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Multiple gains and losses of *Wolbachia* symbionts across a tribe of fungus-growing ants

C. L. FROST,* H. FERNÁNDEZ-MARÍN,† J. E. SMITH*‡ and W. O. H. HUGHES*

**Institute of Integrative and Comparative Biology, University of Leeds, Leeds, LS2 9JT, UK, †Smithsonian Tropical Research Institute, PO Box 0843-03092, Balboa, Ancón, Panama, ‡School of Environment and Life Sciences, University of Salford, M5 4WT, UK*

Abstract

Although the intracellular bacterium *Wolbachia* is ubiquitous in insects, it has a unique relationship with New World ants on which particular bacterial strains have specialized. However, data are from distantly related hosts and detailed phylogenetic information which could reveal transmission dynamics are lacking. Here, we investigate host–*Wolbachia* relationships in the monophyletic fungus-growing ant tribe Attini, screening 23 species and using multilocus sequence typing to reliably identify *Wolbachia* strains. This technique reduces the significant problem of recombination seen using traditional single gene techniques. The relationship between *Wolbachia* and the fungus-growing ants appears complex and dynamic. There is evidence of co-cladogenesis, supporting vertical transmission; however, this is incomplete, demonstrating that horizontal transmission has also occurred. Importantly, the infection prevalence is frequently different between closely related taxa, with the *Acromyrmex* leaf-cutting ants appearing particularly prone to infection and there being no consistent relationship with any of the major life history transitions. We suggest that infection loss and horizontal transmission have driven epidemics or selective sweeps of *Wolbachia*, resulting in multiple gains and losses of infection across the fungus-growing ants.

Keywords: Attini, multilocus sequence typing, phylogenetics, social insect, symbiosis, *Wolbachia*

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Introduction

Wolbachia are maternally inherited symbionts in a wide range of organisms including all major orders of insects, as well as some isopods, mites, spiders, scorpions and nematodes (Werren *et al.* 2008). This transmission pattern is associated with the evolution of a number of phenotypic effects by *Wolbachia* (induction of parthenogenesis, feminization of genetic males, male killing and cytoplasmic incompatibility) which enhance the symbiont's infection rate either by increasing the proportion of females in a population or restricting the reproduction by uninfected females (Werren *et al.* 2008). However, more direct effects on host fitness can also occur. Beneficial effects, such as increased resistance to viruses, are seen in arthropods (Teixeira *et al.* 2008; Bian *et al.* 2010). Detrimental effects have also been demonstrated, including a reduction in host immunocompetence induced by some *Wolbachia* strains (Fytrou *et al.* 2006; Braquart-Varnier *et al.* 2008). *Wolbachia* as a genus can therefore modify the reproductive capabilities of its host and also have direct effects on host survival. It has consequently received a great deal of interest as a potential biological control agent, as well as being an intriguing model of host–symbiont interactions because of its potential role in speciation (Shoemaker *et al.* 2000; Negri *et al.* 2009).

While the mode of transmission was once thought to be entirely vertical, recent studies suggest that *Wolbachia* may be characterized by a combination of horizontal and vertical transmission (Baldo *et al.* 2006, 2008; Raychoudhury *et al.* 2008). Sporadic horizontal transmission is suggested by incongruence between host and parasite phylogenies (Vavre *et al.* 1999), while the fact that phylogenetically similar hosts tend to share more

Correspondence: Crystal Frost, Fax: +44 113 3432835;
E-mail: bsclf@leeds.ac.uk

similar *Wolbachia* strains than more distantly related arthropods indicates vertical transmission (Baldo *et al.* 2008; Russell *et al.* 2009). *Wolbachia* is intracellular (though it can survive in the intercellular space without replication; Rasgon *et al.* 2006) and it is therefore thought that close relationships between hosts are required for horizontal transmission to occur, such as predator–prey or particularly host–parasitoid relationships (Heath *et al.* 1999; Vavre *et al.* 1999; Noda *et al.* 2001). Comparison of host and parasite phylogenies is a powerful tool for investigating the transmission dynamics of parasites. However, the gene most prominently used in *Wolbachia* phylogenetics, *wsp*, has recently been shown to undergo diversifying selection and frequent recombination, making it unreliable as a phylogenetic tool (Jiggins 2002; Baldo *et al.* 2005, 2006). To solve this problem, multilocus sequence typing (MLST) has recently been designed for *Wolbachia* which utilizes five housekeeping genes: *gatB*, *coxA*, *hcpA*, *fbpA* and *ftsZ* (Baldo *et al.* 2006). These genes have a single copy within the *Wolbachia* genome and are subject to strong stabilizing selection, so are not as prone to recombination. In addition, they are spread across the *Wolbachia* genome meaning that one recombination event is unlikely to affect more than a single marker (Baldo *et al.* 2006).

