Survey of *Wolbachia* Frequency in Nashville, Tennessee Reveals Novel Infections

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ABSTRACT
Wolbachia (Rickettsiales: Anaplasmataceae) are maternally transmitted intracellular bacteria that infect approximately half of all insect species. These bacteria commonly act as reproductive parasites or mutualists to enhance their transmission from mother to offspring, resulting in high prevalence among some species. Despite decades of research on *Wolbachia*'s global frequency, there are many arthropod families and geographic regions that have not been tested for *Wolbachia*. Here, arthropods were collected on the Vanderbilt University campus in Nashville, Tennessee, where *Wolbachia* frequency has not been previously studied. The dataset consists of 220 samples spanning 34 unique arthropod families collected on the Vanderbilt University campus. The majority of our samples were from the families Blattidae (Blattodea), Pulicidae (Siphonaptera), Dryinidae (Hymenoptera), Aphididae (Hemiptera), Paronellidae (Entomobryomorpha), Formicidae (Hymenoptera), Pseudococcidae (Hemiptera), Sphaeroceridae (Diptera), and Coccinellidae (Coleoptera). PCR-based techniques were used to assign infection states and, from these data, the first cases of *Wolbachia* in the Paronellidae springtails, Lithobiidae (Lithobiomorpha) centipedes, Lonchopteridae (Diptera) spear-winged flies, Sepsidae (Diptera) black scavenger flies, Cryptocercidae (Blattodea) wood roaches, and Lauxaniidae (Diptera) acalyptrate flies were identified. Within-family infection frequencies ranged from 17-100% when *Wolbachia* was observed; however, numerous families tested did not reveal evidence of infection. These results expand on the field's understanding of *Wolbachia*'s frequency in Nashville, Tennessee, and among arthropod families broadly, and is the first report of *Wolbachia* in centipedes.

KEYWORDS
Wolbachia; Infection Frequency; Endosymbiont; Tennessee; Centipede; Arthropod; Polymerase Chain Reaction; Nashville

INTRODUCTION

Wolbachia is a genus of obligate intracellular bacteria that commonly infect arthropods and nematodes.[1–4] They often reside in reproductive tissue cells, are vertically inherited from ova to offspring[5] and are occasionally transferred horizontally between arthropods.[6–9] *Wolbachia* frequently interact with their hosts as mutualists[10] and/or reproductive parasites[11] to encourage their proliferation through host populations.[12–17] *Wolbachia* can increase or decrease host longevity,[18–22] suppress pathogen replication,[23–27] provide essential nutrients to their host,[28, 29] and cause reproductive parasitism phenotypes such as cytoplasmic incompatibility, male-killing, feminization, and parthenogenesis.[11, 30–34]

Studies agree that *Wolbachia* are common among arthropod species.[1–4] However, estimates for the percentage of infected species range from 16.9%[1] to as high as 66%,[2] with other estimates reporting 40%[3] and 52%.[4] The variance in these estimates can be attributed to differences in testing methodologies and data sets used in these analyses. Each analysis leverages PCR-based screens for bacterial symbionts, but each differ in how they handle species that have small or large sample sizes since high sample sizes are more likely to reveal infections. Intra-species infection frequencies can be below 10% to as high as 100%,[3, 35] and these frequencies can vary based on geography.[36–39]

Despite decades of research aimed at elucidating *Wolbachia's* frequency among arthropods, estimates remain variable partly due to the lack of sampling of some arthropod families and in various geographic regions. Here, we aim to characterize *Wolbachia* infection frequency in arthropod families collected in Nashville, Tennessee, which, to our knowledge, has not been studied in the context of *Wolbachia* frequency. We collect arthropods, extract DNA, and use PCR-based techniques to describe infection states. We report that 73% of the families we collected are infected, identify *Wolbachia* in six families previously not reported with an infection, and describe infection frequencies ranging from 17-100% within infected families. These results expand our knowledge of the infection frequency of *Wolbachia* in a variety of arthropods in Nashville, Tennessee.

METHODS AND PROCEDURES

Arthropod collection and identification.

