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SPECIALIZATION AND GEOGRAPHIC ISOLATION AMONG *WOLBACHIA* SYMBIONTS FROM ANTS AND LYCAENID BUTTERFLIES

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Wolbachia **are the most prevalent and influential bacteria described among the insects to date. But despite their significance, we lack an understanding of their evolutionary histories. To describe the evolution of symbioses between *Wolbachia* and their hosts, we surveyed global collections of two diverse families of insects, the ants and lycaenid butterflies. In total, 54 *Wolbachia* isolates were typed using a Multi Locus Sequence Typing (MLST) approach, in which five unlinked loci were sequenced and analyzed to decipher evolutionary patterns. AMOVA and phylogenetic analyses demonstrated that related *Wolbachia* commonly infect related hosts, revealing a pattern of host association that was strongest among strains from the ants. A review of the literature indicated that horizontal transfer is most successful when *Wolbachia* move between related hosts, suggesting that patterns of host association are driven by specialization on a common physiological background. Aside from providing the broadest and strongest evidence to date for *Wolbachia* specialization, our findings also reveal that strains from New World ants differ markedly from those in ants from other locations. We, therefore, conclude that both geographic and phylogenetic barriers have promoted evolutionary divergence among these influential symbionts.**

KEY WORDS: **Bacteria, coevolution, insects, phylogenetics, specialization, symbiont.**

Many unrelated bacteria have independently evolved symbiotic lifestyles, living in intimate and prolonged association with a broad range of eukaryotes. These associations have played integral roles in the ecology and evolution of both microbes and hosts, shaping their development, diets, diversification, and even their genomes (Margulis and Fester 1991; Charlat et al. 2003). Whereas

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several groups of symbiotic bacteria have evolved exclusive and irreversibly specialized symbioses (Moran and Baumann 1994; Peek et al. 1998; Moran and Wernegreen 2000), others are comparatively generalized, moving between a wide range of species by way of horizontal transmission (Werren 1997; Zchori-Fein and Perlman 2004; Kaeding et al. 2007). Despite this latter trend, it remains possible that these apparent "generalists" are specialized on particular host lineages (e.g., Jeong et al. 1999), moving primarily between related eukaryotes. This would not only reveal limitations in host range, but it would also indicate that host-associated phylogenetic barriers promote divergence and diversification among symbiotic bacteria.

Among the most widespread and prevalent symbionts described among the eukaryotes are maternally transmitted bacteria from the genus *Wolbachia* (Alphaproteobacteria: Rickettsiales). Since the first symbiosis between *Wolbachia* and invertebrates was established nearly 200 million years ago (Rousset et al. 1992), these intracellular microbes have proliferated, colonizing an estimated 66% of all insect species (Hilgenböecker et al. 2008), along with several other groups of arthropods and nematodes (Werren et al. 1995a; Jeyaprakash and Hoy 2000; Werren and Windsor 2000; Fenn and Blaxter 2004). The remarkable success of *Wolbachia* symbionts is due, in large part, to their manipulations of arthropod reproduction, which favor their spread within populations (see O'Neill et al. 1997 and Werren 1997 for review). *Wolbachia* also owe their prevalence to their abilities to infect multiple hosts. Since phylogenetic analyses first revealed that they move horizontally between species (e.g., Werren et al. 1995b; Shoemaker et al. 2002, but see Fenn and Blaxter 2004), subsequent experiments have verified that *Wolbachia* are indeed capable of establishing stable infections in novel species (e.g., Grenier et al. 1998; McGraw et al. 2002). In spite of this, their persistence in novel hosts is often ephemeral, and some strains cannot efficiently manipulate reproduction in these novel backgrounds (e.g., Grenier et al. 1998; Rigaud et al. 2001; Riegler et al. 2004). This suggests that *Wolbachia* are specialized to some degree, possessing a capacity to maintain infections in only limited range of related species.

Previous studies have supported the predictions of specialization, observing that similar *Wolbachia* strains infect related hosts (typically those from the same genus, for example van Meer et al. 1999; Jiggins et al. 2002). However, most have drawn their conclusions from analyses of one or two genes, including the commonly studied *Wolbachia* surface protein (*wsp*) gene. Due to extensive recombination in *wsp* (Baldo et al. 2005) and throughout the genome at large (Jiggins et al. 2001; Werren and Bartos 2001; Baldo et al. 2006a), it is now clear that previous attempts to reconstruct the clonal histories of *Wolbachia* symbionts have been insufficient, and that prior conclusions about evolution and specialization must be reassessed. With these issues in mind, Baldo and colleagues developed a Multi Locus Sequence Typing (MLST) system, which provides a standardized and rigorous framework for studies of *Wolbachia* evolution (Baldo et al. 2006b). Combined with extensive sampling from related hosts, this MLST approach has been used to demonstrate that closely related *Wolbachia* infect related spiders (from the genus *Agelenopsis*; Baldo et al. 2008) and scorpions (from the genus *Opistophthalmus*; Baldo et al. 2007). We do not currently know whether this is a general trend among *Wolbachia* strains found across the arthropods. And, because we have not identified the responsible mechanisms, we do not know whether this pattern is driven by specialization on related hosts or increased opportunities for transfer among relatives.

To further elucidate the patterns and processes that characterize their transmission and evolution, we performed extensive screening and MLST typing (Enright and Spratt 1998; Maiden et al. 1998) of *Wolbachia* strains from cosmopolitan collections of ants (Hymenoptera: Formicidae) and lycaenid butterflies (Lepidoptera: Lycaenidae). Overall, 54 new strains were typed using MLST. Analyses of data from these strains and previously typed strains from other insects allowed us to determine: (1) whether related *Wolbachia* infect related hosts (a trend we term "host association") and (2) whether related strains are found in geographic proximity. To link observed patterns with mechanistic processes, we reviewed and analyzed results from the *Wolbachia* literature, exploring the possibility that these symbionts can most readily infect relatives of their current hosts. This would indicate that *Wolbachia* are specialized on particular host taxa, providing novel insight into the processes that shape the evolution of these prevalent and "influential passengers" (O'Neill et al. 1997).

Materials and Methods

SURVEYED HOSTS

To determine whether related *Wolbachia* are found in related hosts or in close geographic proximity, we extensively surveyed cosmopolitan collections of two insect groups: the ants (Hymenoptera: Vespoidea: Formicidae) and the lycaenid butterflies (Lepidoptera: Papilionoidea: Lycaenidae). Because lycaenids commonly interact with ants (Pierce et al. 2002), we were also interested in determining whether they harbor related *Wolbachia* symbionts and, thus, whether these ecological associations provide a conduit for horizontal transfer.

In total, we screened ants from 329 collections spanning over 200 species, 137 genera, 43 tribes, and 17 of 21 subfamilies within the Formicidae. Collections included 82 ants from the genus *Pheidole* (Formicidae: Myrmicinae: Pheidolini), representing at least 66 species, and 100 ants from the Khao Chong National Forest in the Trang province of Thailand (henceforth referred to as Thai ants). Screening and sequence analyses of strains from these samples allowed us to determine whether closely related ants harbor

host-specific *Wolbachia* lineages and whether *Wolbachia* strains are isolated across localized geographic scales.

In addition, we screened 95 lycaenid butterflies (95 species), spanning 89 genera, 27 of 33 tribes, and seven of seven subfamilies within the Lycaenidae. Our surveys also included five additional lepidopterans and three hymenopteran outgroups. Information on insect samples, including their taxonomy and *Wolbachia* infection status, is presented in Supporting Table S1. Geographic locales for hosts of MLST-typed *Wolbachia* strains are deposited in the MLST database (http://www.pubmlst.org/wolbachia).

DNA EXTRACTIONS AND TEMPLATE QUALITY ASSAYS

DNA was typically extracted from single, ethanol-preserved insects using the DNeasy Tissue Kit (Qiagen Inc., Valencia, CA) according to the manufacturers' protocol (see Supporting Table S1). We verified the quality of lycaenid and Thai ant extractions through amplification of a portion of the insect COI gene using the primers Ben and Jerry (Simon et al. 1994). All other templates were previously deemed of suitable quality through PCR amplification with primers for nuclear and mitochondrial genes (Moreau et al. 2006; Moreau 2008; C. S. Moreau, unpubl. data).

