considered the specimen as carrying multiple infections. Only specimens that showed no sign of multiple infections were included in analyses that made use of the MLST data, because phasing of multiple infections is difficult to impossible unless strains are also present as single infections, which would require denser intraspecific sampling. In some OTUs, we detected different *Wolbachia* strains in different individuals or multiple infections in some but not in other individuals. Given that there was a chance of our PCR approach having

failed to detect multiple infections in these cases, we repeated all statistical analyses that use MLST identities after excluding these species.

To determine the identity of the *Wolbachia* strains found in Australian ichneumonids, we performed a concatenated phylogenetic analysis of all strain types available on the MLST database (as of 26 October 2016, 470 strain types) together with all the specimens for which we obtained sequences from at least three of the five MLST genes. In a first step, we used the ML program RAxML 8.2 (Stamatakis, 2014) both to construct an ML tree and to obtain 1000 bootstrap iterations to assess clade support under the GTR + Γ + I model, with genes partitioned into first plus second vs. third codon positions. The resulting tree was then reduced to include all strain types from the database that showed $\leq 1\%$ distance on the ML tree (across the MLST genes) from any of our samples, plus the one strain with the minimal distance in cases where it was $> 1\%$. To this end, we used R scripts (R Core Team, 2014) and the function 'cophenetic' from the APE package (Paradis *et al.*, 2004). In addition, uncorrected pairwise distances were calculated using the 'dist.dna' function in APE, and information on the isolates reported to contain a certain *Wolbachia* strain was extracted from the isolates database on the MLST server (<http://pubmlst.org/wolbachia/>, accessed on 26 October 2016).

The phylogeny of the *Wolbachia* strains obtained from Australian ichneumonids and their closest counterparts in the database was then re-estimated under ML and Bayesian inference, partitioning by gene and codon positions one plus two vs. three. As *Wolbachia* are known to undergo extensive recombination (e.g. Baldo *et al.*, 2006a), we also estimated the relationships among strains using ClonalFrame, a program that takes recombination into account (Didelot & Falush, 2007). We undertook two independent runs, one from a random coalescent and one from a UPGMA starting tree, for 100 000 iterations after a 50 000 iteration burn-in, sampling every 1000 iterations, and using default priors. The *Wolbachia* trees resulting from these analyses were uploaded to TreeBase (www.treebase.org, study accession <http://purl.org/phylo/treebase/phyloids/study/TB2:S21501?x-access-code=d70380e91ad6be9b00e172c7e9a4f4f8&format=html>).

TESTING FOR PATTERNS IN WOLBACHIA INFECTIONS

To test for patterns in *Wolbachia* infections among ichneumonid wasps, we used several approaches. First, we tested for a correlation between (1) infection status and (2) parasitoid lifestyle in a series of separate analyses considering (1) ecto- vs. endoparasitism and (2) idiobiosis vs. koinobiosis. Correlations were assessed in the program BayesTraits version 2 using

the ‘Discrete’ method, which assesses the evolution of pairs of traits (binary state) across a phylogenetic tree to determine whether they have evolved independently or in a dependent (correlated) manner (Pagel & Meade, 2006). Obtaining likelihood values for these two models (independent vs. dependent) allowed model testing under a likelihood ratio and a Bayes factor test. Fixing specific evolutionary rates to be equal (e.g. *Wolbachia* gain and loss rates in endo- vs. ecto-parasitoids) facilitated further insight into the evolutionary dynamics of the system (see Results section). We conducted Markov chain Monte Carlo (MCMC) analyses over the space of combined or separately estimated rate parameters, both on the single best ML wasp tree and on 1000 trees from the Bayesian inference analysis to account for phylogenetic uncertainty. Finally, we applied a reversible-jump (RJ-MCMC) algorithm, which permits the direct determination of the number of independent rates of character change in a Bayesian context. In all ML analyses, 1000 ML attempts were conducted on the ML tree, and 100 on each of the 1000 Bayesian inferred trees. The RJ-MCMC chains were run for 10 million iterations, with 1 million as a burn-in, and under an exponential prior on the evolutionary rates, with the distribution parameter drawn from a uniform distribution between zero and 100.

