Wolbachia in the spittlebug Prosapia ignipectus: Variable infection frequencies, but no apparent effect on host reproductive isolation

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Funding information
National Institute of General Medical Sciences, Grant/Award Number: R35GM124701

Abstract
Animals serve as hosts for complex communities of microorganisms, including endosymbionts that live inside their cells. Wolbachia bacteria are perhaps the most common endosymbionts, manipulating host reproduction to propagate. Many Wolbachia cause cytoplasmic incompatibility (CI), which results in reduced egg hatch when uninfected females mate with infected males. Wolbachia that cause intense CI spread to high and relatively stable frequencies, while strains that cause weak or no CI tend to persist at intermediate, often variable, frequencies. Wolbachia could also contribute to host reproductive isolation (RI), although current support for such contributions is limited to a few systems. To test for Wolbachia frequency variation and effects on host RI, we sampled several local Prosapia ignipectus (Fitch) (Hemiptera: Cercopidae) spittlebug populations in the northeastern United States over two years, including closely juxtaposed Maine populations with different monomorphic color forms, “black” and “lined.” We discovered a group-B Wolbachia (wPig) infecting P. ignipectus that diverged from group-A Wolbachia—like model wMel and wRi strains in Drosophila—6 to 46 MYA. Populations of the sister species Prosapia bicincta (Say) from Hawaii and Florida are uninfected, suggesting that P. ignipectus acquired wPig after their initial divergence. wPig frequencies were generally high and variable among sites and between years. While phenotyping wPig effects on host reproduction is not currently feasible, the wPig genome contains three divergent sets of CI loci, consistent with high wPig frequencies. Finally, Maine monomorphic black and monomorphic lined populations of P. ignipectus share both wPig and mtDNA haplotypes, implying no apparent effect of wPig on the maintenance of this morphological contact zone. We hypothesize P. ignipectus acquired wPig horizontally as observed for many Drosophila species, and that significant CI and variable transmission produce high but variable wPig frequencies.

KEYWORDS
Cercopidae, cytoplasmic incompatibility, endosymbiosis, host–microbe interaction, speciation
1 INTRODUCTION

Animals interact with microorganisms that influence their behavior, physiology, and fitness (Brownlie et al., 2009; Fredericksen et al., 2017; Gould et al., 2018; Hague, Caldwell, et al., 2020; Hurst & Jiggins, 2000; McFall-Ngai et al., 2013). These include associations between hosts and vertically transmitted endosymbionts that live inside their cells (McCutchon et al., 2019). Hosts may acquire endosymbionts cladogenically from common ancestors (Koga et al., 2013; Raychoudhury et al., 2009; Toju et al., 2013), from sister species via hybridization and introgression (Cooper et al., 2019), or horizontally in ways that are not fully understood (Ahmed et al., 2015; Huigens et al., 2000; O’Neill et al., 1992; Turelli et al., 2018). While few examples exist, endosymbionts can contribute to host reproductive isolation (RI) (Coyne & Orr, 2004; Matute & Cooper, 2021), highlighting the importance of discovering and characterizing endosymbiont-host associations.

Maternally transmitted Wolbachia bacteria are widely distributed (Weinert et al., 2015; Werren et al., 2008; Zug & Hammerstein, 2012), infecting many arthropods and two groups of parasitic nematodes (Bandi et al., 1998), making Wolbachia the most common known endosymbionts in nature. In Drosophila, introgressive and horizontal Wolbachia acquisition seem to predominate (Conner et al., 2017; Cooper et al., 2019; Turelli et al., 2018), but cladogenic acquisition during host speciation has been observed in other taxa (Gether & Bleidorn, 2017; Raychoudhury et al., 2009).

Many Wolbachia manipulate host reproduction to propagate in host populations. For example, many strains cause cytoplasmic incompatibility (CI) that reduces the egg hatch of uninfected embryos fertilized by Wolbachia-infected sperm (Hoffmann & Turelli, 1997). However, if females are also infected, the embryos survive, “rescuing” CI and promoting Wolbachia spread to high frequencies (Barton & Turelli, 2011; Hoffmann et al., 1990; Kriesner et al., 2013; Turelli & Hoffmann, 1995).