PCR SCREENING FOR *WOLBACHIA* INFECTION

PCR screening was performed using *Wolbachia*-specific primers for two genes: the cell surface protein-encoding gene, *wsp* (*wsp* 81F and *wsp* 691R, from Zhou et al. 1998) and the cell division gene, *ftsZ* (*ftsZ*_F1 and *ftsZ*_R1 from Baldo et al. 2006b). PCR cycling conditions for both genes were: (1) 94˚C for 2 min; 35 cycles of 94˚C for 1 min, 56˚C for 1 min, and 72˚C for 2 min; and a final extension step of 72˚C for 10 min. All screening reactions were performed at 10 μl volumes, consisting of 5.32 μl water, 1 μl Qiagen 10× Taq polymerase buffer (Mg^{++} at 15 mM), 1 μl dNTP mix (2.5 mM of each nucleotide), 0.6 μl $MgCl_2$ (25 mM), 0.8 μl of each primer (at 5 μM), 0.08 μl of Qiagen Taq polymerase (5 units/μl), and 0.4 μl of DNA template. We applied the same protocol for PCR reactions used for DNA sequencing, scaling reagents up to a total volume of 25 μl. DNA from *Wolbachia*-infected ants was used as a positive control in all screening reactions to verify the success and reliability of our amplifications. A negative control reaction, containing water in place of DNA, was included for each PCR reaction to test for the possibility of contamination. Insects were scored as positive for *Wolbachia* infection if they amplified with either *wsp* and/or *ftsZ* primers and results from both primer sets were combined for our tallies of infection frequency.

PCR products were electrophoresed on 1% agarose gels and visualized under UV light. Most products were purified by adding 1 μl of Antarctic phosphatase (New England Biolabs, Ipswich, MA), 1 μl of Antarctic phosphatase buffer, and 0.6 μl of *E. coli* exonuclease I (Fermentas, Burlington, Canada), then running these samples on a thermocycler at 37˚C for 35 min, followed by 80˚C for 20 min. Alternatively, when multiple bands were observed for a single reaction, products of the expected size were excised from gels and purified using the QIAquick Gel Purification Kit (Qiagen Inc.) according to the manufacturers' protocol. Cleaned PCR products were sequenced using ABI PRISM BigDye Terminator Cycle Sequencing Kits version 3.1 (Applied Biosystems, Foster City, CA) on an ABI PRISM 3100 Genetic Analyzer. Sequences were edited using Sequencher version 4.2 (GeneCodes 2003).

The identity of nearly all PCR positives was confirmed through sequence and BLASTn analyses. Sequences from *wsp* or *ftsZ* genes from three lycaenids and 32 ants yielded multiple peaks in sequence chromatographs, suggesting the presence of multiple *Wolbachia* infections. Strains from these hosts were excluded from MLST typing.

MLST TYPING

The recently developed MLST system (Baldo et al. 2006b) provides us with a standardized typing scheme, established protocols, and a large amount of data, all of which should greatly increase our capacity to study *Wolbachia* evolution. The use of multiple loci should also enable us to identify recombination events, which could confound attempts to measure clonal relationships between *Wolbachia* strains. The five analyzed housekeeping genes for this MLST system—*gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA*—are separated by >100,000 nucleotides in the wMel genome. These also evolve under purifying selection (Baldo et al. 2006b), making them ideal candidates for phylogenetic analyses.

We used PCR to amplify these five loci according to previously published protocols (Baldo et al. 2006b). Sequences from isolates successfully typed across all five MLST loci were deposited in the MLST (Baldo et al. 2006b; http://www.pubmlst.org/wolbachia) and GenBank databases (Accession #'s EU127553-EU127822). For all subsequent analyses, we included only one strain per sequence type (ST, defined below) per species.

In accordance with the MLST protocol, we assigned a unique number to each unique allele across all five loci. Each *Wolbachia* strain was, thus, characterized by five integers, representing its allelic profile. Each unique allelic profile was defined as an ST and assigned a number. The resulting matrix of allelic profiles allowed us to measure the degree of identity among STs in different host species, by comparing the number of shared alleles.

The program eBURST version 3 (Feil et al. 2004) was used to identify closely related strains based on the matrix of their allelic profiles. This analysis defines mutually exclusive groups of closely related strains based on the number of shared alleles and attempts to determine the founding genotype within each group. All STs assigned to the same group, or clonal complex,

shared ≥ 3/5 identical alleles at MLST loci with at least one other group member. The founding or ancestral genotype was identified as the ST with the highest number of single locus variants (SLVs); when successfully identified, the ST number of this genotype was used to name its respective clonal complex. All other complexes were assigned temporary, alphabetical names. Members of clonal complexes are assumed to be recently derived and highly related based on their genetic similarity; however, variation introduced by recombination at other genes cannot be excluded without further sequencing.

PHYLOGENETICS

As eBURST clusters strains based on allelic identity, it may not reveal relatedness among closely related, but nonidentical strains. To provide further insight into relatedness among *Wolbachia* strains, we performed phylogenetic analyses using two different approaches (i.e., parsimony and Clonal Frame), expanding our analyses to include all relevant published and unpublished MLST-typed *Wolbachia* strains.

MLST sequences were downloaded and aligned with our sequences using ClustalW (http://www.ebi.ac.uk/clustalw). Manual adjustments were then made using MacClade version 4.06 (Maddison and Maddison 2003). Taxa from two divergent *Wolbachia* lineages—Supergroups A and B—were then separated into two alignments to enable separate analyses. In each case, the wBm strain from the unrelated Supergroup D was used as the outgroup to root our trees.

Consensus parsimony phylogenies were constructed from Supergroup A and B alignments using the *dnapars* and *consense* tools of Phylip (Felsenstein 1989). Given that traditional phylogenetic analyses do not account for recombination as a driving force of sequence evolution, it was unclear whether these analyses would provide an accurate estimation of the organismal *Wolbachia* phylogeny. To overcome this obstacle, we analyzed strain relationships using ClonalFrame (Didelot and Falush 2007; http://bacteria.stats.ox.ac.uk/).

ClonalFrame was developed to handle MLST data, providing an alternative to more traditional means of visualizing relatedness of MLST-typed bacteria (e.g., Sheppard et al. 2008). In essence, this program uses a Bayesian approach to (1) estimate clonal relationships among bacteria and (2) identify the locations and instances of recombination. In doing so, ClonalFrame estimates the probability of (T,M,R,C |A), where T = phylogeny, M = rates and lengths of mutation and recombination events, R = locations of recombination events, C = sequences at ancestral nodes, and A = the data.

For ClonalFrame analyses, we performed three separate runs on each dataset, executing 1,000,000 MCMC iterations each (500,000 burn-in iterations, and 500,000 post-burn-in iterations). In all cases, we started with a random tree, using the default estimates for all model parameters. Phylogenies were sampled every 100 iterations after the burn-in. We used the resulting 5001 sampled trees to construct 50% majority consensus phylogenies, which were used in the subsequent analyses.

Representation of *Wolbachia* hosts was biased due to extensive sampling from the ants and lycaenid butterflies. Specifically, ingroup taxa within Supergroup A analyses consisted of 39 ant-associates and 21 *Wolbachia* strains from hosts outside the Formicidae. Ingroup taxa within Supergroup B analyses consisted of 10 lycaenid-associates (16 lepidopteran-associates) and 20 associates from non-lycaenids (14 from non-lepidopterans). To avoid spurious phylogenetic patterns arising from this sampling scheme, we constructed ClonalFrame phylogenies with additional unpublished MLST data from strains harbored by non-lycaenid and non-ant hosts (Julie Stahlhut et al., unpubl. data). This increased our sample sizes to 112 and 41 strains for Supergroups A and B, respectively. Finally, it is likely that phylogenetic relationships near the base of the phylogeny have been obscured by recombination. To account for this, we performed additional Analysis of Traits statistics on phylogenies in which basal nodes had been collapsed (see below).