To investigate whether *Wolbachia* infections are more common in certain groups of ichneumonid wasps (i.e. if there is a pattern with respect to the wasp phylogeny), we used tests for phylogenetic signal as implemented in the PICANTE (Kembel *et al.*, 2010) and GEIGER (Harmon *et al.*, 2008) packages in R. Both the K statistic (Blomberg, Garland & Ives, 2003) and the Lambda statistic (Pagel, 1999) have been developed for continuous traits under the Brownian-motion model; however, the only approach to test for phylogenetic signal in discrete characters that we are aware of (Maddison & Slatkin, 1991) ignores branch length information, which we consider crucial in this context, given the high rates of *Wolbachia* infection dynamics in comparison to speciation times. We tested the presence or absence of *Wolbachia* infections, single and multiple infections, and finally, strain group identity. For the strain group identity, we performed hierarchical clustering in the R package CLUSTER on a distance matrix obtained from the ML tree, trying a different target number of clusters until a version was obtained with reasonable cluster sizes (between one and 14 species per cluster). Each cluster with multiple species included was then tested for phylogenetic signal separately (see also Gerth, Rthe & Bleidorn, 2013).

If horizontal transmission of *Wolbachia* bacteria between parasitoid wasps and their hosts is common, then one could expect to find that strains closely related to

the parasitoid’s *Wolbachia* are found more often than expected by chance in their host groups. To test this hypothesis, we compared host orders of Australian ichneumonid wasps with the isolates from which the closest *Wolbachia* strains have been reported based on the MLST database. As host records are available for only a very small portion of Australian ichneumonids, we predicted host orders from related species. To this end, we mined the Taxapad database for host records from the same subfamily, tribe or genus. Within most higher taxonomic levels, host relationships are sufficiently stable for such an approach, with many subfamilies found exclusively on a single host order. Relevant exceptions are the species-rich Cryptinae, for which we investigated genus-level data, and coded host relationships as unknown in doubtful cases. The predicted host orders for each specimen are shown in the Supporting information (File S1). To test for host-related patterns, we used a custom permutation test implemented in R. For each typed strain from an ichneumonid wasp, we identified the closest match in the MLST database using either uncorrected p-distances or the distances on the ML phylogeny. We then extracted the orders from which the respective *Wolbachia* strain has been reported and counted the number of times there was an overlap with the parasitic wasps’ host order. The significance of the obtained overlap score was then tested by obtaining 1000 permutations of the host records among ichneumonid wasps. As we found a few instances in which multiple individuals of what we treated as single OTU harboured different *Wolbachia* strains and it is thus possible that we overlooked multiple infections in some of them, we also re-ran the analysis without these OTUs.

RESULTS

PREVALENCE OF INFECTION IN AUSTRALIAN ICHNEUMONIDS

Testing 348 specimens of 228 putative ichneumonid species for *Wolbachia*, we found evidence for infection in 72 individuals from 53 putative species, corresponding to infection rates of 23.2% of the species and 20.7% of the individuals tested (Fig. 1; Table 2). These values are very similar to what has been found in equivalent screening studies across different arthropod groups (Table 1). In species for which we sampled more than one individual (22 species with two to five individuals sampled), we found 76% infection on average, indicating that intermediate infection frequencies might be common in ichneumonids. Considering parasitoid life-history traits of the wasps, the small difference in infection rates that was found between ectoparasitoids (19.6%) and endoparasitoids (24.2%) was not

Figure 1. Phylogenetic tree of Australian ichneumonid parasitoid wasps screened for *Wolbachia*, with indication of parasitoid lifestyle and infection status (see key). The tree was split (A and B) because of its size. The tree is the result of a maximum likelihood analysis of a partitioned dataset containing 1924 bp from three genes (Andrew Bennett, unpublished data), with the Australian taxa represented by *CO1*. Values next to nodes represent bootstrap percentages and Bayesian posterior probabilities, respectively. Taxon names are preceded by the first three letters of the subfamily (key in B) and individual numbers.