Wolbachia may contribute to host RI (Coyne & Orr, 2004; Matute & Cooper, 2021), with the best evidence coming from Drosophila. Wolbachia contribute to assortative mating and postzygotic isolation between co-occurring D. paulistorum semispecies (Miller et al., 2010), and to reinforcement of isolation between uninfected D. subquinaria and Wolbachia-infected D. recens (Jaenike et al., 2006; Shoemaker et al., 1999). In contrast, Wolbachia do not contribute to RI in the D. yakuba clade, which includes wYak-infected D. yakuba, wSan-infected D. santomea, and wTei-infected D. teissieri (Cooper et al., 2017). Thus, while some results from Drosophila strongly support contributions of Wolbachia to RI, and interest in the possibility of such effects remains high, it is unknown whether Wolbachia effects on RI are common in nature.

Wolbachia frequencies differ significantly among infected host taxa, ranging from very low to obligately fixed infections (Bandi et al., 1998; Cooper et al., 2017; Kriesner et al., 2013; Miller et al., 2010). Wolbachia effects on reproduction (e.g., CI) and fitness (e.g., fecundity effects, Weeks et al., 2007), in combination with imperfect maternal transmission, govern its frequencies in host populations (Caspari & Watson, 1959; Hoffmann et al., 1990). Intensive sampling of a few systems has revealed both stable and variable Wolbachia frequencies within host populations. Wolbachia that cause intense CI, like wRi in Drosophila simulans, persist at high and relatively stable frequencies, balanced by imperfect maternal transmission (Kriesner et al., 2013; Turelli et al., 2018). In contrast, Wolbachia that cause weak or no CI tend to occur at variable intermediate frequencies (Cooper et al., 2017; Hamm et al., 2014; Hoffmann et al., 1996; Kriesner et al., 2016; Meaney et al., 2019) via effects on host fitness that are mostly unknown (Brownlie et al., 2009; Hague et al., 2021; Teixeira et al., 2008; Weeks et al., 2007). These strains include wMel-like Wolbachia with frequencies that vary spatially in D. melanogaster and D. yakuba (Hague, Caldwell, et al., 2020; Kriesner et al., 2016), and temporally in D. yakuba and D. santomea (Cooper et al., 2017; Hague, Mavengere, et al., 2020). In all but a few other systems, limited sampling has left a gap in knowledge about whether Wolbachia frequency variation is common (Cattel et al., 2016; Hamm et al., 2014; Hughes, Allsopp, et al., 2011; Hughes, Ren, et al., 2011; Ross et al., 2020; Schuler et al., 2016).

Prosapia ignipectus (Fitch) (Hemiptera: Cercopidae) is one of about 14 species of Prosapia and one of two commonly found in the United States, the other being its sister species P. bicincta (Say) (Hamilton, 1977). P. ignipectus occurs in southern Ontario, Canada, and the northeastern United States from Minnesota to Maine (Carvalho & Webb, 2005; Hamilton, 1977, 1982; Peck, 1999; Thompson & Carvalho, 2016). These species vary in male genital morphology and in associations with host plants, with P. ignipectus monophagous on the late season C4 perennial grass Schizachyrium scoparium (Little bluestem) (Hamilton, 1982; Thompson, 2004) and P. bicincta polyphagous on a variety of C4 grasses, but not including Little bluestem (Fagan & Kuiter, 1969; Thompson, 2004). Both species have conspicuous dorsal coloration, standing out against their respective host plants. All P. bicincta individuals have a single narrow transverse orange line across the widest part of the pronotum and a pair of narrow orange lines across the elytra. Most P. ignipectus individuals have a solid black dorsal surface, but in Maine some P. ignipectus have P. bicincta-like coloration (Figure 1). Notably, only 10 km separate monomorphic black and monomorphic lined P. ignipectus populations in western Maine, with little evidence of a hybrid zone and no obvious physical barriers to mixing across the boundary (Thompson & Carvalho, 2016). This morphological contact zone has persisted for at least 90 years. About 45 km southwest of this abrupt transition between aposematic color forms, three other P. ignipectus populations were found to be polymorphic with both black and lined forms—these populations are surrounded by monomorphic black populations. It has been hypothesized that Wolbachia may contribute to host RI and to preservation of the sharp Maine morphological contact zone (Thompson & Carvalho, 2016).