As part of an Introduction to Biology Laboratory course at Vanderbilt University, arthropods were collected from January to March 2018 on the Vanderbilt University campus in Nashville, Tennessee **(Fig. 1A)**. Samples were collected inside buildings, outside buildings on the campus grounds, and in the Vanderbilt University greenhouse **(Fig. 1B).** Each sample represents a single arthropod. A variety of collection methods were used, including Berlese funnels, food and chemical attractants, pitfall traps, color traps, sticky traps, and active collection. Arthropods were removed from traps within 24 h and were individually frozen at -20°C in sterile 1.5 mL Eppendorf tubes (Eppendorf, Hamburg, Germany), 15 mL conical tubes, or 50 mL conical tubes. The family of each arthropod was determined using dichotomous keys and pictorial guides.[40–42] Sub-family level identification was not conducted due to time restrictions of the class, and all specimens were destroyed during downstream processing.

Determining Arthropod Infection Status.

DNA was extracted from all arthropod samples using the Gentra Puregene Tissue Kit (QIAGEN, Hilden, Germany) slightly modified from the manufacturer's protocol. For larger samples, the posterior end of the arthropod was dissected for DNA extraction since *Wolbachia* are transmitted maternally and are likely to be present in these tissues. To determine infection state, PCR was conducted using WSpec-forward (5'-CAT ACC TAT TCG AAG GGA TAG-3') and WSpec-reverse (5'-AGC TTC GAG TGA AAC CAA TTC-3') primers targeting the *Wolbachia* 16S rRNA gene,[1, 43] using the following cycling conductions: 94℃ for 2 m, 30 cycles at 94℃ for 30 s, 49℃ for 45 s and 72℃ for 1 min, and a final extension at 72℃ for 10 m. Positive controls and negative controls were *Wolbachia*-infected and uninfected *Drosophila melanogaster* respectively from laboratory stocks generously donated by the Bordenstein lab at Vanderbilt University. PCR products were run on a 1% agarose gel and visualized under UV light after treatment with GelRed (Biotium, Fremont, CA). A sample was considered infected if it produced a 436 bp-long fragment as expected for the WSpec amplicon.

Analyses, and figure creation.

All analyses were conducted and graphs generated in GraphPad Prism 8 (GraphPad Software, San Diego, CA), maps were generated in ArcGIS Online (Esri, Redlands, CA), and figure aesthetics were edited in Affinity Designer 1.7 (Serif, Nottingham, United Kingdom).

RESULTS

Arthropods (n=220) were collected on the Vanderbilt University campus in Nashville, Tennessee (Fig. 1A). Samples were collected in three clearly different environments, either inside buildings, not including the Vanderbilt University greenhouse (n=21), outside buildings (n=84), or in the Vanderbilt University greenhouse (n=115), and most families collected were only found in one location type. The Psychodidae (Diptera) and Sphecidae (Hymenoptera) were only collected inside campus buildings; the Aphididae, Armadillidiidae (Isopoda), Cryptocercidae, Paronellidae, Pholcidae (Araneae), Pseudococcidae, and Thripidae (Thysanoptera) were only found in the greenhouse. The Acrididae (Orthoptera), Anthomyzidae (Diptera), Ceratopogonidae (Diptera), Dryinidae, Latridiidae (Coleoptera), Lauxaniidae, Lithobiidae, Lonchopteridae, Pulicidae (Siphonaptera), Salticidae (Araneae), Sarcophagidae (Diptera), Sciaridae (Diptera), Sepsidae, Sphaeroceridae (Diptera), Tetranychidae (Trombidiformes), Tipulidae (Diptera), and Vespidae (Hymenoptera) were only found on campus grounds (Fig. 1B). Additionally, Blattidae specimens were found in the greenhouse and non-greenhouse buildings, and Coccinellidae, Drosophilidae (Diptera), Formicidae, Muscidae (Diptera), Pentatomidae (Hemiptera), and Theridiidae (Araneae) specimens were found in both indoor and outdoor locations **(Fig. 1B).** The Phoridae (Diptera) were the only family found both in the greenhouse and on campus grounds **(Fig. 1B).**