Table 2. Prevalence of *Wolbachia* infections among Australian ichneumonid wasps

	Species			Individuals		
	Total	Infected	Percentage	Total	Infected	Percentage
All ichneumonids	228	53	23.2	348	72	20.7
Ectoparasitoids	46	9	19.6	68	13	19.1
Endoparasitoids	182	44	24.2	280	59	21.1
Idiobionts	70	15	21.4	106	22	20.8
Koinobionts	158	38	24.1	242	20	20.7

statistically significant, as shown by a permutation test ($P > 0.40$). Likewise, there was no strong difference in the prevalence of the detected multiple infections (6.5% in ecto- and 8.7% in endoparasitoids). Idiobiont parasitoids, which paralyse and quickly consume their hosts, did not exhibit significantly lower infection prevalence than koinobionts, which stay with the host over moulting events (21.4 vs. 24.1%, $P > 0.52$). The lack of discernible patterns between wasp phylogeny, *Wolbachia* infection status and parasitoid life style is illustrated in Fig. 1.

The largest portion of our samples (271 specimens) were from latitudes between 31 and 35°S, and they show an infection rate of 19.7%. Only 15 specimens from 10 species were sampled north of the Tropic of Capricorn (23.4°S), and they show a much lower infection prevalence (6.7%), but this, again, is probably an artefact of small sample size. In any case, here we can report prevalent *Wolbachia* infections in ichneumonids also from higher latitudes in Australia.

We detected *Wolbachia* infections in 12 of the 18 subfamilies included in the present study (Acaenitinae, Anomaloninae, Banchinae, Campopleginae, Cremastinae, Cryptinae, Ichneumoninae, Labeninae, Mesochorinae, Metopiinae, Pimplinae and Tersilochinae; Supporting information, File S1). Of the remaining six subfamilies, three are represented by a single species only (Brachycyrtinae, Ctenopelmatinae and Diplazontinae), whereas five to 12 species were screened in the remaining three (Ophioninae, Orthocentrinae and Tryphoninae). As the absence of *Wolbachia* in these groups might well be explained by the small sample size, we tested for a phylogenetic pattern in the infection status using the wasp phylogeny and two different tests for phylogenetic signal. We found no significant phylogenetic patterns in the infection status (K statistic on ML tree, $P = 0.67$; on 1000 Bayesian trees, mean $P = 0.72$; Pagel's Lambda = 0.000, corresponding to a star phylogeny), nor in the prevalence of single vs. multiple infections (K statistic on ML tree, $P = 0.83$; on 1000 Bayesian trees, mean $P = 0.72$). The power of both these comparisons is limited by our sampling and the phylogenetic uncertainty encountered in the host tree. To address at

least the latter, we also tested whether any of the 1000 Bayesian trees showed significant signal, which was not the case (minimum $P = 0.34$ for infection status and 0.52 for single vs. multiple infections).

WOLBACHIA INFECTIONS AND PARASITOID LIFE-HISTORY TRAITS

We used a Bayesian phylogenetic approach to test for a correlation between infection status and parasitoid life-history traits and to estimate rates of loss and gain of *Wolbachia* infections. The approach compared two models, one assuming independent evolution of the two characters and the second considering them as dependent or correlated (Pagel & Meade, 2006). For ecto- vs. endoparasitoids, the dependent model had a slightly better likelihood, both on the ML tree and on the 1000 trees from the Bayesian analysis, but this difference was far from significant (on ML tree, likelihood ratio statistic LRT = 2.49, d.f. = 4, $P = 0.65$; on Bayesian trees, median LRT = 1.46, median $P = 0.83$, maximum LRT = 6.7, minimum $P = 0.15$; Table 3). Very similar results, with no evidence for a pattern in infections with respect to parasitism mode, were obtained for the comparison between idiobiont vs. koinobiont parasitoids (Table 3; all P -values non-significant).

