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Variable Infection Frequency and High Diversity of Multiple Strains of *Wolbachia pipientis* in *Perkinsiella* Planthoppers▽†

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This survey of *Wolbachia* infections in populations of the planthoppers *Perkinsiella saccharicida* and *Perkinsiella vitiensis* revealed variable frequencies, low-titer infections, and high phylogenetic diversities of strains. These observations add to the growing realization that *Wolbachia* infections may be extremely common within invertebrates and yet occur infrequently within populations and at low titer within individuals.

Wolbachia pipientis is a maternally inherited endosymbiotic bacterium that infects a wide range of arthropods and nematodes. *Wolbachia* is renowned for inducing dramatic reproductive phenotypes, such as cytoplasmic incompatibility (44) and parthenogenesis (35), that manipulate host reproduction to enhance *Wolbachia* transmission. However, recent papers have uncovered an alternative and more cryptic mode of life for these bacteria: infections that occur at low densities within hosts and at a low frequency within and among populations (1, 2, 15).

In the course of examining the delphacid planthoppers *Perkinsiella saccharicida* and *Perkinsiella vitiensis* for symbionts that might be utilized in future paratransgenic approaches targeting *Fiji disease virus* (FDV) transmission, we encountered a number of novel *Wolbachia* strains associated with these species. Several DNA extraction techniques were used to determine if a particular extraction method was optimal for *Wolbachia* detection. Genomic DNA was isolated from individual surface-sterilized planthoppers (19) by using CTAB (cetyltrimethylammonium bromide) (31), Holmes Bonner (13), rapid release preparation (40), STE (27), salt (23), and Chelex (42) DNA extractions and a Puregene DNA extraction kit (Gentra Systems, MN). The *Wolbachia* surface protein (*wsp*) gene was amplified with either New England BioLabs *Taq* (NEB, Beverly, MA) or Takara *Taq* (Takara Bio, Inc., Japan) polymerase using primers 81F/691R (4, 17). Twenty microliters of PCR product was run on a 1% agarose gel stained with ethidium bromide and visualized under a UV transilluminator. PCR products were TA cloned into pGEM-T Easy vectors and sequenced. When a negative PCR result was encountered, the integrity of the DNA was verified by amplification of the 12S rRNA gene for insect mitochondria (27). PCRs were repeated on those negative *Wolbachia* samples that had positive 12S amplification, after diluting the template either 1/10 or 1/100 to account for PCR inhibitors (45). Although spiking the *Wolbachia*-positive template with *Perkinsiella* host DNA did not appear to interfere with amplification, these inhibition experiments were not quantitative, and small changes in amplification efficiency may be critical when the template concentration is at the limit of amplification. *Wolbachia* was detected in an additional 8 samples when the PCR product was diluted. A Puregene DNA extraction kit (Gentra Systems, MN) combined with amplification using Takara *Taq* polymerase appeared to be the most successful method to amplify these bacteria from planthoppers (see Table S1 in the supplemental material).

Wolbachia was detected in 45 of the 302 planthoppers assayed. *Wolbachia* strains within this planthopper appear to maintain infection densities that are below the threshold for detection by direct hybridization techniques (7) (see Fig. S1 in the supplemental material) and are at the limit of detection by PCR, as faint bands were recorded in the majority of cases. More-sensitive long PCR techniques (15) did not amplify *Wolbachia* in planthoppers from the Woodford region, QLD, Australia. This finding was similar to that of Sun et al. (37), where nested PCR failed to increase the *Wolbachia* detection level in flies.

The frequencies of infection of *Wolbachia* in planthoppers varied between populations, from 4% to 100% (Fig. 1). In concordance with the findings in this study, geographic variability in *Wolbachia* infection frequencies was also observed in the planthopper *Tagosodes orizicolus* (37 to 100%) (12). The variable infection frequencies observed in this study may be a true reflection of the infection rate in the population, or alternatively, density levels between individuals may fluctuate beyond the sensitivity of PCR, accounting for this variation. The latter scenario would mean that *Wolbachia* infections are more prevalent in the insect population than previously thought.