Here, we use collections of P. ignipectus from several sites in the northeastern United States across two years, in combination with collections of P. bicincta from Hawaii and Florida, United States, to assess modes of Wolbachia acquisition and to test for Wolbachia frequency variation through space and time. By sampling monomorphic
black and lined populations and typing both Wolbachia and mtDNA haplotypes, we also test for contributions of Wolbachia to the P. ignipectus morphological contact zone. Finally, we generate whole genome Wolbachia data for phylogenetic analysis and to search for loci associated with inducing and rescuing CI (Beckmann et al., 2017; LePage et al., 2017; Shropshire et al., 2018). While we cannot currently test P. ignipectus for CI in the laboratory, CI-causing Wolbachia are predicted to occur at high infection frequencies and to have specific loci associated with CI in their genomes.

2 | METHODS

2.1 | Sampling

We netted specimens from Little bluestem; sorted them by species, sex, and color form; and preserved them in 95% ethanol. The 2019 specimens (N = 4 sites) were collected on August 23. The 2020 specimens (N = 9 sites) were collected on August 9 (Silver Lake, NH), August 17 (Wonalancet, NH), and August 20 (all Maine localities) (Table S1). Collection sites were on the verges of public rights of way or privately owned land. In two cases (New Vineyard and New Portland), they correspond to sites reported in Thompson and Carvalho (2016). Specimens were collected near the height of abundance for P. ignipectus, which starts to emerge in adult form in late July and early August. We also sampled three additional spittlebug species at these sites: Lepronia quadrangularis (Say) (N = 25), Philaenus spumarius (L.) (N = 5), and Philaenarchys killa (Hamilton) (N = 24), all of the family Aphrophoridae. Like, P. ignipectus, P. killa is monophagous on Little bluestem. L. quadrangularis is a polyphage but often abundant on Little bluestem. P. spumarius is an extreme polyphage, with a preference for forbs (herbaceous perennial dicots) but is occasionally collected from Little bluestem in the company of P. ignipectus. By screening them for Wolbachia, we tested for the possibility of horizontal Wolbachia transfer through plant interactions (Chrostek et al., 2017). Lastly, because identification of infections in sister hosts enables formal analysis of modes of Wolbachia acquisition (Conner et al., 2017; Cooper et al., 2019; Raychoudhury et al., 2009; Turelli et al., 2018), we also obtained samples of the sister species P. bicincta from Hawaii (N = 60) and Florida (N = 40) to screen for infections. P. bicincta is native to the southeastern United States (Fagan & Kuitert, 1969; Thompson & Carvalho, 2016), but has recently been introduced into the Kona Region of Big Island, Hawaii (Thorne et al., 2018).

2.2 | Wolbachia typing

We generated whole-genome Wolbachia data to type the Wolbachia infecting P. ignipectus and to search for loci associated with CI. We extracted 800ng of high molecular weight DNA (Qiagen Genomic-tip 20/G; Qiagen, Germany) from one black New Vineyard female (see below), and then input and sequenced it (Ligation Sequencing Kit, SQK-LSK109; FLO-MIN106 flow cell) for 48 hr (Oxford Nanopore Technologies). We mapped raw nanopore reads (5.8Gb of data) to all known Wolbachia sequences (NCBI taxid 953) with BLASTn and extracted reads where at least 60% of their length mapped (qcovs ≥ 60). We then corrected and assembled reads using canu 2.1.1 (Koren et al., 2017, 2018; Nurk et al., 2020) and polished the Wolbachia assembly using nanopolish 0.13.2 (Loman et al., 2015). We annotated our Wolbachia assembly plus the genomes of model group-A (wMel, Wu et al., 2004; and wRi, Klasseon et al., 2009) and group-B (wPip-Pel, Klasson et al., 2008; and wMau, Meaney et al., 2019) strains using Prokka v1.11 (Seemann, 2014). We used only genes present in single copy and with identical lengths in all genomes. To assess the quality of our assembly, we excluded wPig and repeated this with only wMel, wRi, wPip, and wMau.