Using Bayes factor tests and an RJ-MCMC over different rate restriction schemes to estimate rates of gain and loss of *Wolbachia* in ecto- vs. endoparasitoids, the independent model was supported by a Bayes factor of 9.2 (very strong support). Under the dependent model, equal rates for both parasitism modes were strongly preferred (posterior probability of symmetric model = 0.97; Table 4); relative rates of gain and loss of the infection were estimated as one and 3.3, respectively, for both ecto- and endoparasitoids (Table 4). Almost identical results were obtained for idiobionts and koinobionts, and this was not merely attributable to a large overlap in the two ways of categorizing parasitoid lifestyle. In fact, ~13% of cases represent idiobiont endoparasitoids (especially many Cryptinae) or koinobiont ectoparasitoids (Tryphoninae and the *Polysphincta* genus group in Pimplinae).

Table 3. Maximum likelihood comparisons of different evolutionary models in BayesTraits

Parasitoid lifestyle		Ecto/endo*		Idio/koino*	
Models†	Trees‡	LRT§	P-value	LRT§	P-value
Dependent > independent	Maximum likelihood tree	2.49	0.65	0.31	0.99
Dependent > independent	Bayesian trees: median	1.46	0.83	0.16	0.99
	Bayesian trees: minimum	6.7	0.15	2.97	0.56
Unrestricted > restricted	Bayesian trees: median	0.81	0.94	-0.31	0.99
	Bayesian trees: minimum	6.11	0.19	-5.38	0.25

*Parasitoid lifestyle characteristics tested: (1) ectoparasitoidism vs. endoparasitoidism; (2) idiobiosis vs. koinobiosis.

†Test whether the model assuming dependent evolution between *Wolbachia* infections and an aspect of parasitoid lifestyle is preferred over the independent model, or a model with unrestricted vs. symmetrically restricted rate of gain and loss of *Wolbachia*.

‡Trees used for the testing, and whether median or minimum P-values across the 1000 Bayesian trees are reported.

§Likelihood ratio test statistic ($2 \times$ the difference in log likelihood); negative values mean a (in all cases not significant) superiority of the restricted models.

Table 4. Reversible-jump Markov chain Monte Carlo over rate restrictions

	Ecto/endo*	Idio/koino*
BF support for dependent model†	-9.24	-5.76
pp of symmetric model‡	0.97	0.99
Relative rate of gain§	1.00/1.00	1.00/1.00
Relative rate of loss§	3.34/3.34	3.34/3.34

*Parasitoid lifestyle characteristics tested: (1) ectoparasitoidism vs. endoparasitoidism; (2) idiobiosis vs. koinobiosis.

†Bayes factor test favouring the dependent model. Negative because of support for the independent model, with all values being highly significant (smaller than -2).

‡Posterior probability (pp) of a model with equal rates of *Wolbachia* gain and loss between the ecological categories, as estimated by reversible-jump Markov chain Monte Carlo.

§Relative rates of gain and loss of *Wolbachia* bacteria, for both categories, median of posterior over different models.

DIVERSITY OF *WOLBACHIA* IN AUSTRALIAN ICHNEUMONIDS

Most of the *Wolbachia* strains found in Australian ichneumonids represented new strains not yet present in the MLST database; unfortunately, we cannot currently assess whether this is attributable to the taxonomic group or geographical region, as very few data exist from both the family Ichneumonidae and from wild populations in Australia. There were, however, notable exceptions of known strain types (Supporting information, [File S2](#)). *Mesochorus* sp. A (a hyperparasitoid of Braconidae, Ichneumonidae or Tachinidae in a probably lepidopterous primary host) harbours a *Wolbachia* strain that is identical in all five MLST markers to a strain that has previously been reported from two species of Pteromalidae (Chalcidoidea) parasitic wasps (strain type ST 23; although the sequence of the *wsp* gene shows some differences). Five ichneumonid species representing five different subfamilies all carry a common strain that has previously been

recorded from butterflies, ants, weevils and braconid wasps from all over the world (ST 19, with identical *wsp* sequences). Finally, there were two new strain types found in the present study that are shared by four or five Australian ichneumonids, respectively, which all parasitize Lepidoptera but are from a range of different subfamilies ([Fig. 2](#); Supporting information, [File S2](#)).

Phylogenetic relationships among the *Wolbachia* strains isolated from Australian ichneumonids, together with the most closely related strains from the online *Wolbachia* database, are shown in [Fig. 2](#). Concatenated analyses and those taking recombination into account recovered similar trees (Supporting information, [File S3](#)), except for a few conflicts, which were restricted to the (often poorly supported) backbone nodes, as can be expected for recombining organisms. Given that our analyses aimed to identify closely related strains, we continue with the maximum likelihood tree.