Social Hymenoptera (ants, bees and wasps) represent a particularly intriguing group for the study of *Wolbachia*. Female workers are functionally sterile, making them, as well as males, an evolutionary dead end for *Wolbachia*. *Wolbachia* can therefore only transmit vertically if the host colony is sufficiently successful to produce new queens. The effects of *Wolbachia* are also more complex than in solitary organisms because they can apply at both the level of the individual and of the colony. For example, minor effects at the individual level may accumulate over many individuals to result in a significant colony-level cost that will reduce the production of sexuals and thus the transmission of *Wolbachia*. Little is known about *Wolbachia* in bees and wasps, but it appears to be widespread in ants, with 50% of Indo-Australian ant species, eight out of nine Central American leaf-cutting ant species and 29% of worldwide ant species being infected (Wenseleers *et al.* 1998; Van Borm *et al.* 2001; Russell *et al.* 2009). These social insect *Wolbachia* appear to show a number of phenomena that are unknown in other insects. There is specialization of a *Wolbachia* clade on New World ants (Russell *et al.* 2009), and two ant species appear to show natural loss of infections in workers (Van Borm *et al.* 2001; Wenseleers *et al.* 2002), suggesting that the biology of social insect hosts has resulted in symbiont specialization.

However, the social insect species used in previous studies are for the most part distantly related, limiting inferences about how host life history may affect the dynamics of infection. An approach focusing on a more closely related group of taxa may thus be informative. The fungus-growing ant tribe, Attini, is an ideal model group for such a study. These ants are increasingly important models in sociobiology and evolutionary ecology. The tribe is monophyletic with a number of important evolutionary transitions which may be expected to impact host–symbiont relationships (Schultz & Brady 2008). These include distinct transitions from obligate monandry to high polyandry, monomorphic to polymorphic workers, smaller to a larger antibiotic-producing metapleural glands and fungal agriculture, as well as gradual changes in colony size and antibiotic-producing bacterial mutualists (Fig. 1) (Weber 1972; Villesen *et al.* 2002b; Hughes *et al.* 2008; Schultz & Brady 2008; Fernandez-Marin *et al.* 2009; Sen *et al.* 2009). The terminal clade which consists of the leaf-cutting ants, *Atta* and *Acromyrmex*, has been shown to have prevalent, and sometimes multiple, *Wolbachia* infections, and includes one species, *Acromyrmex echiniator*, in which natural curing/loss of *Wolbachia* appears to occur (Van Borm *et al.* 2001). However, the incidence of *Wolbachia* in other members of the tribe is unknown.

Here, we investigate the occurrence and phylogenetics of *Wolbachia* infections across the tribe Attini. We determine whether the high level of infection seen in the leaf-cutting ants is mirrored across the entire clade or whether infection rates map with life history changes across the host tribe. We then use multilocus sequence typing to establish whether there is congruence between *Wolbachia* and host phylogenies, which would suggest transmission is almost exclusively vertical, or whether there is evidence of horizontal transmission.

Materials and methods

Workers from twenty sympatric species representing eight of the twelve attine genera were collected from colonies in Gamboa, Panama, between 2005 and 2009 (see Supporting information). Workers from a further three species of *Acromyrmex* and *Atta* were collected from Juiz de Fora, Brazil, in 2008 to provide a phylogeographic perspective for these genera. Samples were stored in 100% ethanol at −20 °C. Workers were either collected directly in the field or from colonies maintained in the laboratory. Large worker and forager castes (1.8–2.4 mm head width) were utilized for *Acromyrmex* and *Atta*, respectively. We screened 8 individuals per colony where available (see Supporting information). This sampling effort had only a 2% chance of false negatives (i.e. incorrectly concluding a colony was uninfected) for infection rates such as those found previously in leaf-cutting ants (Van Borm *et al.* 2001). Gynes were also screened, where available, for