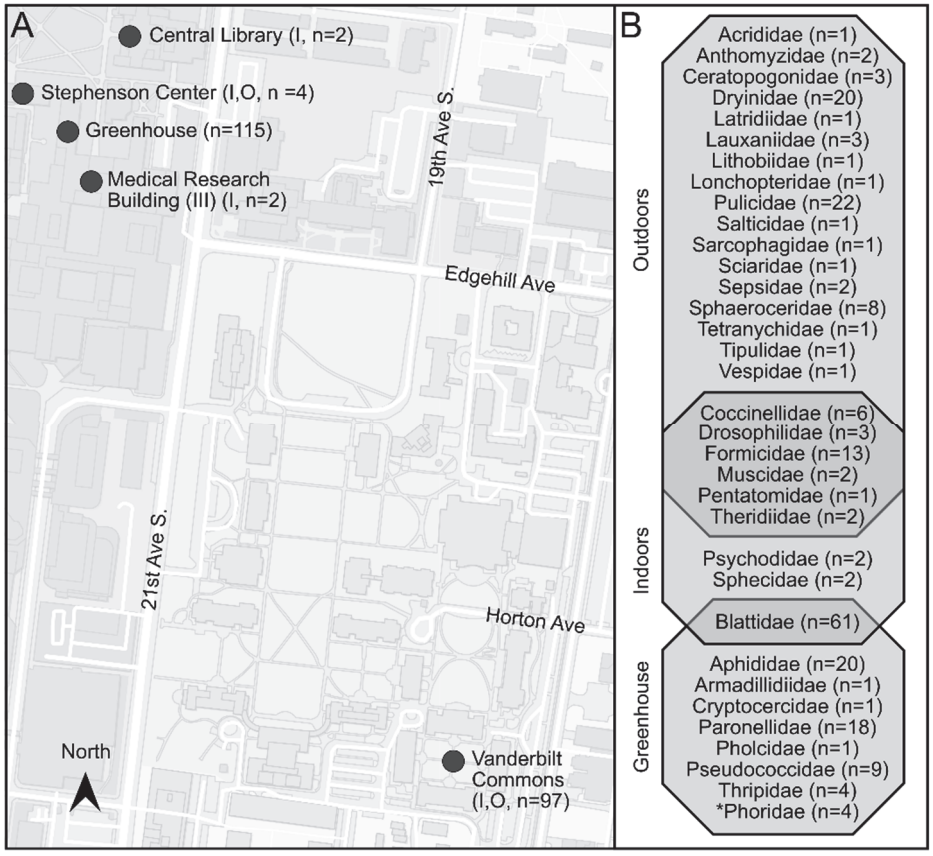

Figure 1. Samples representing 34 arthropod families were collected outdoors, indoors, and in a greenhouse on the Vanderbilt University campus in Nashville, TN. (A) Dark grey dots represent sampling locations. "I" indicates that samples were collected from inside buildings (excluding the greenhouse). "O" indicates that samples were collected outside and around campus buildings. The sample size is shown beside each sampling location. (B) Venn diagram shows arthropod families found in each sampling location. 17 families were only sampled from outdoor locations, two families only from indoor locations, and seven families only from the greenhouse. Some families are found both outside and inside, and some are found both inside and in the greenhouse. Only the Phoridae (denoted with an *) was found in both the greenhouse and outdoors. The number of samples collected for each family are shown to the right of the family name.

To characterize *Wolbachia* infection states, DNA was extracted from samples and PCR was used to amplify for a segment of the *Wolbachia* 16S rRNA gene using the WSpec primer set.[36, 43] We identified *Wolbachia* 16S rRNA fragments in 49% of specimens and in 73% of families. *Wolbachia* was not detected in the families Latridiidae (n=1), Pentatomidae (n=1), Salticidae (n=1), Tipulidae (n=1), Vespidae (n=1), Anthomyzidae (n=2), Sphecidae (n=2), Theridiidae (n=2), and Thripidae (n=4) **(Fig. 2**). However, aside from the Thripidae, all of the uninfected families in our study had two or fewer samples each, making it impossible to conclude whether these infection states can be generalized to the family as a whole. Additionally, *Wolbachia* was found in the families Acrididae (n=1, 100% infected), Armadillidiidae (n=1, 100%), Cryptocercidae (n=1, 100%), Lithobiidae (n=1, 100%), Lonchopteridae (n=1, 100%), Pholcidae (n=1, 100%), Sarcophagidae (n=1, 100%), Sciaridae (n=1, 100%), Tetranychidae (n=1, 100%), Muscidae (n=2, 50%), Psychodidae (n=2, 50%), Sepsidae (n=2, 50%), Ceratopogonidae (n=3, 33%), Drosophilidae (n=3, 67%), Lauxaniidae (n=3, 100%), Phoridae (n=4, 75%), Coccinellidae (n=6, 17%), Sphaeroceridae (n=8, 50%), Pseudococcidae (n=9, 100%), Formicidae (n=13, 31%), Paronellidae (n=18, 67%), Aphididae (n=20, 20%), Dryinidae (n=20, 70%), Pulicidae (n=22, 32%), Blattidae (n=61, 52%) **(Fig. 2)**. Among infected families, the infection frequency was as low as 17% in the Coccinellidae to as high as 100% in the families Acrididae, Armadillidiidae, Cryptocercidae, Lithobiidae, Lonchopteridae, Pholcidae, Sarcophagidae, Sciaridae, Tetranychidae, Lauxaniidae, and Pseudococcidae **(Fig. 2)**. Notably, we do not claim that *Wolbachia* has reached fixation in any of these families since all families with 100% infection frequency had low sample sizes.