Of the *Wolbachia* infections identified in our study (successfully typed for at least three of the five genes of the MLST scheme), all but one belong to supergroup A. The exception was a strain isolated from a specimen of the campoplegine genus *Venturia*, known to be a koinobiont endoparasitoid of mostly Lepidoptera ([Yu et al., 2012](#)), that belongs to supergroup B (Cam_211 *Venturia* sp. B). This supergroup is known to be more common among Lepidoptera ([Werren & Windsor, 2000](#)); therefore, if their hosts are a major source of infection for the ichneumonid parasitoids, one may expect the subfamilies attacking Lepidoptera to have a higher proportion of these strains. However, most ichneumonid species included here presumably attack Lepidoptera (blue strains in [Fig. 2](#)) and, thus, this single correspondence with supergroup B cannot be viewed as evidence for *Wolbachia* transmission from the parasitized host to the ichneumonid wasp.

Figure 2. Relationships among *Wolbachia* strains from Australian ichneumonids and their closest counterparts from the online database, as based on the five genes of the multilocus sequence typing scheme. The maximum likelihood tree is shown with bootstrap support values > 50%, and Bayesian posterior probabilities from the concatenated analysis (MrBayes) and from an approach taking recombination into account (ClonalFrame). Strains from ichneumonids are marked with the first three letters of the subfamily followed by the specimen number, genus and morpho-species designation. Strain type numbers from the MLST database (ST) are followed by the order(s) from which they were isolated.

In a few instances of our identified strains from supergroup A, the most closely related strain from the MLST database has been isolated from the same insect order or arthropod group that also serves as a host for the parasitoid wasps. This is the case in the only included representative of the subfamily

Acaenitinae (Aca_464 *Yezoceryx* sp. B), which attack Coleoptera, and in several species of the cremasine genus *Pristomerus* that all harbour strains very closely related to those isolated from Lepidoptera (ST 352 and 373; Fig. 1). However, there are many more counter-examples in our dataset, with ichneumonid

parasitoids of Lepidoptera finding their closest match to *Wolbachia* strains among isolates from Diptera or other Hymenoptera. We investigated this further by undertaking a customized permutation test to explore correlations between the ichneumonid's host order and the *Wolbachia* host of the most closely related bacterial strain from the MLST database. The test revealed no significant pattern ($P = 0.42$, or $P = 0.43$ when OTUs with differing infections in different individuals were excluded). We also tested whether several *Wolbachia* strains are more likely to be found in certain groups of ichneumonids by delimiting clusters of bacteria and searching for a phylogenetic signal in the wasp tree. Again, no significant pattern could be discerned ($P > 0.16$, both with and without OTUs with differing infections in different individuals).

DISCUSSION

ICHNEUMONID PARASITIDS ADHERE TO THE GLOBAL EQUILIBRIUM HYPOTHESIS

In the most extensive examination of *Wolbachia* infections in a group of parasitic wasps to date, including the first records for many subfamilies and genera, we detected endosymbionts in 23% of 228 provisional Australian ichneumonid species. The incidence of *Wolbachia* in this group is thus about the same as detected in other screenings of insects (summarized in Table 1; Werren *et al.*, 1995a; West *et al.*, 1998; Werren & Windsor, 2000; Kittayapong *et al.*, 2003; Duron *et al.*, 2008; Russell *et al.*, 2009). Based on the high consistency of infection prevalence estimates in different screening studies across different taxa and geographical regions, Werren & Windsor (2000) suggested that *Wolbachia* are at a global equilibrium in most host groups, with rates of loss of the bacteria outweighing rates of gain by ~4:1 on evolutionary time scales. Using Bayesian phylogenetic methods, we found very similar rates of loss and gain in ichneumonids (3.3:1), thus supporting the notion of a global equilibrium for the Australian taxa.