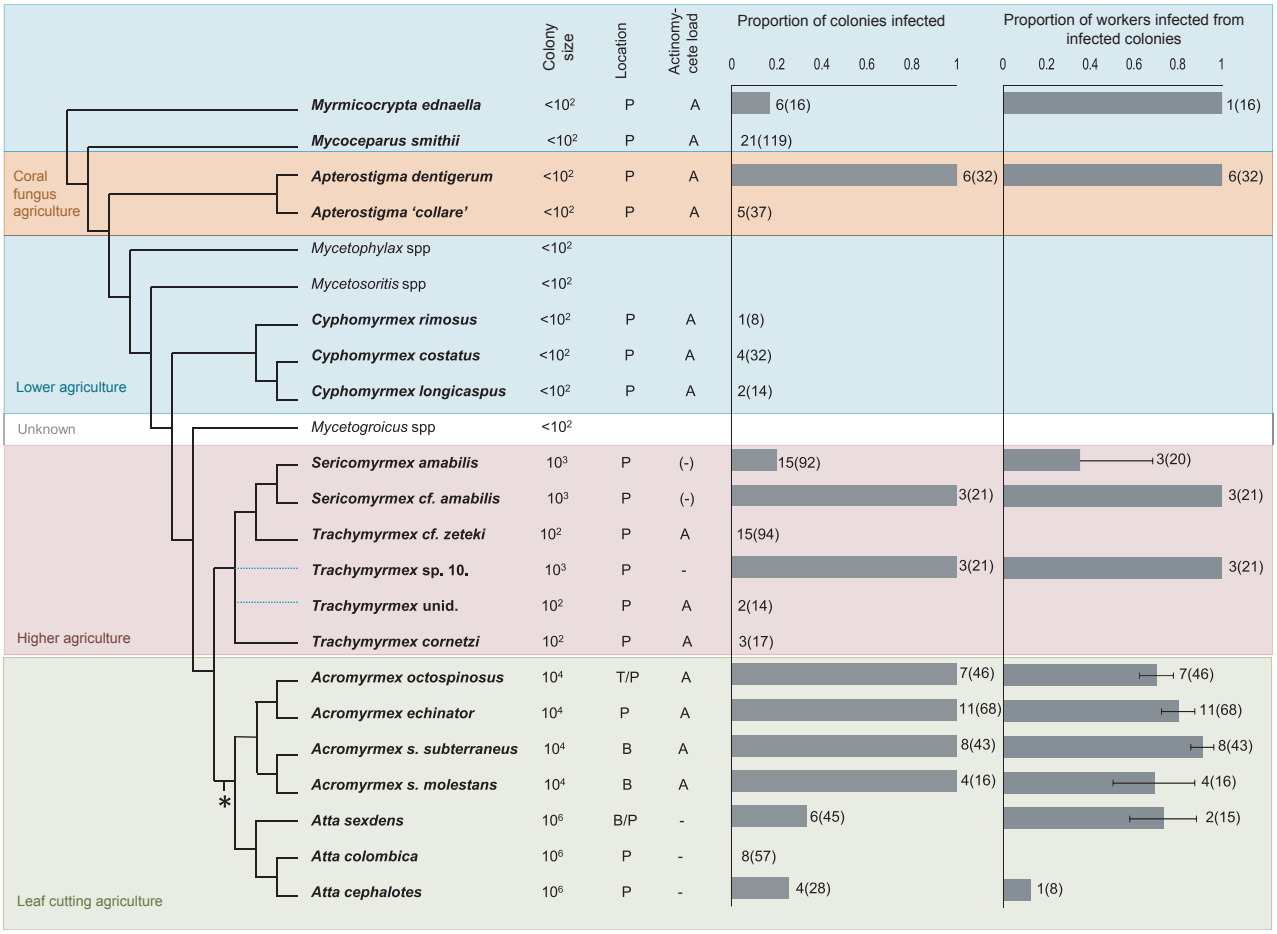


Fig. 1 Phylogeny of the tribe Attini mapped with *Wolbachia* infection prevalence and life history traits. Phylogeny modified from Schultz & Brady (2008) and Sumner *et al.* (2004). Species investigated during this study are highlighted using bold with blue dashed lines used in areas where the phylogeny is unclear. Typical mature colony sizes and presence of symbiotic actinomycete bacteria are noted in columns beside the phylogeny along with location of sampling for each species (B-Brazil, P-Panama, T-Trinidad); agricultural status is indicated by coloured boxes, and other transitions are noted by an asterisk on the phylogeny at the point where transition occurred (transition to polyandry/polymorphism/larger metapleural glands; Hughes *et al.* 2008; Fernandez-Marin *et al.* 2009; Sen *et al.* 2009). The proportion of colonies infected with *Wolbachia* of attine species investigated is shown beside this phylogeny. The *Wolbachia* individual-level infection frequencies for infected colonies for each of the infected attine species investigated is also shown on the far right of the figure (mean ± SE). On both graphs, the total number of colonies screened is noted beside the relevant bars with the total number of individuals screened in parentheses.

species negative or intermediate for *Wolbachia* infection. These included eight *Atta cephalotes* gynes collected from a mating swarm, eight *Atta cephalotes* gynes from a single colony, 13 *Apterostigma collare* (two colonies), two *Mycoceparus smithii* (one colony), 30 *Trachymyrmex cornetzi* (four colonies) and 38 *Trachymyrmex zeteki* (nine colonies).