PHYLOGENETIC STATISTICS

We used the Analysis of Traits program within the Phylocom package (Webb et al. 2006) to test whether related symbionts: (1) infect related hosts from the same families or orders and (2) are found in the same geographic regions (New World versus non-New World locations). In doing so, we coded the taxonomy and geographic locations of invertebrate hosts as characters of *Wolbachia* strains. We then addressed whether changes in these characters occur less often than expected by chance. Statistically significant results would reveal that (1) *Wolbachia* are most often or most successfully transferred between related hosts and (2) that *Wolbachia* migration has been limited by physical distance or geographic barriers.

In summary, the Analysis of Traits program reconstructs ancestral trait values, computing *D*: the root mean square deviation of trait values at descendant nodes, relative to trait values at ancestral nodes. This *D* statistic provides a measure of constraint for the character of interest: low values reveal that ancestors resemble their descendants, and that character state changes are rare. A tree-wide estimate of *D* was obtained by taking the average for all individual nodes, revealing the overall pattern of evolutionary constraint across the phylogeny. The significance of tree-wide statistics was computed by comparing *D* estimated from the actual dataset to values obtained from 100,000 randomized datasets in which trait values were shuffled across the given topology. To calculate *P*-values, and thus determine whether our character was more constrained than expected by chance, we divided the number of randomizations for which *D* was less than that observed in

our dataset by the total number of randomizations plus one (for the actual dataset).

The names of all host taxa in our phylogenies were entered into a tab delimited "traits" file that contained values for the characters of interest. Because Analysis of Traits statistics cannot handle discrete multistate traits, we were limited to comparing *Wolbachia* from one group of related or geographically localized hosts (coded as "1" in the traits file) to *Wolbachia* from unrelated or geographically distant hosts (coded as "0"). For example, to determine whether the ant-associates were closely related, we coded symbionts from the family Formicidae as "1" in our traits file and symbionts from all other hosts as "0." Outgroup taxa were coded as 0's or 1's in separate analyses; to provide conservative statistical estimates, we only present findings from the analysis yielding the highest value of *D*. This approach is well suited for the biases in our current dataset, which result from extensive sampling from ants and lepidopterans and comparatively little from other individual taxa. As a result, our focus on relatedness of *Wolbachia* strains from these two taxa (compared to strains from all unrelated hosts) caters to the strength of the current sampling regime.

Because Analysis of Traits cannot handle missing data, we pruned our phylogenies when measuring the effects of geographic distance, eliminating strains from hosts with no recorded collection location (five from Supergroup A and 10 from Supergroup B). To disentangle the effects of geographic and taxonomic barriers we pruned phylogenies by removing strains from particular host taxa or geographic locales, performing separate Analysis of Traits statistics on these smaller datasets.

POPULATION STRUCTURE

To identify population structure within *Wolbachia* supergroups, we executed analysis of molecular variance (AMOVA) statistics (Excoffier et al. 1992) in Arlequin (Excoffier et al. 2005) using the Kimura two-parameter model for pairwise distance estimations. Given the results of preliminary analyses and our hypothesis of host association, we drew candidate group and population boundaries between strains based on their hosts' taxonomy and/or geographic locations. In doing so, we examined whether population structure was characterized by isolation between strains from unrelated and/or geographically distant arthropods. Based on an approach adopted by several previous studies (Stanley et al. 1996; Pérez-Lezaun et al. 1999; Hammer et al. 2001; Rasgon et al. 2006), we ran separate analyses that varied in the designation of boundaries between populations and groups containing these populations. We then identified analyses with the lowest amount of within population variation—these elucidate the clearest identifiable boundaries between symbiont populations, providing us with insight into the forces that limit *Wolbachia* spread, thereby promoting their divergence.

LITERATURE SEARCH ON TRANSINFECTION SUCCESS

To determine whether genetic specialization can drive the patterns of host association observed in our analyses, we surveyed the *Wolbachia* literature for studies describing the abilities of *Wolbachia* strains to infect novel hosts after experimental transfer. In total, we summarized and quantified data collected from 25 different publications by determining whether: (1) *Wolbachia* were transmitted to offspring of the recipient host, (2) transmission efficiency was higher than 80 or 90%, and (3) symbionts persisted for at least five or 10 generations after transfer. After coding the taxonomic distance of the donor and recipient hosts as an ordered categorical variable (Supporting Table S4), we used likelihood-ratio tests to analyze the effect of host relatedness on *Wolbachia* persistence after experimental transfer.

Results

WOLBACHIA PREVALENCE ACROSS THE ANTS AND LYCAENID BUTTERFLIES

Within the Hymenoptera, diagnostic PCR screening detected *Wolbachia* in one pompilid wasp and 96 of 329 ants (Supporting Table S1). The estimate of a 29.1% infection frequency was lower than that from a previous study that found *Wolbachia* in 50% (25/50) of Indo-Australian ant species (Wenseleers et al. 1998; $\chi^2 = 8.657$, P-value $= 0.0033$). Our studies differed with respect to the PCR primers used for screening and the number of ants used for each extraction. As such, we cannot rule out the possibility that observed frequency differences arose in part from different methodologies.

Among the butterflies, screening with *wsp* primers identified *Wolbachia* in one nymphalid and in 18 of 95 (18.9%) lycaenids. Previous studies estimated that 24.7–44.9% of lepidopterans are infected with *Wolbachia* (Tagami and Miura [2004]—*wsp* screening; West et al. [1998]—*ftsZ* screening). However, given that (1) many of our surveyed lycaenid templates were obtained from extractions of butterfly legs, and (2) *Wolbachia* may be absent from some somatic tissues (Dobson et al. 1999), our findings should not be considered as strong evidence for a lower infection rate in lycaenids.

DIFFERENCES BETWEEN *WOLBACHIA* OF ANTS AND BUTTERFLIES

A total of 54 *Wolbachia* strains from ants, butterflies, and one wasp were fully typed by MLST and assigned to an ST. Comparisons to previously published MLST-characterized *Wolbachia* enabled us to classify our strains into predefined lineages known as supergroups. Of 39 MLST-typed ant-associates, 37 (95%) belonged to Supergroup A, and one each to Supergroups B and F. This pattern was consistent with previous observations on the relative abundance of Supergroup A within the ants (Wenseleers

et al. 1998). In contrast, 10 of 13 (77%) MLST-typed *Wolbachia* from lycaenid butterflies hailed from Supergroup B. The remaining three belonged to Supergroup A. This was also consistent with previous trends observed among the Lepidoptera, which indicated that Supergroup B is the most common *Wolbachia* type within this insect order (West et al. 1998; Werren and Windsor 2000; Tagami and Miura 2004).

Interestingly, the relative abundances of A and B strains in ants and lycaenids were significantly different ($\chi^2 = 33.041$, $P < 0.001$). Because we used diagnostic PCR to detect *Wolbachia*, it is conceivable that we have failed to detect more divergent strains from other supergroups. But given that (1) this result is consistent with those of other studies using different methodologies and primers and, (2) most strains in ants and lycaenids were identified with the same *wsp* primers, we find it unlikely that our methods have produced a systematic difference in the abundances of Supergroups A and B. We thus conclude that the lack of relatedness between strains harbored by ants and butterflies reveals that symbiotic associations between these insects have not commonly served as a conduit for *Wolbachia* transfer.

COMPLEXES OF RELATED SYMBIONTS

Of 106 MLST-typed *Wolbachia* strains from all supergroups (including our data and published sequences), eBURST analyses revealed that roughly half (52) grouped into one of 15 different clonal complexes (Table 1). Three additional pairs of strains from different host species were identical (one from our dataset) at all five loci. In total, clonal complex size varied from two to 12 strains. The largest (complex ST-19) consisted of 12 strains, including eight that were identical across all 2079 sequenced nucleotides. Combined, these findings reveal that horizontal transfer outpaces recombination, enabling us to decipher patterns of common ancestry among closely related strains from different hosts.