The fact that Australian ichneumonids show the same prevalence of *Wolbachia* as other insect groups is unexpected given the potential role of parasitoids as vectors for these endosymbionts, which *a priori* could be expected to lead to a higher infection rate and thus a greater endosymbiont prevalence in this ecological guild (Cook & Butcher, 1999). However, the establishment of *Wolbachia* within a species depends on many factors other than transmission rates, including potential positive effects of the endosymbiont and, of course, its ability to manipulate the reproduction of its host. In ichneumonids, the phenotypic consequences of *Wolbachia* infections have not yet been studied and

therefore we do not know about its influence on reproductive manipulations. But it is noteworthy that the sex determination system in this family might preclude certain types of manipulations. As far as is known, ichneumonids use a complementary sex-determination mechanism (CSD) which is also found in some other groups of Hymenoptera (Asplen *et al.*, 2009), and CSD is incompatible with parthenogenesis induction through gamete duplication (Beukeboom & Pijnacker, 2000), which in turn is the only known mechanism used by *Wolbachia*. Furthermore, CSD might cause decreased fitness under cytoplasmic incompatibility (Cook & Butcher, 1999). Whether these effects result in lower rates of establishment of *Wolbachia* in CSD species is unknown, but the order Hymenoptera would represent an ideal study system to test this, given the many lineages that have seen multiple transitions from CSD to an arrhenotokous facultative haplodiploid sex determination system (HDS; Asplen *et al.*, 2009).

Although no parasitoid wasp group has been extensively screened for *Wolbachia*, other groups of Hymenoptera have been examined. The ants (Formicidae) have received the most thorough screening, with 34% of 455 species shown to be infected (Russell, 2012), which has been attributed to their eusocial lifestyle. Gall wasps (Cynipidae) were also for some time believed to have a generally higher level of *Wolbachia* incidences (Plantard *et al.*, 1999), but this has been shown to be the case only for tribes where the bacteria also induce parthenogenesis (Rokas *et al.*, 2002). Even higher infection frequencies (59–67%) have been found in fig wasps communities around the globe (Haine & Cook, 2005; Ahmed *et al.*, 2013); elevated transmission rates within the closed environment of the fig fruits have been cited as a possible reason. In a large screening effort of different families of bees, Gerth *et al.* (2015) also found a very high prevalence of *Wolbachia* (63%, or 107 of 170 species), but could not identify any clear ecological correlates, although they mention trophallaxis (exchange of liquids between adults) as a possible factor, which is common in the group having the highest infection frequencies in the study (Halticidae, 90%) and has been proposed as being responsible for the exchange of microbiota (reviewed by Wcislo, 2016). Of the hymenopteran groups showing very high levels of infection, both ants and bees have CDS, which contradicts the idea that this sex determination system poses a real obstacle for establishment of *Wolbachia*.

NO INDICATION OF HORIZONTAL TRANSMISSION BETWEEN HOSTS AND PARASITIDS

Scanning Australian ichneumonids for *Wolbachia* showed no phylogenetic signal in infection prevalence,

indicating both a fast turnover rate with respect to the time scale of the phylogeny and a similar relationship between the rate of gain and loss across the ichneumonid tree. Furthermore, the infection prevalence is very similar across parasitoids with different lifestyles, i.e. ectoparasitism vs. endoparasitism and idiobiosis vs. koinobiosis (Askew & Shaw, 1986). We also find no differences in gain and loss rates of the bacterium between ecological groups, nor do we find any evidence of shared *Wolbachia* strain types with potential host groups.