Molecular analysis

Worker abdomens were crushed in 200 μL of 5% Chelex 100 (BioRad) suspended in 10- μM Tris buffer. Samples had 4 μL of Proteinase K added (5 μL/mL), were incubated overnight at 56 °C and then boiled for 15 min. After spinning down, the DNA extract (supernatant) was taken as aliquot and frozen at −20 °C prior to use. To determine the infection status, 10 μL PCRs using the GoTaq® Flexi DNA Polymerase system were used. Each PCR contained 1 μL of DNA template, 1× buffer, 0.5 mM of each primer and 0.25 u of Go Taq DNA polymerase. $MgCl_2$ and DNTP concentrations were optimized for each primer as were the annealing temperatures (see Supporting information). All PCR cycles were 94 °C for 2 min, 30 cycles of 94 °C for 2 min, annealing temp for 45 s, 72 °C for 2 min, finishing with 7 min at 72 °C. General *Wolbachia* primers, *wsp*, were used to determine the presence of *Wolbachia* (Braig *et al.* 1998). Host DNA controls using the

ant-specific microsatellite *cypho15B_16b* were included for each sample to ensure that negatives were not owing to poor DNA extraction (Villesen *et al.* 2002a). All PCR products were visualized on 1% agarose gels with ethidium bromide. Negative samples were run twice to confirm negativity. Positive and negative controls were included in each run.