Preliminary analysis of a few loci placed the P. ignipectus Wolbachia in group-B (see below), but we performed Bayesian analyses using the GTR + I + M model for sequence evolution using whole-genome data to confirm this (Höhna et al., 2016). Genes were concatenated and partitioned by codon position, with a rate multiplier, σ, assigned to each partition to accommodate variable substitution rates. We used flat, symmetrical Dirichlet priors on the stationary base frequencies, π, and the relative rate parameters, η, of
the GTR model (i.e., Dirichlet(1,1,1...)). As in Turelli et al. (2018), we used a $\Gamma(2,1)$ hyperprior on the shape parameter, $\alpha$, of the discrete-$\Gamma$ model (adopting the conventional assumption that the $\beta$ rate parameter equals $\alpha$, so that the mean rate is 1 (Yang, 1994). The $\Gamma$ model for rate variation assigns significant probability near zero when the $\alpha < 1$ (accommodating invariant sites). The $\Gamma(2,1)$ hyperprior on $\alpha$ assigns 95% probability to the interval (0.36, 4.74), allowing for small and large values. Four independent runs for each gene set produced concordant topologies. We diagnosed MCMC performance using Tracer 1.7 (Rambaut et al., 2014).

2.3 | Wolbachia and mtDNA haplotyping of black and lined color morphs

To confirm that the same Wolbachia strain infects different P. ignipectus populations and color morphs, we amplified and Sanger sequenced five protein-coding Wolbachia genes (coxA, hcpA, fbpA, ftsZ, and wsp) in both directions (Eurofins Genomics LLC, Louisville, Kentucky)(see below, Table S2). We also amplified and Sanger sequenced gatb but sequence quality was consistently too low to include in our analyses. Samples included one infected female of each color form (black or lined), from each of the four populations (Carthage, New Portland, New Vineyard, and Strong) sampled in both years (Table S1).

To specifically assess whether Wolbachia might contribute to the morphological contact zone between New Vineyard (monomorphic black) and New Portland (monomorphic lined) P. ignipectus, we amplified and Sanger sequenced the cytochrome Oxidase I (Col) mitochondrial locus from one male and one female from these populations, with the exception of one (New Vineyard black male) that did not produce a usable sequence. We also produced Col sequences for one black and one lined female from the polymorphic Strong population. Covariance of Wolbachia and mtDNA haplotypes with P. ignipectus color forms would support a potential role for Wolbachia in maintaining the morphological contact zone.

We visually inspected each sequence for quality and ambiguities, and consensus sequences were used as queries for a BLASTn search and the NCBI “nr” database to confirm that orthologous genes were amplified (Altschul et al., 1990). We then used the “multiple locus query” function of the multi locus sequence typing (MLST) database to type Wolbachia (Baldo et al., 2006). Together, these data enable us to test for differentiation in Wolbachia and mtDNA between populations and color forms, including between populations monomorphic for different color forms separated by only 10 km in Maine.