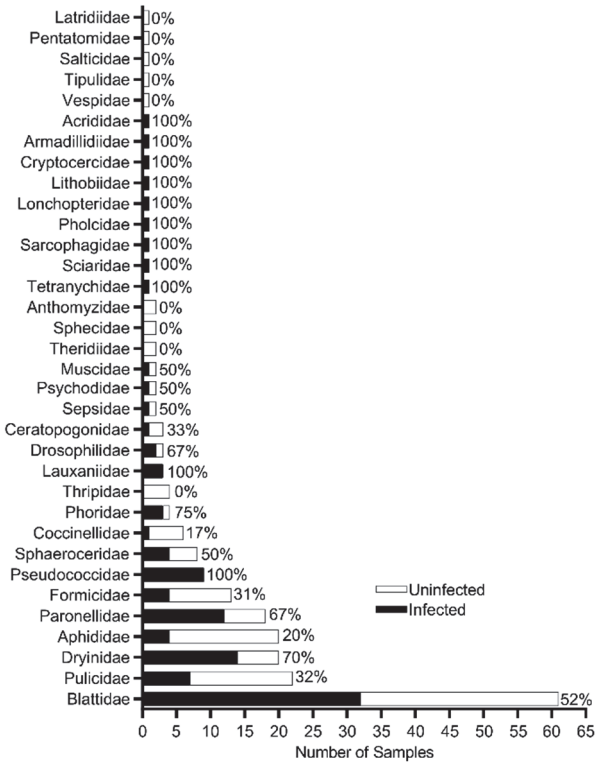

Figure 2. *Wolbachia* infection frequency varies between arthropod families. Of the 34 families collected, 25 were infected with *Wolbachia*. All of the samples were infected in ten families and all were uninfected in nine. The remaining families had infection frequencies between 17-67%. White bars represent uninfected samples and black bars represent infected samples. The percentage to the right of each bar represent the percent of infected samples per family.

DISCUSSION

We report *Wolbachia* infection states for 220 arthropod samples spanning 34 families, of which 25 are infected. To our knowledge, six of these infected families did not have prior reports of infection in the literature **(Table 1)**: Paronellidae elongate springtails, Lithobiidae stone centipedes, Lonchopteridae spear-winged flies, Sepsidae black scavenger flies, Crytptocercidae wood roaches, and Lauxaniidae acalyptrate flies. We found the infection frequency for these families to be 78% in the Paronellidae (n=18), 50% in the Sepsidae, and 100% in the Lithobiidae (n=1), Lonchopteridae (n=1), Cryptocercidae (n=1), and Lauxaniidae (n=4). Conclusions about infection frequencies can only be drawn from the Paronellidae, which had a relatively robust sample size. While not reported in the Paronellidae, *Wolbachia* has been reported in seven other springtail families spanning four orders including the order Entomobryomorpha, which contains the Paronellidae.[44–46] These results suggest that *Wolbachia* might be more common among this subclass than it would seem from literature.

The infection in Lithobiidae centipedes is of particular interest since, to our knowledge, *Wolbachia* has not been previously reported in centipedes. In fact, two prior surveys have proposed negative infection states for centipedes in the family Lithobiidae[36] and in the order Scolopendromorpha.[47] However, it remains possible that our positive infection state is the result of a false-positive infection. For instance, the carnivorous lifestyle of centipedes may increase the rate of false-positives since they may feed on *Wolbachia*-infected insects whose DNA would then contaminate the sample. Future studies that collect more samples and dissect specific tissue for *Wolbachia*-infection assays will help confirm the frequency of infection among this arthropod order. Additionally, while *Wolbachia* are common among Diptera and Blattodea,[12, 36, 48–50] we report the first cases of infection in the Lonchopteridae, Sepsidae, Lauzaniidae, and Cryptocercidae, which have been mostly overlooked. These results validate the premise that additional sampling and *Wolbachia* infection testing is necessary to identify *Wolbachia* in arthropod groups that are under sampled.