Patterns of complex membership clearly revealed that related *Wolbachia* infect related hosts. Specifically, strains from 12 of 15 clonal complexes (80%) were confined to arthropods from the same order, family, or genus. Five of these complexes consisted entirely of ant-associates (family: Formicidae) and four were comprised solely of strains from butterflies and moths (order: Lepidoptera). Two previously identified complexes consisted of *Wolbachia* from the genus *Drosophila*, and a third contained strains from the spider family Agelenidae. Of the three remaining complexes, two were predominantly comprised of strains from a single insect taxon (complex ST-13: 3/4 *Drosophila*; complex ST-19: 9/12 from Formicidae). Host association within the ant family Formicidae did not extend to lower taxonomic levels such as subfamilies, tribes, or genera (data not shown). Most notably, 9 of 13 strains from *Pheidole* ants grouped into four different complexes; and none of these was exclusive to the *Pheidole* genus.

Wolbachia strains from Thai ants did not typically belong to exclusive complexes, providing little evidence for geographic differentiation on a localized scale. Yet several lines of evidence suggested a trend of host-specific geographic isolation emerging at a larger scale. First, members from all ant-exclusive clonal complexes were restricted to either the New World or to locations outside the New World. Furthermore, although 16 of 367 pairs of ant-associates from the same region had identical sequences, all pairs of ant-associates from different regions (New World vs. non-New World) differed by at least 26 of 2079 nucleotides (374 comparisons).

In stark contrast, complex membership among *Wolbachia* from non-ant hosts provided little evidence for geographic isolation. In fact, identical or highly related strains from complexes ST-i, ST-a, and ST-13 were found in both New World and non-New World locations. These trends remained after adding unpublished MLST-typed strains to our analyses. So in summary, *Wolbachia* from ants show a unique pattern of divergence across the Atlantic and Pacific Oceans.

PHYLOGENETIC ANALYSES

Evidence for host association and geographic isolation within Supergroup A

Three independent runs of ClonalFrame on the Supergroup A alignment yielded consensus trees with similar topologies. In fact, all but one of 34 nodes in the tree from Figure 1A was recovered in all three runs. The Supergroup A parsimony tree was less similar, differing from the presented topology at 10 of 34 nodes. Of the three ClonalFrame runs, run #1 had the highest likelihood score ($-\ln L = 7069.8$ vs. 7156 .6 for run #2 and 7103.2 for run #3). For simplicity, we therefore base most of our subsequent discussion on this topology (presented in Fig. 1A).

Although several *Wolbachia* strains from unrelated hosts were closely related, the trends within this phylogeny provided evidence for host association at the family level: 19 of 39 strains (49%) from the Formicidae fell into one of five ant-specific clades with two to eight members each. The largest clade contained symbionts from broadly distributed New World ants, including *Azteca* sp. (Ecuador), *Dorymyrmex elegans* (Florida), *Pheidole vistana* (Mexico), *P. obtusospinosa* (Arizona), *P. micula* (Arizona), *P. vallicola* (Arizona), *Solenopsis invicta* (Argentina), and *Wasmannia* sp. (Peru).

It is interesting to note that the aforementioned ants come from two different subfamilies (Dolichoderinae and Myrmecinae), which are not sister taxa. This pattern applied to other clades of ant associates, illustrating a lack of specificity at this lower taxonomic level. Similarly, 12 strains from the genus *Pheidole* were distributed across the phylogeny, typically showing close relatedness to associates from other ant genera. As such, we found no evidence for codivergence between ants and *Wolbachia*.

Table 1. Distributions of closely related *Wolbachia* complexes across hosts and geography.

Host species (strain name)	ST[2]	Host family	Country of origin	Clonal complex[1]
Drosophila melanogaster	**1**	Drosophilidae	not recorded	ST-13$^+$
Drosophila innubila	**10**	Drosophilidae	USA	ST-13$^+$
Drosophila recens	**13**	Drosophilidae	USA	ST-13$^+$
Drosophila simulans (wAu)	**14**	Drosophilidae	Australia	ST-13$^+$
Nasonia longicornis	**24**	Pteromalidae	USA	ST-13$^+$
Ephestia kuehniella	**19**	Pyralidae	not recorded	ST-19
Leptogenys sp.	**19**	Formicidae	Thailand	ST-19
Leptomyrmex sp.	**19**	Formicidae	Australia	ST-19
Ornipholidots peucetia	**19**	Lycaenidae	South Africa	ST-19
Pheidole plagiara	**19**	Formicidae	Thailand	ST-19
Pheidole planifrons	**19**	Formicidae	Thailand	ST-19
Pheidole sauberi	**19**	Formicidae	Thailand	ST-19
Technomyrmex albipes	**19**	Formicidae	Philippines	ST-19
Myrmecorhynchus sp.	**54**	Formicidae	Australia	ST-19
Hypolimnas bolina	**91**	Nymphalidae	French Polynesia	ST-19
Ochetellus glaber	**112**	Formicidae	Australia	ST-19
Pheidole sp.	**118**	Formicidae	Indonesia	ST-19
Drosophila neotestacea	**11**	Drosophilidae	USA	ST-a$^+$
Drosophila orientacea	**12**	Drosophilidae	Japan	ST-a$^+$
Drosophila simulans (wMa)	15	Drosophilidae	Tanzania	ST-b$^+$
Drosophila simulans (wNo)	16	Drosophilidae	Seychelles	ST-b$^+$
Solenopsis invicta	29	Formicidae	Argentina	ST-c
Pheidole coloradensis	114	Formicidae	USA	ST-c
Pheidole micula	115	Formicidae	USA	ST-c
Pheidole vistana	116	Formicidae	Mexico	ST-c
Jamides alecto	38	Lycaenidae	Malaysia	ST-d
Iraota rochana	110	Lycaenidae	Malaysia	ST-d
Azteca sp.	46	Formicidae	Ecuador	ST-e
Wasmannia sp.	47	Formicidae	Peru	ST-e
Pheidole obtusospinosa	117	Formicidae	USA	ST-e
Stenamma snellingi	45	Formicidae	USA	ST-f
Myrmica incompleta	49	Formicidae	USA	ST-f
Polyergus breviceps	50	Formicidae	USA	ST-f
Pseudomyrmex apache	44	Formicidae	USA	ST-g
Pheidole minutula	55	Formicidae	French Guiana	ST-g
Solenopsis sp.	**122**	Formicidae	Thailand	ST-h
Monomorium chinense	**123**	Formicidae	Thailand	ST-h
Azanus mirza	41	Lycaenidae	Ghana	ST-i
Celastrina argiolus	41	Lycaenidae	USA	ST-i
Nacaduba angusta	41	Lycaenidae	Malaysia	ST-i
Spalgis epius	42	Lycaenidae	Malaysia	ST-i
Thersamonia thersamon	109	Lycaenidae	Russia	ST-i
Hypolimnas bolina	125	Nymphalidae	French Polynesia	ST-i
Nasonia vitripennis	26	Pteromalidae	not recorded	ST-j
Lycaeides idas	36	Lycaenidae	USA	ST-j
Ostrinia scapulalis	**27**	Crambidae	not recorded	ST-k
Anthene emolus	**37**	Lycaenidae	Malaysia	ST-k
Libythea myrrha	113	Lycaenidae	Malaysia	ST-k
Acraea eponina	4	Nymphalidae	Africa	ST-l
Horaga onyx	39	Lycaenidae	Malaysia	ST-l

Continued.

Table 1. Continued.

Host species (strain name)	ST[2]	Host family	Country of origin	Clonal complex[1]
Agelenopsis aptera	66	Agelenidae	USA	ST-m
Agelenopsis aleenae	66	Agelenidae	USA	ST-m
Agelenopsis longistyla	66	Agelenidae	USA	ST-m
Agelenopsis pennsylvanica	70	Agelenidae	USA	ST-m
Barronopsis texana	70	Agelenidae	USA	ST-m
Anoplolepis gracillipes	52	Formicidae	Philippines	
Lophomyrmex sp.	52	Formicidae	Thailand	
Muscidifurax uniraptor	23	Pteromalidae	not recorded	
Nasonia vitripennis	23	Pteromalidae	not recorded	
Culex pipiens pipiens	9	Culicidae	USA	
Culex pipiens quinquefasciatus	9	Culicidae	USA	

[1]**Clonal-complex: *Wolbachia* isolates were assigned to the same complex if they shared at least three identical alleles with one other complex member. Complexes were named after the ST (sequence type) of the primary founder, where identified by eBURST; alternatively, complexes were given temporary alphabetical names. The STs of strains belonging to complexes with a more stringent cut-off of four identical alleles are presented in bold font. "+" indicates that complexes were reported in Baldo et al. (2006b).**

[2]**ST, or sequence type = a unique identifier for each allelic profile. Identical ST numbers indicate that strains are identical across all five MLST loci.**

When considering geographic distributions of related *Wolbachia*, we did not observe evidence for isolation within New World or non-New World regions. Perhaps most demonstrative was our finding that 11 strains from the geographically restricted Thai ants were distributed across five non-exclusive clades. These clades consisted of 19 additional strains originating from distant locations within the Old World and Oceania. In contrast, when considering a broader scale, the ClonalFrame phylogeny provided clear evidence for geographic isolation: *Wolbachia* strains from New World and non-New World locations regularly grouped into separate clades.