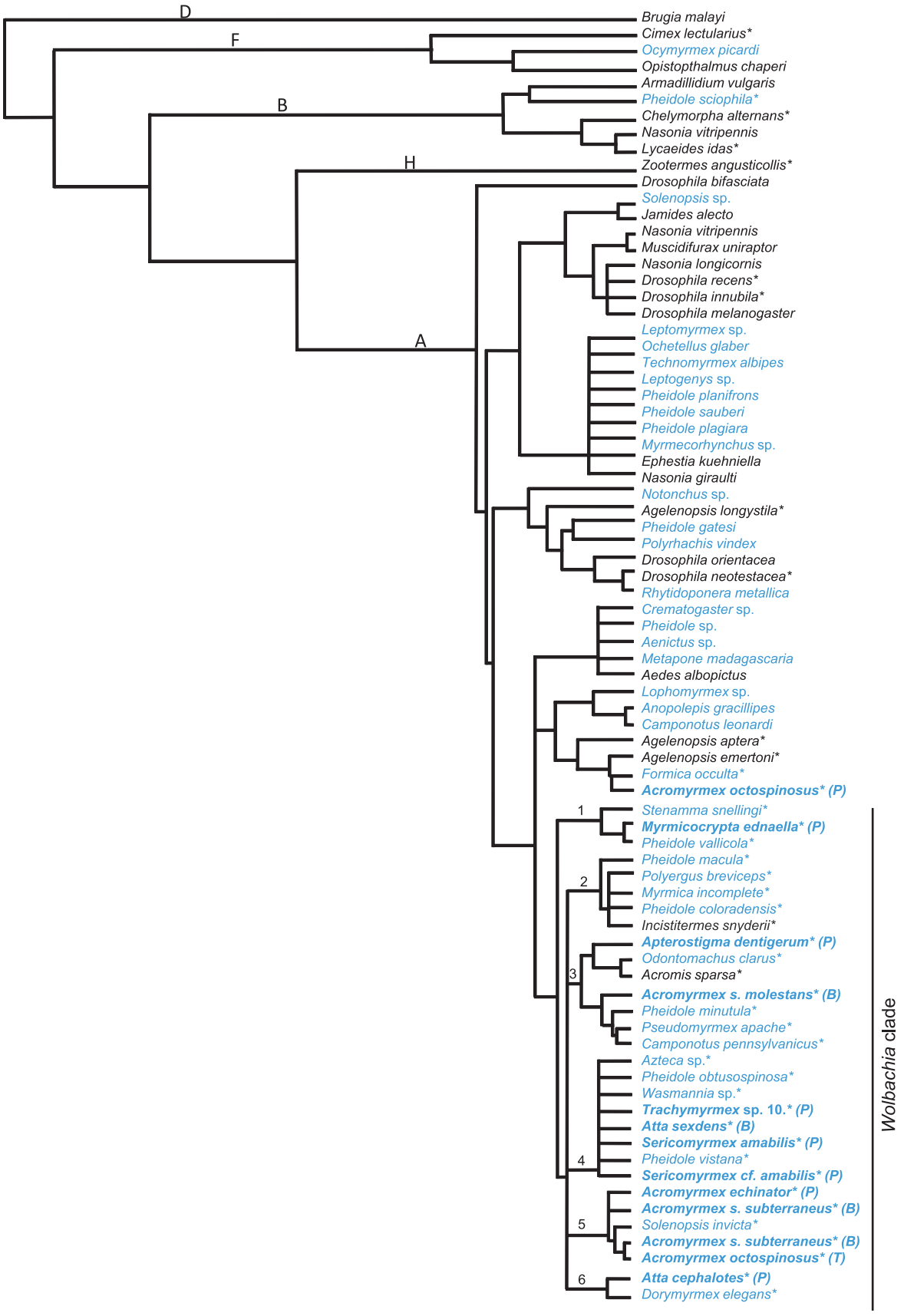
Wolbachia-positive samples were sequenced using *wsp* for multiple colonies of each host species to assess whether multiple bacterial strains were found within a single species. Depending on this intraspecific diversity, at least one individual per species was then used for MLST sequencing. To obtain sequences for phylogenetic analysis, 50 μL PCR amplifications were carried out for *wsp* and MLST primers (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*; Baldo *et al.* 2006) and purified using the Qiaquick PCR purification kit® (Qiagen). Sequencing was performed using the ABI Dye Terminator Labelled Sequencing system with an ABI 3130*xl* capillary sequencer. Colonies that appeared to have multiple infections from their *wsp* sequences were excluded from MLST analysis. Results were manipulated in ClustalX and Bioedit. Both forward and reverse primers were utilized, with a reliable sequence produced after manual correction of the forward and reverse pairs (MLST sequences produced are deposited in GenBank under accession numbers HM211007–HM211071). Phylogenies were produced using ClonalFrame, a programme specifically designed for use with multilocus data which accounts for both point mutation and homologous recombination, thus providing more reliable clonal relationships (Didelot & Falush 2007). Convergence was assessed using the ClonalFrame comparison tool which compares independent repeat trees, as well as determination of Gelman-Rubin statistic. It was found that 700 000 iterations was sufficient for convergence; the first 350 000 were discarded as burn-in with the second half sampled every 200 iterations. Five independent trees based on 1939 bases were run, four of these trees had no significant topological differences and the fifth had only three nodes with discordance, providing significant confidence for the topology of the tree with the highest likelihood ($r/m = 1.79$, $\rho/\theta = 0.34$, Fig. 2). Significant differences were seen between *wsp* and MLST phylogenies when a subset of isolates were analysed (see Supporting information). We therefore produced a phylogeny using the MLST data alone for the attine *Wolbachia*, combined with those previously submitted to the *Wolbachia* MLST database. Fisher's exact tests and generalized linear models were carried out in R 2.8.1 (R Development Core Team 2005) using the cbind function, fitted with a binomial error structure, with the response variable being the number of infected to uninfected individuals per colony. The correlation between life history changes in the host, and infection prevalence at both the colony and the individual levels were then assessed using the method of phylogenetically independent contrasts, calculated using the PDAP module of the Mesquite program (Midford *et al.* 2003; Maddison & Maddison 2006).