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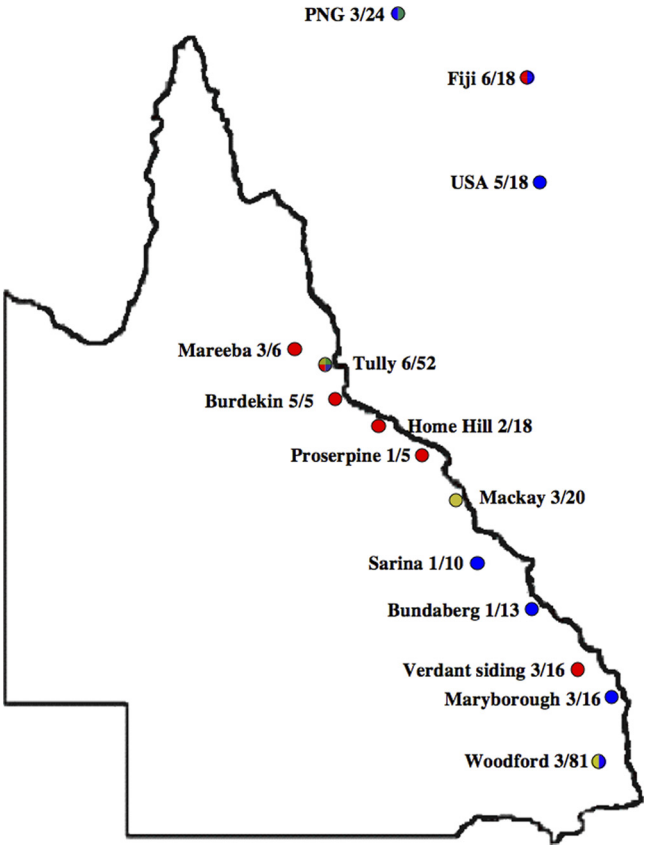

FIG. 1. Map of Queensland, Australia, showing populations of *P. saccharicida and P. vitiensis* assayed for *Wolbachia* infection. Colors indicate *Wolbachia* strains based on the phylogenetic groupings from the Bayesian trees shown in Fig. 2. Numbers indicate *Wolbachia*-positive planthoppers/total number of insects screened at each site.

Given the low-titer infections observed in *Perkinsiella* planthoppers, it seems unlikely that *Wolbachia* would be able to induce reproductive phenotypes like cytoplasmic incompatibility (14, 25). Indeed, reciprocal crosses between Tully and Woodford planthoppers were fertile (30). Our data, together with recent studies indicating that low-density *Wolbachia* infections exist in other host species (1, 2, 15), suggest that *Wolbachia* utilizes mechanisms other than reproductive parasitism to maintain itself within these populations and that these mechanisms may be at least as common and important to *Wolbachia* as reproductive parasitism. There is emerging evidence that *Wolbachia* can confer fecundity advantages under certain situations (5, 8, 43). Moreover, recent studies have also shown that *Wolbachia* may function to provide protection against pathogens in insects (11, 16, 24, 39). *Wolbachia*'s ability to improve host fitness suggests that in some instances its biology may be more similar to that of a mutualistic secondary symbiont than that of an exclusive reproductive parasite.

The diversity of strains infecting these *Perkinsiella* species is much greater than the diversity observed for *Wolbachia* strains infecting other planthopper species (12, 18, 25). Bayesian phylogenetic analysis of the *wsp* gene indicates there are multiple groups of *Wolbachia* strains in *Perkinsiella* planthoppers (Fig. 2) (29, 32). Phylogenetic trees constructed from either the whole *wsp* segment or the *wsp* segment with the highly variable repeats (HVR) removed (3) group the taxa similarly (see Fig. S2 in the supplemental material), and both divided the *Wolbachia* strains into four main clades. Three of these clades clustered with diverse sequences from supergroup B, including sequences from other *Wolbachia* strains within planthoppers. The fourth group of sequences clustered with *Wolbachia* strains from the cockroaches *Supella longipalpa* and *Blattella* sp., tentatively classified as supergroup F (41). Recombination analysis using RDP with default parameters (22) identified this group of sequences as possible recombinants, but the parental, minor, and major sequences could not be ascertained with confidence. It seems likely that the cockroach F group is parental and the planthopper *Wolbachia* sequences are recombinant, as the F group is supported by sequence data from four independent loci (41). No other evidence for recombination was observed in the B group sequences, precluding the possibility that recombination was the cause of the high phylogenetic variation seen within these strains. PCRs were performed on surface-sterilized planthoppers, the *Wolbachia* sequences are each the consensus of five individual clones of a PCR product, and all sequences are novel. Taken together, these factors suggest that the strain variation seen in planthoppers is authentic and not due to environmental or laboratory contaminants or sequencing errors.