2.4 | Analysis of Cl loci

Recent work has identified Cl-causing factors (cifs) associated with WO prophage in Wolbachia genomes (Beckmann et al., 2017; LePage et al., 2017; Shropshire & Bordenstein, 2019; Shropshire et al., 2020; Shropshire et al., 2018). Two genes (cifA/B) transgenically expressed in male D. melanogaster induce CI, while one gene (cifA) expressed in females rescues it. To identify cif loci, we used BLASTn to search for cif homologs in our whole-genome raw reads, querying the Type 1 cif pair in wMel, the Type 2 pair in wRi, the Type 3 pair in wNo, the Type 4 pair in wPip, and the Type 5 pair in wStri (Bing et al., 2020; Lindsey et al., 2018; Martinez et al., 2020). We later broadened our search for Type 1 pairs by querying wPip and wNPa pairs (Gerth & Bleidorn, 2017; Klasson et al., 2008). For each Type, we extracted raw reads that covered at least 40% of the genes. We then corrected and assembled the reads with canu 2.1.1 (Koren et al., 2017, 2018; Lindsey et al., 2018; Martinez et al., 2020). We later broadened our search for Type 1 pairs by querying wPip and wNPa pairs (Gerth & Bleidorn, 2017; Klasson et al., 2008). For each Type, we extracted raw reads that covered at least 40% of the genes. We then corrected and assembled the reads with canu 2.1.1 (Koren et al., 2017, 2018; Nurk et al., 2020), producing sequences with about a 1% error rate. We limit our analyses to the discovery of cif types, since we did not generate additional sequence data to further correct the long reads. The assembled genes were compared to those in Martinez et al. (2020).

2.5 | Analysis of Wolbachia frequency variation

To test for Wolbachia frequency variation, we extracted DNA from many individuals from each collection using a standard squish buffer protocol and identified Wolbachia infections using polymerase chain reaction (PCR) (Simpliamp ThermoCycler; Applied Biosystems, Singapore) (Meany et al., 2019). We amplified the Wolbachia surface protein (wsp) (Braig et al., 1998) and arthropod-specific 28S rDNA, which served as a positive control (Baldo et al., 2006) (Table S2). PCR products were visualized using 1% agarose gels. Assuming a binomial distribution, we estimated exact 95% confidence intervals for Wolbachia frequencies for each collection. We used Fisher’s exact test (FET) to determine differences in frequencies among sites, between years, between sexes, and between color forms.

3 | RESULTS

3.1 | Prosapia ignipectus likely acquired its group-B Wolbachia following initial divergence from P. bicincta

Across all samples, Wolbachia infection frequency ($p$) in P. ignipectus is high ($p = 0.93 [0.90, 0.95]$; $N = 486$). Based on five Sanger sequenced loci, the multiple sequence query of the MLST database supports that a group-B strain, most closely related to Wolbachia in Chloropidae (Diptera) (ID 93, ST 104), infects our P. ignipectus samples—we call this strain wPig. Preliminary phylogenetic analyses using only our five Sanger sequenced genes also placed wPig in group B. Our draft wPig assembly size (1.32Mb, N50 = 91,011) falls in the range of complete Wolbachia genomes (e.g., wMel at 1.26 Mb and wRi at 1.44 Mb), despite its fragmentation (50 contigs). In total, we extracted 65 single-copy homologs of equal length (43,473 total bp) for our phylogenetic analysis, which also places wPig in group B (Figure 2). When excluding the wPig genome, we were able to
extract an additional 135 homologs (167,241 bp) from wMel, wRi, wPip, and wMau. This indicates that significant residual error in the wPig assembly reduces the number of homologs meeting our equal length criteria for inclusion. Finer placement of wPig among group-B strains will require the generation of short-read data to further correct our draft wPig assembly. Thus, we do not attempt to place wPig precisely among group-B strains.

None of the *P. bicincta* samples from Hawaii and Florida were *Wolbachia* infected. Even if some *P. bicincta* are *Wolbachia* infected, as previously reported for one individual used as a PCR control in another study (Anderson et al., 2019), *Wolbachia* infection frequency \(p\) must be very low across the *P. bicincta* range, given our species estimate and credible interval \(p = 0.0 [0.0, 0.04]; N = 100\), keeping in mind the possibility that the Hawaiian population may have experienced a recent bottleneck during introduction and may not be representative of the species in the native range. Very low frequency *Wolbachia* infections in global *P. bicincta* populations, in combination with generally high wPig frequencies in *P. ignipectus*, indicate that *P. ignipectus* likely acquired wPig after its initial divergence from *P. bicincta*, although it is also possible that *P. bicincta* lost its resident *Wolbachia* following cladogenic acquisition. Because testing predictions about modes of *Wolbachia* acquisition requires formal analysis of *Wolbachia*, host nuclear, and host mtDNA phylogenics and chronograms, we are unable to distinguish between introgressive and horizontal wPig transfer (Conner et al., 2017; Cooper et al., 2019; Gerth & Bleidorn, 2017; Raychoudhury et al., 2009; Turelli et al., 2018). We discuss this further below.