Results

Infection prevalence

Colony infection rate differs significantly across the Attini (Fishers exact test with simulated *P*-value based on 2000 replicates: $P = 0.0005$; Fig. 1). The species examined can be broadly classified as highly infected (*Apterostigma dentigerum*, *Sericomyrmex* cf. *amabilis*, *Trachymyrmex* sp. 10 and all *Acromyrmex* species, in which all colonies are infected), infected to a lower extent (*Atta sexdens*, *Atta cephalotes*, *Myrmicocrypta ednaella* and *Sericomyrmex amabilis*, where infection occurs in 16–33% of colonies) or apparently devoid of infection (*Mycocepurus smithii*, all other species of *Trachymyrmex*, all species of *Cyphomyrmex* and *Atta colombica*). The additional gyne samples run for uninfected species were also uninfected, supporting the validity of the low and null infection rates seen in the relevant species. It appears that colony infection prevalence is sporadic in all host clades except for the *Acromyrmex* where *Wolbachia* is found in all colonies of all species. No correlation was found between infection prevalence and any of the host life history traits investigated ($P < 0.05$ in all phylogenetically independent contrasts).The two *Atta sexdens* colonies that were found to be infected with *Wolbachia* were both collected from Brazil, whereas the four collected in Panama were all uninfected, demonstrating that there can be significant differences in infection rates across populations. The same was not seen in *Acromyrmex* which have similar infection prevalence at both the colony and individual level across all three locations. The infection rates of individuals within infected colonies also differed across the Attini (ANOVA

Fig. 2 *Wolbachia* phylogeny based on multilocus sequence typing (MLST) sequence data using host species as labels, with sequences produced during this study highlighted in bold. Ants are shown in blue with non-ants shown in black. Supergroups are labelled with their corresponding letters. New World samples are denoted with an* and samples where the location is unknown are marked with an†. The isolates discovered here also have an additional letter in parentheses to denote country of sampling (B-Brazil, P-Panama, T-Trinidad). A 'New World ant' clade is labelled to highlight the fact that New World ants are frequently infected by *Wolbachia* from this clade, with six subclades within this also marked.



(General Linear Model), $P < 0.001$, df = 38; Fig. 1). Individual infection rates for infected colonies of *A. echinator* and *A. octospinosus* workers were higher than found in an earlier study by Van Borm *et al.* (2001) in the same area (81% vs. 35% and 67% vs. 43%, respectively), and, contrastingly, much lower for Panamanian *Atta cephalotes* workers (12.5% vs. 53.9%, with only one worker from one *Atta cephalotes* colony found to be infected from Panama during this study).

Phylogeny

Each species appeared to have a single, predominant *Wolbachia* strain based on *wsp* sequences, except for *Acromyrmex subterraneus subterraneus* (note, though, that individuals with multiple strains were excluded from phylogenetic analysis). All *Wolbachia*, except for one *A. octospinosus* isolate, from the fungus-growing ants examined with MLST, fall within a single lineage which consists of *Wolbachia* isolated mainly from New World ants (Fig. 2). This includes two 'non-ant' *Wolbachia*, one of which is from the beetle, *Acromis sparsa*, whereas the other interestingly is from a social insect, the termite *Incistitermes snyderii*. The strains within this clade can be subdivided into six smaller subclades which include attine isolates. Mapping the distribution of *Wolbachia* against its host (Fig. 3) reveals significant relationships between host and parasite phylogenies, particularly between the *Acromyrmex* and clade 5 *Wolbachia* (Fig. 3). However, these relationships are incomplete with non-attine genera interspersed with the strains acquired during this study. It is notable that *Acromyrmex* not only has a higher prevalence of *Wolbachia*, but also the highest diversity of strains. Panamanian *Acromyrmex* have *Wolbachia* representatives in two of the six subclades within the New World ant clade, as well as the one attine isolate which is external to the New World strains, implying that lineage switching has occurred with the introduction of new strains. It is interesting to note that *Acromyrmex* from Brazil and Trinidad have the same *Wolbachia* strain as some of their Panamanian counterparts. In contrast, others, such as the *Atta sexdens* isolates from Brazil, have a different strain to those found in the Panamanian *Atta cephalotes* (Fig. 2).