Typically, only one strain of *Wolbachia* is present in each species of delphacid planthopper (12, 18, 25). Here, we observe a variety of strains infecting different *Perkinsiella* populations. In the majority of cases, only a single strain was detected in individual planthoppers, but on two occasions, individuals from Fiji and Verdant Siding, QLD, Australia, each possessed two strains of *Wolbachia*. Mapping *Wolbachia* strains to their locations shows that there is no distinct relationship between geographic region and strain type (Fig. 1).

Previous studies have also detected variable frequencies of low-titer infections with diverse *Wolbachia* strains, for example, in the fly *Bactrocera dorsalis* (37). Even in this system, however, the phylogenetic diversity of *Wolbachia* strains was not as dramatic as that found in *Perkinsiella*. If *Wolbachia* acts as more of a mutualist toward its host, infection with diverse strains may allow the host to respond to various environmental conditions or pathogens. In light of recent discoveries of *Wolbachia*-mediated pathogen interference (11, 16, 24, 39), it would be particularly interesting to examine pathogen-*Wolbachia* interactions to see if there is an advantage to *Wolbachia* infection. Additionally, as antipathogen protection can differ between strains (28), it would be intriguing to compare antipathogen effects in planthoppers infected with different *Wolbachia* strains. Indeed, the interplay between pathogens and *Wolbachia* could help to maintain strain diversity.

Other factors may contribute to the strain diversity and prevalence of infection identified in *Perkinsiella* planthoppers. Sintupachee et al. (33) suggested that *Wolbachia* may be transmitted horizontally via plants. Increases in strain diversity may be the result of *Wolbachia* adapting to the plant host in order to survive. However, it seems unlikely that the many diverse strains observed in our data could be due to repeated transient infection of the gut by plant-acquired *Wolbachia*, given the previous lack of evidence for *Wolbachia* transmission via feeding. Alternatively, and possibly more likely, low-density *Wolba-*