Finally, while there are reports of infection in the Pentatomidae,[51] Salticidae,[52] Theridiidae,[53, 54] Thripidae,[55–57] Tipulidae,[58] and Vespidae[59] in the literature **(Table 1)**, we did not find evidence of infection in these families in Nashville. However, we are cautious to make firm conclusions on these data due to low sample sizes and numerous alternative explanations for negative results. For example, PCR-based techniques can miss low-density *Wolbachia* infections if they are below a detection threshold,[60] failures in DNA extraction and/or PCR can result in false-negatives due to insufficient or low quality DNA, and since the WSpec primers used here were designed for Supergroup A and B *Wolbachia* it is plausible that our techniques would not detect highly divergent *Wolbachia* strains.[36, 43] Further work is necessary to confirm that *Wolbachia* do not reside in these families in Nashville, but if these results hold

to larger sampling it may suggest differences in *Wolbachia*'s frequency in Nashville relative to other regions that have been sampled. However, since we only identified samples to family, it is possible that our samples belong to different species than have been reported in other studies.

Family	Infected?	Literature reports of infection?	Reference
Acrididae	Yes	Yes	61
Aphididae	Yes	Yes	62–64
Armadillidiidae	Yes	Yes	65
Blattidae	Yes	Yes	48, 49
Ceratopogonidae	Yes	Yes	66, 67
Coccinellidae	Yes	Yes	68
Drosophilidae	Yes	Yes	12, 69, 70
Dryinidae	Yes	Yes	71
Formicidae	Yes	Yes	72–74
Muscidae	Yes	Yes	75
Pholcidae	Yes	Yes	54
Phoridae	Yes	Yes	76
Pseudococcidae	Yes	Yes	77
Psychodidae	Yes	Yes	78–80
Pulicidae	Yes	Yes	81
Sarcophagidae	Yes	Yes	82
Sciaridae	Yes	Yes	83
Sphaeroceridae	Yes	Yes	59
Tetranychidae	Yes	Yes	84
Lithobiidae	Yes	No	36
Sepsidae	Yes	No	85
Cryptocercidae	Yes	---	N/A
Lauxaniidae	Yes	---	N/A
Lonchopteridae	Yes	---	N/A
Paronellidae	Yes	---	N/A
Pentatomidae	No	Yes	51
Salticidae	No	Yes	52
Theridiidae	No	Yes	53, 54
Thripidae	No	Yes	55–57
Tipulidae	No	Yes	58
Vespidae	No	Yes	59
Sphecidae	No	No	36
Anthomyzidae	No	---	N/A
Latridiidae	No	---	N/A

Table 1. Family level *Wolbachia* infection status compared to the literature. Bold family names represent families for which the infection status in the literature disagrees with those reported in this study. Bold and underlined families had not been previously screened for *Wolbachia* infection.

In summary, we describe *Wolbachia*'s frequency among arthropods in Nashville, Tennessee and report the first instance of *Wolbachia* in several arthropod families, including in the Lithobiidae centipedes. Additionally, we provide data for infection frequency within numerous other families, often overlooked by the current literature. This research will inform studies aimed at understanding *Wolbachia*'s global spread and distribution, by adding Nashville, Tennessee, to the *Wolbachia* pandemic map.

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Author contributions

JDS designed research; SP, PW, AB, AC, and JDS performed research and analyzed data; SP, PW, AB, and JDS wrote the paper.

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PRESS SUMMARY

Wolbachia are intracellular and maternally inherited bacteria that infect roughly half of all arthropod species. Since arthropods account for approximately 85% of all animals, *Wolbachia* is thought to be the world's most common animal-associated bacterial infection. However, estimates of *Wolbachia* infection frequency range from as low as 17% to as high as 66% of species. Additionally, strong arthropod family-biases and geographical-biases exist in the current literature. It is thus important to survey new species and different geographic regions to better understand *Wolbachia's* global frequency. Here, we report a dataset of 220 arthropod samples, spanning 34 families, that have been tested for *Wolbachia* using PCR-based techniques. These samples were collected by undergraduate students as part of an Introduction to Biology Laboratory at Vanderbilt University in Nashville, Tennessee. We confirm literature reports of infections in 25 arthropod families and novel infections in six, and put Nashville on the map of locations tested for *Wolbachia* frequency.