Discussion

While caution is needed in interpreting data on some of the species examined to imply they are completely uninfected, given that low prevalence infections can occur in the Attini, it appears that in some the infection rates are at least very low if not absent. All 21 colonies of *Mycocepurus smithii* appear uninfected for example, as was also found for 15 colonies examined by Himler *et al.* (2009). The colony infection frequencies agree with previous studies for the Panamanian *Acromyrmex echinator* and *A. octospinosus* (Van Borm *et al.* 2001), as well as *Mycoceparus smithii* (Himler *et al.* 2009).

The phylogeny produced here agrees with that of Russell *et al.* (2009), with all but one of the attine *Wolbachia* being within the New World ant clade, supporting the hypothesis that specialization in this host group has occurred. A degree of congruence is seen between some attines and *Wolbachia,* suggesting that vertical transmission is important in this system. These relationships are not complete, for example a *Wolbachia* isolate found in *A. sexdens*, which is one of the more derived Attini, is very similar to that found in *Sericomyrmex* and *Trachymyrmex* species, which are more basal. While this could be explained by a widespread *Wolbachia* which infected

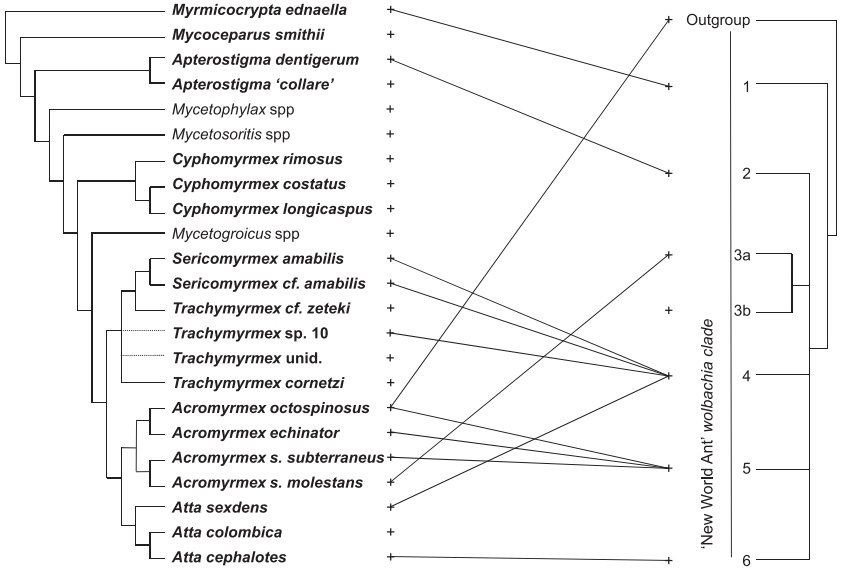


Fig. 3 Comparison of host and symbiont phylogenies. *Wolbachia* isolates from the reduced *Wolbachia* phylogeny on the right are conjugated to those of the host species in which they were found (host phylogeny shown on the left, dashed lines used in areas where the host phylogeny is unclear, species looked at in this study are highlighted in bold font).

the lineage in the past being lost in some lines and not others, the discovery of multiple *Wolbachia* strains in this single tribe of ants and evidence of horizontal transmission in others insects, both suggest that horizontal transmission most likely explains the sharing of *Wolbachia* strains by phylogenetically distant host taxa. This pattern of broad-scale relationships with apparently sporadic horizontal transmission can also be explained by horizontal transmission being more frequent between closely related attines. Such a pattern is seen in other insects as well as the wider New World ants (Baldo *et al.* 2006, 2008), but our results show that it can extend down to the genus-level within the Attini. *Wolbachia* is an intracellular symbiont so an intimate relationship between hosts is required for horizontal transmission. Predator–prey transmission seems unlikely in these fungivorous ants, but host–endoparasite transmission is more probable. Ants in general are particularly prone to parasitoid attack (Boomsma *et al.* 2005). There are numerous records of parasitoid attacks on *Atta* spp. and also single records of parasitoids from *Cyphomyrmex* and *Acromyrmex* (Braganca *et al.* 1998; Fernandez-Marin *et al.* 2006; Silva *et al.* 2008). Both the activity levels of new queens upon nest founding, and the size of a colony may also affect exposure to parasitoids and thus infection by *Wolbachia*. This could help to explain why *Acromyrmex* are more prone to *Wolbachia* infection. *Acromyrmex* have at least 10-fold larger colonies than lower and more basal higher attines, and, while their sister taxa *Atta* have larger colonies still, the queens of *Atta* are claustral so will not leave the nest once excavated, unlike *Acromyrmex* queens which leave their incipient nest to forage.