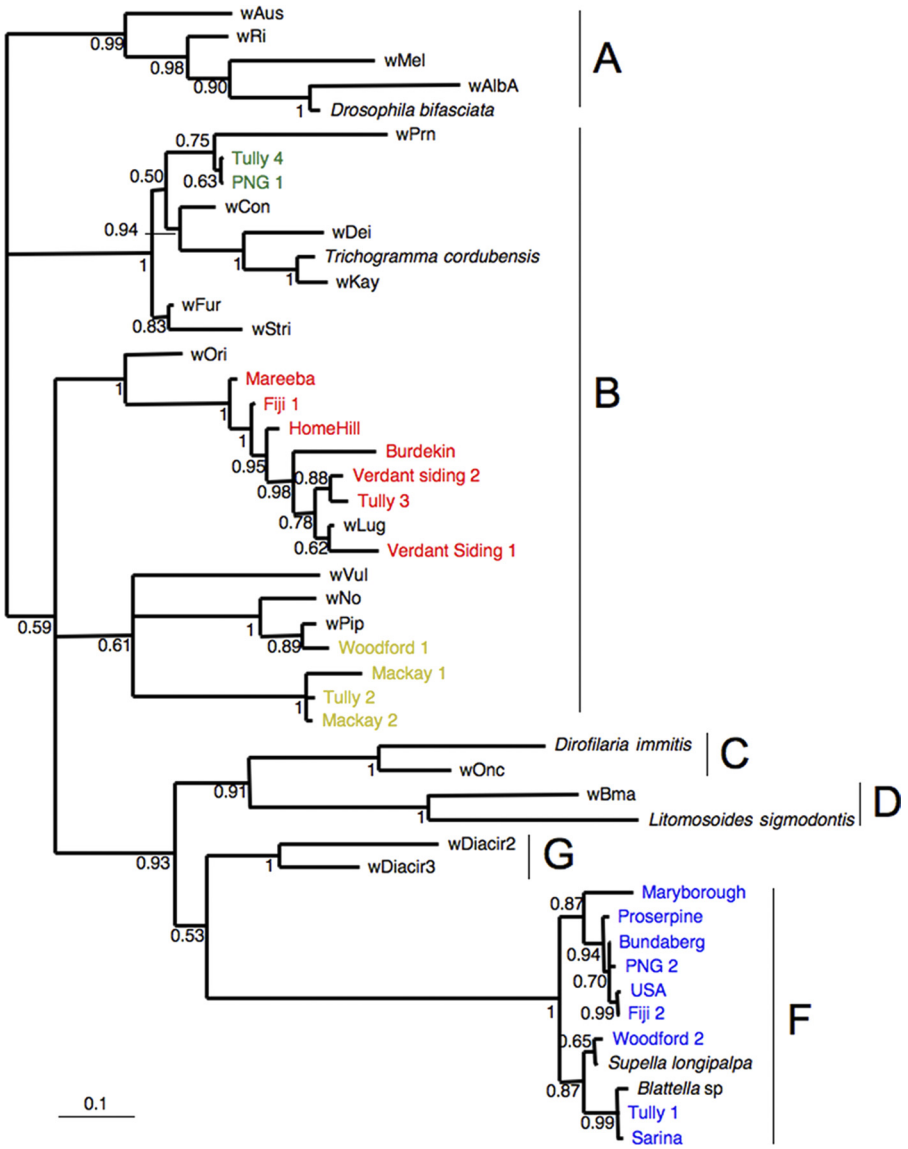

FIG. 2. Bayesian phylogenetic tree of the *wsp* gene, constructed using MrBayes (32). Analysis was run in duplicate (4 chains each), with default heating (0.2) for 1 million generations and with samples collected every 100 generations. Bayesian posterior probabilities were calculated by computing a 50% majority rule consensus of the trees remaining from the duplicate runs after discarding the burn-in that represented 25% of trees. *Wolbachia* strain names are used for reference taxa; where no strain name exists, the name of the host is used. *Wolbachia* supergroups (A to D, F, and G) are indicated on the tree. Colors represent groupings based on phylogenetic analysis. The colored groups correspond to those shown in Fig. 1. Numbers below branches are Bayesian clade confidence values. Planthoppers surveyed from Fiji and PNG were *P. vitiensis* (also a vector of FDV).

chia infections may be the result of the interaction with other microbial flora within the insect. Planthoppers are known to harbor *Asaia* species (38) and yeast-like symbionts (26, 36), and both of these microorganisms have been identified in *Perkinsiella* planthoppers (G. L. Hughes, unpublished data). Antagonism between symbionts has been demonstrated in ticks infected with *Rickettsia* (6, 21), while yeasts have been shown to reduce symbiotic bacteria in ants and displace bacterial symbionts within aphids (9, 20). The dynamic and low-titer *Wolbachia* infections may be shaped in part by positive or negative interactions with other members of the symbiont community.

This study shows a high diversity of *Wolbachia* strains occurring at low density and variable infection frequencies within *Perkinsiella* planthoppers. These results add to an emerging understanding that *Wolbachia* may be more pervasive than currently accepted, due to cryptic infections that occur in few individuals within a population and at low infection densities within these hosts. If these cryptic *Wolbachia* infections are shown to be widespread, then we may come to see reproductive parasitism as the exception and not the general rule for *Wolbachia*.

Nucleotide sequence accession numbers. All *Wolbachia wsp* gene sequences were submitted to GenBank under accession numbers GU190767 to GU190788.

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