Given these trends and the results of previously described analyses, we used Analysis of Traits statistics to address two specific hypotheses: (1) *Wolbachia* from ants are closely related, and (2) *Wolbachia* from New World hosts are not related to strains from non-New World hosts.

Analysis of Traits statistics is presented in Table 2 and Supporting Table S2. Statistics on host taxonomy revealed that the trait of ant association was constrained on all Supergroup A phylogenies produced by ClonalFrame (e.g., run #1: $D = 0.250$, $P = 0.00091$) and parsimony analyses ($D = 0.209$, $P = 3 \times 10^{-5}$). This trend remained strong and significant even when analyzing phylogenies: (1) with collapsed basal nodes (data not shown) or, (2) constructed from a larger dataset containing unpublished isolates (see Supporting Table S2). These findings reveal that trait of ant association is constrained on the phylogeny, indicating that related *Wolbachia* infect related hosts.

The trait of geographic location (New World vs. non-New World) also showed significant constraint on ClonalFrame and parsimony phylogenies (e.g., parsimony analysis: $D = 0.184$, $P < 1 \times 10^{-5}$; ClonalFrame run #1: $D = 0.219$, $P = 8 \times 10^{-5}$). These findings support our visual observations, suggesting that related *Wolbachia* strains are typically confined to New World or non-New World locations. To further examine observations from our analyses of clonal complexes, we pruned our Supergroup A phylogenies, yielding alternative trees that contained only ant- or non-ant associates. As expected, the trait of geographic origin was highly constrained on phylogenies of ant-associates (ClonalFrame run #1: $D = 0.164$, $P < 1 \times 10^{-5}$). This trait was constrained to a lesser extent on the non-ant *Wolbachia* phylogenies ($D = 0.247$, $P = 0.0251$), due in part to monophyly of strains from New World spiders (family Agelenidae). In spite of this, we observed two instances whereby highly related strains from *Drosophila* showed cosmopolitan distributions. So in accordance with our studies of clonal complex membership, these findings suggest that oceanic barriers are less formidable for *Wolbachia* in at least some non-ant taxa.

No statistical phylogenetic evidence for geographic isolation or host association in Supergroup B

Three independent runs of ClonalFrame on the Supergroup B alignment yielded consensus trees with similar topologies—only three of the 22 nodes in Figure 1B were not recovered in all three analyses. The parsimony tree showed a larger number of differences, disagreeing with 8 of 22 of the nodes in the tree from Figure 1B. Of the three ClonalFrame runs, run #1 had the highest likelihood score ($-\ln L = 5455.5$ vs. 5492.3 for run #2 and 5456.1 for run #3). As such, we present the phylogeny from this run in Figure 1B, focusing on this topology in our discussion.

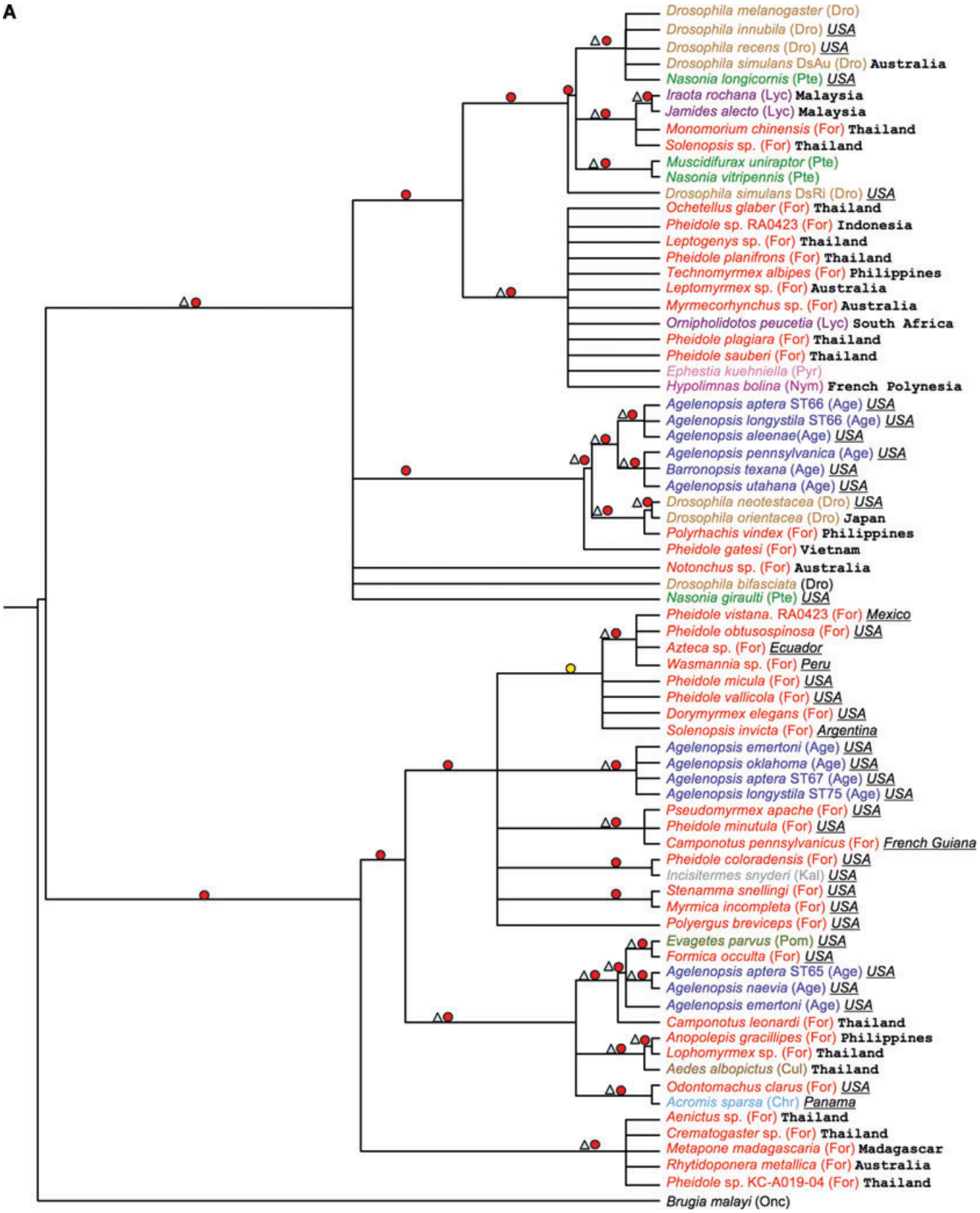
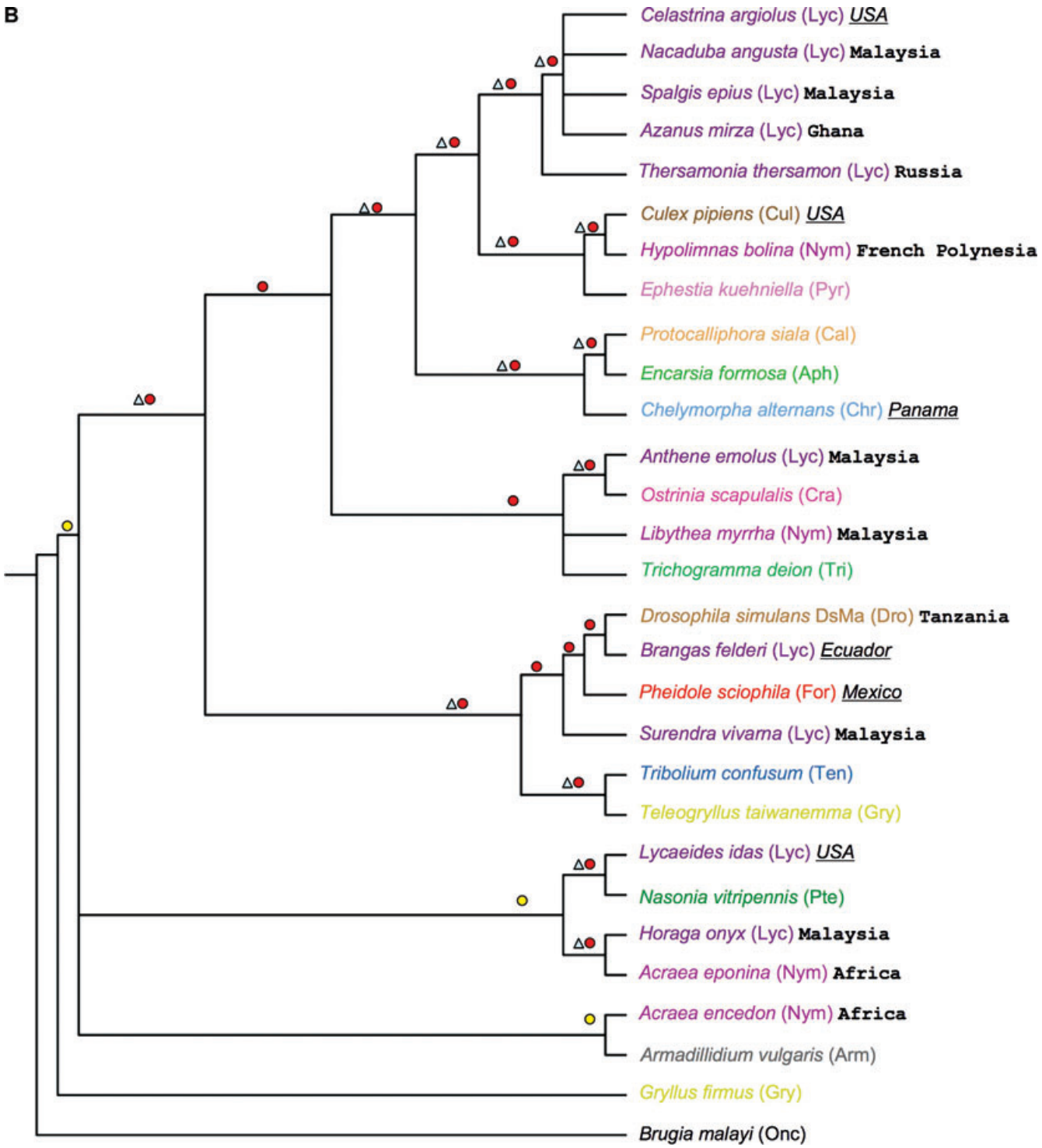