Infection prevalence is seen to be sporadic across the tribe Attini with highly infected species and genera being closely related to uninfected members of the tribe. The exception is *Acromyrmex* which have both a high prevalence and diversity of *Wolbachia* suggesting that they are particularly prone to infection. Neither colony nor individual infection rates appeared to correlate consistently with morphological worker castes, mating frequency, metapleural gland size, presence of symbiotic actinomycete bacteria or agriculture in the Attini, but infection rates at both levels vary dramatically. This result suggests that some taxa are refractory to infection or that some *Wolbachia* may have lower vertical transmission rates, as demonstrated in other insects (Kassem & Osman 2007; Narita *et al.* 2007). Such loss of infection could explain the low within-colony infection rates. Natural curing of *Wolbachia* has uniquely been proposed in workers of two ant species, (Van Borm *et al.* 2001; Wenseleers *et al.* 2002), including *Acromyrmex echiniator* in which Van Borm *et al.* (2001) demonstrate that adult workers harbour significantly lower infection rates than males, gynes and worker pupae (45% vs. 95%, 94% and 87% respectively). Alternatively, it may be that *Wolbachia* does not replicate as well in workers as in gynes, something which could be an adaptation to maximize vertical transmission or simply because of the regression of worker ovaries. In tandem with this, it has been shown that *Wolbachia* density can decrease with the age of an insect (Unckless *et al.* 2009), so the infection rate of a colony may change with the age of the queen as well as the age structure of the worker population.

It is hard to explain the contrasting differences in *Acromyrmex* and *Atta* infection prevalence between the current study and that of Van Borm *et al.* (2001) as being attributed to differences in screening protocols and detection error. It can be explained by cycling of the *Wolbachia* prevalence through time. The main candidate for this adaption is the ability of workers to lose infection (Van Borm *et al.* 2001; Wenseleers *et al.* 2002). The workers are responsible for all nonreproductive tasks in the colony and are an evolutionary dead end to *Wolbachia*. Loss of *Wolbachia* from these individuals would therefore reduce the overall burden on the colony while still allowing transmission of *Wolbachia* through the reproductives. The ability of workers to clear infections of *Wolbachia*, as with other symbionts, seems likely to depend upon factors such as colony health. For the cycle to be completed, the same strain or a new strain gained from horizontal transmission would have to resurge, the former being indicative of an epidemic and the latter of a selective sweep. Such dynamic transitions in *Wolbachia* infection prevalence in populations have been demonstrated in the butterfly *Hypolimnas bolina* (Hornett *et al.* 2009).

The variation in infection rates and phylogenetics of *Wolbachia* together demonstrates that the relationship between *Wolbachia* and the fungus-growing ants is fluid. The infection pattern across the attines does not match consistently with any of the tribe's life history changes, but it does appear that some genera are more prone and others more refractory to infection. While vertical transmission appears efficient because of high infection rates of some colonies and interactions between host and parasite phylogenies, horizontal transmission between species and genera has also occurred, most plausibly mediated by parasitoids. This appears to cycle with the loss of *Wolbachia* either because of imperfect vertical transmission or because of natural curing, providing convincing evidence for selective sweeps or epidemics of *Wolbachia* in the Attini.

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Supporting information

Additional supporting information may be found in the online version of this article.

Fig. S1 Comparison of *wsp* (left) and MLST (right) phylogenies using a subset of available sequences.

Table S1 Total number of individuals screened and number positive for each colony

Table S2 PCR reagent and annealing conditions for *wsp*, the five *Wolbachia* genes used for MLST and the host gene *cypho15B_16B*

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