However, this lack of signal with respect to parasitoid lifestyle might be more a question of data adequacy and study scale than an indication that host–parasitoid transmission does not take place. The number of *Wolbachia* strains that have been reported from Australia and have made their way into the online MLST database (Baldo *et al.*, 2006b) is very limited, and as several studies have demonstrated geographical patterns in *Wolbachia* strain distribution (Russell *et al.*, 2009; Gerth *et al.*, 2015), our comparison between parasitoid and host group *Wolbachia* strains might be premature given the data available. Furthermore, the molecular data sampling that we used (i.e. the MLST typing scheme) relies on five rather conserved genes and might not provide the necessary resolution to prove horizontal transmission. This also applies to previous reports of horizontal transmission, which relied solely on MLST strain identity, even though adding the *wsp* gene might increase discriminatory power to some extent (e.g. Werren *et al.*, 1995b; Vavre *et al.*, 1999a; Batista *et al.*, 2010; Morrow *et al.*, 2014; Ahmed *et al.*, 2016). In any case, the few reports of host–parasitoid transmission do not allow for an evaluation of the overall importance of this pathway; a more systematic approach would be required to test this. West *et al.* (1998) studied two host–parasitoid communities, each including ~30–40 species, but found no evidence for horizontal transfer. The same was the case in a study of gall wasps and their inquiline (Rokas *et al.*, 2002). Additional, similar studies that sample both host and parasitoid species, preferentially from the same locality, are needed; several ichneumonid groups for which detailed host data are available (e.g. some Pimplinae, Campopleginae and Ichneumoninae; Shaw & Horstmann, 1997; Shaw, 2006; Hinz & Horstmann, 2007) would represent good starting points.

An interesting observation from our data were the two *Wolbachia* strains shared among several ichneumonid species from different subfamilies (Fig. 1; Supporting information, File S2). Additional studies are needed to show whether it is a mere coincidence that these wasps all parasitize Lepidoptera (Gauld, 1984a), or if a shared host could have acted as a vector for *Wolbachia* to infect the parasitoids. Given the

rather low level of specialization of some of the wasps involved, this is a clear possibility, but a lot more data, both on the *Wolbachia* strains and on the host ranges of Australian ichneumonids (Yu *et al.*, 2012), are needed to tackle this question adequately.

NO EVIDENCE FOR A LOWER *WOLBACHIA* PREVALENCE IN AUSTRALIA'S TEMPERATE ZONE

A recent study on Australian tephritid fruit flies (Morrow *et al.*, 2014, 2015) found a strong geographical pattern, with all *Wolbachia* infections recorded in the tropical north and no incident in the temperate south, and a similar latitudinal gradient has been found in a review of *Wolbachia* infections in Lepidoptera (Ahmed *et al.*, 2015a). Although our sampling in the north was rather limited (10 species sampled north of the Tropic of Capricorn), there is clearly no evidence in Australian ichneumonids for infection frequencies being conspicuously low in the south; if anything, we observed the opposite cline, with infection rates increasing in southern latitudes.

MIND THE SCALE: THE NEED TO DISTINGUISH BETWEEN SHORT- AND LONG-TERM DYNAMICS IN *WOLBACHIA* INFECTIONS

The absence of evidence for several patterns we had expected to find in this first broad *Wolbachia* screening of a parasitoid wasp group is probably no real evidence for absence, but rather a matter of scale. Our study, as have several previously, used a species-level sampling to investigate potential patterns related to horizontal transmission pathways. This was done in terms of differences between ecto- and endoparasitoids, between idiobionts and koinobionts, and with respect to related *Wolbachia* strains from potential host groups. As for previous studies (West *et al.*, 1998; Rokas *et al.*, 2002; Yun *et al.*, 2010; Gerth *et al.*, 2013), we failed to recover any evidence for patterns we would expect attributable to horizontal transmissions. Instead of concluding that host–parasitoid transmissions are not common in nature, we would argue that it is important to consider the time scales at which transmission dynamics probably take place. At short time scales and with dense intrapopulation sampling, horizontal transmissions can potentially be traced, at least if the host–*Wolbachia* association is still rather young, as demonstrated for Australian tephritid fruit flies (Morrow *et al.*, 2014, 2015). Horizontal transmissions might be effective in the short term even if the endosymbionts fail to establish themselves at high frequencies and over a longer time period in a species. In other words, it is likely that parasitoids can act as vectors for *Wolbachia* transmissions between host species

even if they act only as transient hosts, and vice versa. This has been demonstrated in a laboratory population of *Drosophila*, where parasitic mites transferred *Wolbachia* to uninfected fly populations in the course of only a few generations (Brown & Lloyd, 2015).