Figure 1. Phylogenies of MLST-typed *Wolbachia*. Of three separate ClonalFrame runs on MLST data for (A) Supergroups A (B) Supergroup B, we present the 50% majority consensus topologies from the runs with the highest likelihood scores. Colored circles are used to indicate agreement with topologies from the two additional runs: yellow circles indicate that the node was supported in one run; red circles



indicate support in both additional runs. Blue triangles reveal agreement with 50% majority consensus trees obtained through parsimony analyses. *Wolbachia* strains are named after their hosts and are color coded to distinguish different host families, which are abbreviated in parentheses (see below). Note that ant-associates are presented in red font whereas associates from lycaenid butterflies are presented in purple font. Geographic origins are presented in black. Strains from locations within the *New World* are presented in underlined and italic Arial font. Those from non-New World locations are presented in Courier New Bold font. ST numbers or strain names are included for some strains to help distinguish between unrelated *Wolbachia* from the same host species. Age, Agelenidae; Aph, Aphelinidae; Arm, Armadillidiidae (isopod); Cal, Calliphoridae; Chr, Chrysomelidae; Cra, Crambidae; Cul, Culicidae; Dro, Drosophilidae; For, Formicidae; Gry, Gryllidae; Kal, Kalotermitidae; Lyc, Lycaenidae; Nym, Nymphalidae; Onc, Onchocercidae (nematode); Pom, Pompilidae; Pte, Pteromalidae; Pyr, Pyralidae; Ten, Tenebrionidae; and Tri, Trichogrammatidae.

Table 2. Analysis of traits statistics on host association and geographic isolation.

Dataset[1]	Method and run[2]	Character[3]	Char. State 1 (sample size)	Char. State 2 (sample size)	D[4]	Rank[5]
Supergroup A	ClonalFrame run #1	Host Taxonomy	ant (40)	Non-ant (33)	0.250	91***
Supergroup A	Parsimony	Host Taxonomy	ant (40)	Non-ant (33)	0.209	3***
Supergroup A	ClonalFrame run #1	Geography	New World (38)	non-New World (30)	0.219	8***
Supergroup A	Parsimony	Geography	New World (38)	non-New World (30)	0.184	1***
Supergroup A, ants only	ClonalFrame run #1	Geography	New World (22)	non-New World (17)	0.164	1***
Supergroup A, ants only	Parsimony	Geography	New World (22)	Non-New World (17)	0.143	2***
Supergroup A, no ants	ClonalFrame run #1	Geography	New World (21)	non-New World (8)	0.247	2510**
Supergroup A, no ants	Parsimony	Geography	New World (21)	non-New World (8)	0.240	4156**
Supergroup B	ClonalFrame run #1	Host taxonomy	lycaenid (10)	non-lycaenid (18)	0.287	12908
Supergroup B	Parsimony	Host taxonomy	lycaenid (10)	non-lycaenid (18)	0.307	20811
Supergroup B	ClonalFrame run #1	Host taxonomy	Lepidoptera (16)	non-Lepidoptera (12)	0.323	23214
Supergroup B	Parsimony	Host taxonomy	Lepidoptera (16)	non-Lepidoptera (12)	0.343	41638
Supergroup B	ClonalFrame run #1	Geography	New World (6)	non-New World (12)	0.396	87927
Supergroup B	Parsimony	Geography	New World (6)	non-New World (12)	0.380	87365

[1]Supergroup A and B datasets were analyzed separately. For geographic and host-specific analyses, trees were pruned and limited to members of the taxa of interest.

[2]Phylogenies were constructed using parsimony (in Phylip) or ClonalFrame analyses. Three separate ClonalFrame runs were performed. In this table we present results from the run with the highest likelihood score.

[3]Taxonomy of bacterial hosts along with their geographic origins were analyzed as characters, and Analysis of Traits statistics determined whether these were constrained on bacterial phylogenies.

[4]*D*, average root mean square deviation of trait values across the phylogeny; provides a measure of phylogenetic constraint, giving us a quantitative measure of whether related taxa share identical character states (e.g., whether they infect members of the same host family).

[5]Rank for the *D* value of our dataset in relation to the null distribution generated by 100,000 character state randomizations (across fixed tree topologies). Significant deviations from random expectations are indicated by: **, 0.05> *P*-value > 0.001, ***, *P*-value < 0.001.

Like the two additional ClonalFrame phylogenies, the tree in Figure 1B reveals a weak pattern of close relatedness among lepidopteran associates: 9 of 16 strains (56%) from moths and butterflies grouped into one of three exclusive clades. However, Analysis of Traits statistics did not provide significant support for host association among lycaenid or lepidopteran *Wolbachia* (Table 2, Supporting Table S2), regardless of whether analyses were performed on the ClonalFrame or parsimony trees, trees with collapsed basal nodes (data not shown), or trees constructed with the larger, unpublished dataset. Analyses also provided no statistical support for geographic isolation within Supergroup B.

POPULATION STRUCTURE

In performing AMOVA analyses on 114 *Wolbachia* isolates from Supergroup A (published and unpublished isolates with recorded geographic information), we focused on the importance of geography and host taxonomy in shaping symbiont evolution and population structure. Of several different analyses (data not shown), the model that identified the largest amount of subdivision, or population structure, contained three unnested populations: (1) strains from New World ants ($n = 17$), (2) strains from non-New World ants ($n = 22$), and (3) strains from all other insects ($n = 75$). Under this grouping, 21.76% of the total genetic variation among strains was accounted for by differences among populations (Table 3), and the estimated ϕ_{ST} value (0.2176) was significantly different from the null expectation ($P < 0.00001$). An otherwise identical model, in which non-ant strains were split into New World and non-New World populations, resulted in a smaller estimate of among-population variation (19.04%), suggesting that *Wolbachia* from other invertebrates show less differentiation between New World and non-New World locations.

To further elucidate structure among the three aforementioned Supergroup A populations, we performed an exact test of population differentiation in Arlequin, according to the methods of Raymond and Rousset (1995). This analysis found significant differentiation among all three populations (Bonferroni-corrected P-values: $P_{\text{non-New World ant vs. non-ant}} < 0.00001$, $P_{\text{New World ant vs. non-ant}} = 0.00135$, $P_{\text{non-New World ant vs. New World ant}} = 0.01815$). As such, it appears that (1) *Wolbachia* from ants are distinct from those in non-ants, and (2) strains from ants belong to at least two isolated populations—those from the New World and those from other regions.

AMOVA analyses within Supergroup B did not identify significant population structure (Table 3). The model with the lowest amount of within-population variation consisted of four unnested populations: (1) strains from New World Lepidoptera

Table 3. AMOVA statistics identify boundaries between *Wolbachia* populations.

Supergroup	Populations	Variance component	Percent variation	ϕ_{ST}	*P*-value[1]
A	(1) New World, ant	among population	21.76	0.2176	<0.001
	(2) non–New World, ant	within population	78.24		
	(3) non-ant				
B	(1) non–New World, Lepidoptera	among population	4.81	0.0481	0.1369
	(2) non–New World, non-Lepidoptera	within population	95.19		
	(3) New World, Lepidoptera				
	(4) New World, non-Lepidoptera				

[1]*P*-values were estimated by comparing the observed ϕ_{ST} values to those obtained in 1023 randomizations.

($n = 11$), (2) strains from New World non-Lepidoptera ($n = 3$), (3) strains from Lepidoptera found outside the New World ($n = 3$), and (4) strains from non-Lepidoptera found outside the New World ($n = 13$). Under this analysis, only 4.81% of total genetic variation was explained by variation among populations. This small ϕ_{ST} value (0.0481) was not greater than that expected by chance ($P = 0.1369$). However, because sample sizes for some populations were extremely small, further sampling is necessary to determine whether our result is a function of low statistical power.

LITERATURE SEARCH REVEALS EVIDENCE FOR SPECIALIZATION

Analyses of data summarized from 25 publications revealed an effect of host relatedness in shaping the success of experimental *Wolbachia* transfer (Supporting Tables S3 and S4). Specifically, likelihood-ratio tests demonstrated a positive correlation between transinfection success and relatedness of donor and recipient host: symbionts transferred between related taxa (e.g., same or genus) were more likely to be transmitted to the F1 generation ($P = 0.0028$), to exhibit transmission efficiencies exceeding 80% ($P = 0.0161$) or 90% ($P = 0.051$), and to persist for at least five ($P = 0.0495$) or 10 ($P = 0.0026$) generations in their novel host. After removing data from intraspecific transfers, we still found significant evidence for a positive effect of relatedness on transmission to F1 ($P = 0.0061$) and persistence for $\geq$10 generations ($P = 0.0043$). Effects on transmission efficiencies and persistence beyond five generations showed nonsignificant trends toward reduced transinfection success with decreasing relatedness.

It is important to note that these studies included some examples of symbionts that successfully infected distant hosts. For example, *Wolbachia* transferred from *Drosophila simulans* (insect order: Diptera) persisted for over 10 generations after transfer to the planthopper, *Laodelphax striatellus* (insect order: Hemiptera) (Kang et al. 2003). Although transmission efficiency was lower than 80% in the novel host, this illustrates an impressive capacity of some strains to establish in distantly related insects.

Discussion

EVOLUTIONARY PATTERNS OF *WOLBACHIA*–INSECT ASSOCIATIONS

In our study, we surveyed a diverse collection of ants (Hymenoptera: Formicidae) and lycaenid butterflies (Lepidoptera: Lycaenidae), characterizing *Wolbachia* strains from single infections with the recently developed MLST method (Baldo et al. 2006b). Through analyses of 2079 sequenced nucleotides from five well-spaced loci, we have provided a thorough examination of the evolutionary histories of interactions between *Wolbachia* and their arthropod hosts.

Several lines of evidence reveal, convincingly, that related symbionts infect related hosts. First, our data indicated that complexes of highly similar *Wolbachia* strains were typically found in exclusive, or nearly exclusive association with members of the same host family (Formicidae: ants) or order (Lepidoptera: butterflies and moths) (Table 1). This pattern supports similar observations made for *Wolbachia* associated with other host taxa, including the genus *Drosophila* (Baldo et al. 2006b) and the family, *Agelenidae* (Baldo et al. 2008). Second, phylogenetic analyses that accounted for both mutation and recombination revealed a significant and robust pattern of close relatedness among *Wolbachia* from different ant species (Fig. 1A; Table 2). Third, AMOVA identified population structure within Supergroup A—strains from non-ant hosts were significantly differentiated from strains found in New World and non-New World ants (Table 3).

Our analyses did not reveal trends of host association at the levels of subfamily or genus within the ant family Formicidae, indicating that *Wolbachia* have not commonly spread through co-divergence or introgression. When looking at higher taxonomic levels, we did not find evidence for close relatedness between strains from ants and those from non-ant hymenopterans. In contrast, strains from the butterfly family Lycaenidae formed

lepidopteran-exclusive complexes with strains from moths and other butterflies (Lepidoptera). Although further sampling is needed to verify the significance of this trend, this difference suggests that the degree of "specificity" may vary between clades of *Wolbachia* and that insects from broad taxonomic groups may harbor groups of related symbionts.

Similar trends of host association have been observed in previous studies, which have typically based their inferences on analyses of single genes. In these cases, "related" strains were found to infect members of the same genus (*Acraea*, *Armadillidium*, and *Trichogramma*), superfamily (Oniscidea), or order (Siphonaptera and Phthiraptera) (Bouchon et al. 1998; van Meer et al. 1999; Jiggins et al. 2002; Dittmar and Whiting 2004; Kyei-Poku et al. 2005). However, inferences of relatedness drawn from most of these studies were based on analyses of just one or two genes, including *wsp*, which is subject to high rates of recombination that obscure phylogenetic reconstruction (Baldo et al. 2005, 2006a,b, 2007). Nevertheless, combined with our MLST analyses and those of Baldo and colleagues (2007, 2008), these findings reveal a common trend of host association, whereby related *Wolbachia* are most often found in related hosts. This suggests the potential for diffuse coevolution between related groups of hosts and bacteria. Future investigations are needed to determine the extent and significance of this coevolution. Furthermore, more research will be necessary to measure the degrees of specificity and whether these vary across clades of hosts or bacteria.

In spite of these results, it is essential to note that highly related, even genetically identical *Wolbachia* strains are occasionally found in distantly related hosts. This was even true for some ant-associates, including those in clonal complex ST-19, which were highly related to strains from lepidopterans. So clearly *Wolbachia* strains are capable of occasional movement between distant relatives. It will be interesting to identify factors that may enable these more distant host-switching events. Do multiple infections permit successful infection by strains from unrelated hosts? Are these large leaps undertaken by strains from generalist clades? Are the hosts of these "generalists" united by virtue of sharing particular physiological attributes? By studying exceptions to the patterns of specialization we will further elucidate the processes that drive host-*Wolbachia* coevolution.

POTENTIAL CAUSES OF HOST ASSOCIATION

In considering why related *Wolbachia* infect related hosts we can envision two non-exclusive explanations: (1) similar ecologies of related hosts facilitate transfer of related microbes, and/or (2) *Wolbachia* are genetically specialized on related invertebrates.