The question of scale is also important with respect to the marker system used to establish the relatedness between different *Wolbachia* strains. We found that two different strain types were shared among Australian ichneumonids and other insects, in the absence of any evidence for an ecological connection between them: strain type 19 in a diverse range of insect orders from around the world, and strain type 23 shared between an Australian ichneumonid hyperparasitoid and two North American parasitic wasps that are too small to act as its hosts (the large taxonomic and geographical distribution of strain type 19 and others has already been noted before; see Ahmed *et al.*, 2016). This can be seen as evidence that the horizontal movement of *Wolbachia* through different hosts, even of different orders or classes, is too fast to be detected reliably using the house-keeping genes of the MLST alone. The *wsp* gene is more promising in this respect (Braig *et al.*, 1998), but we clearly need additional fast-evolving molecular markers to ascertain strain identity at the time scale that horizontal transmissions probably take place. Given recent developments in sequencing technologies, whole-genome sequencing of *Wolbachia* might soon supersede the MLST approach.

At a higher taxonomic level and thus longer time scales, signatures of short-term dynamics, such as horizontal transmissions, have probably already been obscured, but such datasets might instead be used to study long-term evolutionary equilibria in *Wolbachia*–host interactions. Our finding that the overall *Wolbachia* prevalence in ichneumonid parasitoids is very similar to what has been suggested as a general global equilibrium in arthropods (Werren & Windsor, 2000) points in that direction. The existence of such an equilibrium is conceivable given the conflict between the reproductively manipulative *Wolbachia* strains and their hosts' genomes. In addition, there is accumulating evidence indicating that arthropods have indeed evolved counter-measures to limit the negative impact of these endosymbionts (Perrot-Minnot, Guo & Werren, 1996; Hornett *et al.*, 2006). Rates of loss of *Wolbachia* infections seem fairly high in arthropods given the general lack of persistence in the association between *Wolbachia* and its host over speciation events. In contrast to the situation in filarial nematodes (Taylor & Hoerauf, 1999), such co-speciation has been reported only very rarely (Raychoudhury *et al.*, 2009; Gerth *et al.*, 2013). Empirical evidence for counter-mechanisms in the host stems from observations of incomplete vertical transmission or even the complete cure

of a host individual. In the parasitoid wasp *Nasonia vitripennis* (Pteromalidae: Chalcidoidea), the loss of *Wolbachia* strains was observed after a prolonged, cold-induced diapause (Perrot-Minnot *et al.*, 1996); such periods of reproductive dormancy might account for lower infection rates at higher latitudes, such as those identified for Australian *Drosophila* (Kriesner *et al.*, 2016). In contrast, in the butterfly *Hypolimnys bolina*, some populations have evolved to suppress male killing in their *Wolbachia* strain (Hornett *et al.*, 2006).

Long-term evolutionary equilibrium could be moved in favour of *Wolbachia* infections if they provided their hosts with a selective advantage (Bordenstein *et al.*, 2009). Although there is strong evidence that *Wolbachia* primarily act as mutualists in nematodes, with which they also have a very stable relationship over evolutionary time scales (Taylor & Hoerauf, 1999), evidence for positive effects is more equivocal in arthropods (Zug & Hammerstein, 2015). However, a range of effects have been noted, from a simple increase in fecundity or longevity to protection against viruses or fungi, probably through a general upregulation of the immune response (Panteleev *et al.*, 2007). More information is needed on the factors that maintain an equilibrium of *Wolbachia* infections in arthropods and to explain the differences observed between groups. However, our study indicates that horizontal transmissions are not a limiting factor, and the focus should instead be on long-term evolutionary dynamics.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

File S1. Specimen data from Klopstein *et al.* (2017). *Wolbachia* infections in Australian ichneumonid parasitoid wasps (Hymenoptera, Ichneumonidae): evidence for adherence to the global equilibrium hypothesis.

File S2. *Wolbachia* MLST strain types and allele identities. Values in brackets denote uncertain identities due to incomplete or low-quality sequences (usually due to multiple infections). Two values separated by a slash indicate double infections that are a single base pair apart and could thus both be recorded. As multiple infections within one individual cannot be reliably phased, only individuals that showed no sign of multiple infections were included in analyses that required MLST data.

File S3. Consensus tree resulting from the ClonalFrame analysis of the *Wolbachia* MLST sequences.