With regard to the first scenario, it is possible that related *Wolbachia* spread between related hosts by means of shared diets or parasites. Previous studies suggest that *Wolbachia* could spread through consumption of infected or contaminated diets (Kittayapong et al. 2003; Sintupachee et al. 2006; see Purcell et al. 1994; Darby and Douglas 2003 for examples with other heritable symbionts). However, we would only expect this mechanism to generate a pattern of host association among relatives with similar diets. Given the breadth and variability of ant diets (e.g., Hölldobler and Wilson 1990; Davidson et al. 2003), this seems unlikely.

It is, alternatively, conceivable that patterns of host association arise because related hosts share related, symbiont-vectoring parasites. In fact, there is direct (e.g., Jaenike et al. 2007) and indirect evidence (Vavre et al. 1999; Noda et al. 2001; Dedeine et al. 2005) for a role of parasitism in the horizontal transfer of maternally transmitted symbionts (but, see West et al. 1998). In the absence of experimental studies or sampling from parasites of ants or lepidopterans, we cannot rule out this possibility as a partial cause of our observations.

Genetic specialization provides a compelling alternative for our observations of host association. Under this scenario, *Wolbachia* are preadapted to infect related arthropods because they share similar physiologies to their current hosts. Accordingly, previous research has shown that experimentally transferred *Wolbachia* are more likely to persist after transfer between relatives (e.g., van Meer and Stouthamer 1999; Poinsot and Merçot 2001). Rigaud and colleagues (2001) provided a convincing demonstration of this phenomenon through a study of *Wolbachia* that were transferred among four species of isopods. Strains transferred between members of the same genus or species were transmitted to 84–95% of offspring, retaining the ability to feminize their hosts. When these same strains were transferred to hosts from different families, they were found in only 0–9% of offspring. They were, furthermore, unable to feminize their novel and inhospitable hosts.

Our analyses of the literature reveal that this phenomenon is a general property of *Wolbachia* across the arthropods, as both persistence and maternal transmission efficiency decrease with increasing taxonomic distance from their natural hosts (Supporting Tables S3 and 4). This provides the strongest evidence to date for genetic specialization, which has likely favored repeated and prolonged encounters of related groups of hosts and symbionts over evolutionary time.

GEOGRAPHIC ISOLATION IS HOST SPECIFIC

Our findings suggest that unidentified barriers that correlate with host phylogenies promote divergence among *Wolbachia* populations, limiting gene flow and horizontal transfer among unrelated hosts. It also appears that some *Wolbachia* populations are isolated by geographic barriers. Considerations of genetic distances, clonal complex membership, phylogenetic distributions, and AMOVA all revealed a strong split between strains from New World and non-New World ants. These findings resemble those

obtained from a previously published single-gene analysis, which indicated that New World ant-associates were related to one another, but not to *Wolbachia* strains found in ants from non-New World locations (Tsutsui et al. 2003).

At this point, limited sampling and a lack of specific geographic coordinates prevent us from performing a more localized and quantitative analysis of geographic isolation. But in a qualitative sense, we do not see evidence for isolation within New World or non-New World locations, even among the ant-associates. For example, the largest clade of New World ant-associates contained strains originating from Florida, Arizona, Mexico, Ecuador, Peru, and Argentina. Strains from the largest clade of non-New World ant-associates hailed from Australia, Thailand, and Madagascar. Furthermore, *Wolbachia* from ants collected in the Trang province of Thailand were no more closely related to each other than they were to strains from ants in other non-New World locations. So although more localized geographic isolation seems plausible, the only isolation we have identified is mediated by the Atlantic and Pacific Oceans.

It is interesting that geographic isolation is comparatively weak among strains from non-ant hosts, which occasionally shared relatedness that transcended oceanic barriers. What mechanisms could account for this host-specific pattern? We can likely rule out a confounding effect of ant phylogeny, because New World and Old World ants do not form reciprocally monophyletic clades (e.g., Moreau et al. 2006). Moreover, like other insects, ants have been spread worldwide by means of human activity (McGlynn 1999). So it is not immediately clear whether human-mediated spread could drive the observed patterns.

A third possibility for host-specific geographic isolation could extend from intrinsic differences in host mobility. Long-distance movement of ants is governed by queen mating flights, which typically do not exceed a few dozen kilometers (Hölldobler and Wilson 1990). We would expect several other insects to be more mobile, most notably the Lepidoptera, which are capable of flights spanning thousands of miles (Urquhart 1976). To test this hypothesis, it will be necessary to perform future MLST studies on *Wolbachia* from cosmopolitan collections of immobile invertebrates, such as springtails, bristletails, silverfish, arachnids, terrestrial isopods, and secondarily flightless insects. Such investigations will help to disentangle the relative contributions of host mobility, geographic barriers, and symbiont specialization as impediments to symbiont gene flow and host switching.

IMPLICATIONS FOR INSECT AND SYMBIONT INTERACTIONS

Our literature review suggests that traits responsible for symbiont survival and transmission could drive evolutionary patterns of host association, assuming that adaptation to a hosts' physiology will render *Wolbachia* better able to infect that hosts' relatives. But traits involved in bacterial survival and transmission may provide only a partial mechanistic explanation for our findings. Indeed, the effects of these symbionts on their hosts' development and reproduction may also be of significance. In considering this possibility, it is important to consider the range of reproductive manipulations conducted by *Wolbachia* from the ants, moths, and butterflies. Within the Lepidoptera, *Wolbachia* are known to induce male killing, feminization, and cytoplasmic incompatibility (CI) (e.g., Jiggins et al. 2000a; Sasaki and Ishikawa 2000; Fujii et al. 2001; Hiroki et al. 2002). Indirect evidence suggests that *Wolbachia* may execute CI within the ants (van Borm et al. 2001). Given that experimentally transferred symbionts can be inefficient in their manipulations of reproduction (Grenier et al. 1998; Huigens et al. 2004; Riegler et al. 2004), we speculate that specificity could arise, or be reinforced, if symbionts were pre-adapted to most efficiently manipulate reproduction in related hosts.

Another trait of relevance for symbiont persistence is bacterial virulence. *Wolbachia* have been suggested to have negative effects on ant fitness, as worker infection frequencies within colonies of *Formica truncorum* were negatively correlated with the production of sexual males and females (Wenseleers et al. 2002). Interestingly, *Wolbachia* are found at higher frequencies among sexual females than reproductively sterile adult workers of three ant species (van Borm et al. 2001), and it appears that these symbionts are actually lost from workers during development (Wenseleers et al. 2002). Given their effects on colony fitness, it was suggested that loss from workers could represent an adaptive strategy for ant-infection, whereby *Wolbachia* facilitate the production of transmitting hosts (Wenseleers et al. 2002). Although additional studies are required to determine the generality of this phenomenon, it is tantalizing to consider that this unique feature of ant-*Wolbachia* symbioses could be a cause or consequence of specialization.

SUMMARY

Throughout their long histories as symbionts, *Wolbachia* have had substantial ecological and evolutionary impacts, influencing their hosts' sex ratios (Jiggins et al. 2000a; Hiroki et al. 2002; Dyer and Jaenike 2004), mating behavior (Jiggins et al. 2000b; Dyson and Hurst 2004), modes of reproduction (Gottlieb and Zchori-Fein 2001; Huigens and Stouthamer 2003), genome evolution (Ballard et al. 1996; Ballard 2000; Shoemaker et al. 2004; Hurst and Jiggins 2005), and quite possibly, their diversification (Werren 1998; Shoemaker et al. 1999; Bordenstein et al. 2001; Jaenike et al. 2006). Our findings provide compelling evidence that these histories have been characterized by repeated encounters between related lineages of hosts and symbionts and that these are likely driven by genetic specialization that manifests in the form of host-range limitations. Our results also indicate that these host-range limitations act in concert with oceanic barriers, thus promoting

diversification among these prolific and profoundly influential bacteria.

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Supporting Information

The following supporting information is available for this article:

Table S1. Insect taxa screened for *Wolbachia* in this study.

Table S2. Statistics on *Wolbachia* phylogenies reveal host association and geographic isolation.

Table S3. Persistence and transmission of *Wolbachia* after experimental transfer.

Table S4. Effects of host relatedness on *Wolbachia* persistence and transmission in novel hosts.

Supporting Information may be found in the online version of this article.
(This link will take you to the article abstract).

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