Of the additional species we netted from Little bluestem, all *L. quadrangularis* were uninfected \(p = 0.0 [0.0, 0.14]; N = 25\), all *P. spumarius* were infected \(p = 1.0 [0.48, 1.0]; N = 5\), and only one *P. killa* individual was infected \(p = 0.04 [0.001, 0.21]; N = 24\). Because *Wolbachia* that infect *P. spumarius* and wPig in *P. ignipectus* are both at high frequency, we also typed the *Wolbachia* infecting *P. spumarius* to determine whether a wPig-like variant infects this host species. The multiple sequence query in the MLST database supports that a different group-B strain, most closely related to the thrp species *Aptinothrips rufus* (ID 1945, ST 509) infects *P. spumarius*. Generating more sequence data will be required to resolve the phylogenetic relationships of these and other group-B strains, including *Wolbachia* in *P. spumarius* (Li et al., 2015).

### 3.2 No apparent effect of wPig on the maintenance of the morphological *P. ignipectus* contact zone

The Strong, Carthage, and Dixfield *P. ignipectus* populations (Figure 3) were polymorphic for the black and lined forms (Figure 1, Table S1), like three populations close to Rumford, Maine sampled in earlier work (Thompson & Carvalho, 2016). This set of mixed color form populations runs roughly from Rumford northeast to Strong, but not to the sharp boundary dividing the monomorphic black New Vineyard population from the monomorphic lined New Portland population. It has the appearance of a hybrid zone, but one that does not reach the definitive boundary between the forms. The existence of distinct color forms both within and between the populations sampled facilitated investigation of the relationship, if any, between *Wolbachia* infection and patterns of color form occurrence.

We found no evidence for wPig genetic differentiation between *P. ignipectus* populations or color forms. Regions of the five wPig genes we sequenced were identical, except for a single nucleotide position in wsp, where the Strong lined sample differed from all others. In addition to populations sharing wPig type based on MLST loci, wPig frequency did not vary between color forms (black: \(p = 0.93 [0.90, 0.95], N = 338\); lined: \(p = 0.92 [0.86, 0.96], N = 123\); FET, \(P = 0.69\)), between color forms for males only (black: \(p = 0.84 [0.75, 0.90], N = 98\); lined: \(p = 0.90 [0.79, 0.97], N = 51\); FET, \(P = 0.33\)), or among females (black: \(p = 0.97 [0.94, 0.99], N = 55\); FET, \(P = 0.27\))

![Figure 2](image-url)
wPig frequency varies through space and time. Circle size denotes sample size, with outline and fill color denoting sampling year and infection status, respectively. Sample means and 95% binomial confidence intervals are reported for each sample. The dashed back line denotes the geographical separation of monomorphic black and monomorphic lined Prosapia ignipectus populations.

\[ N = 240; \text{lined: } p = 0.93 \pm 0.08, N = 72; \text{FET, } p = 0.19, \] across all samples. wPig frequency also did not differ between New Vineyard (monomorphic black) and New Portland (monomorphic lined) populations (FET, \( p = 0.16 \)).

We found no evidence for differentiation in Col mtDNA haplotype between the New Vineyard and New Portland *P. ignipectus* populations, where all samples were identical across the 680 bp that we recovered. The black and lined females from the polymorphic Strong population also did not differ from each other, or from other populations, across this region. Thus, wPig and mtDNA haplotypes were not differentiated between populations or color forms.

Our mtDNA haplotypes are also very similar to ten *P. ignipectus* samples included in the Barcode of Life Database (BOLD) (Footitt et al., 2014). A single base-pair insertion present in all of our samples is absent from all ten BOLD samples. Four other sites in Col that are polymorphic among the BOLD samples are fixed in our samples for one of the BOLD alleles. mtDNA haplotypes of *P. ignipectus* and *P. bicincta* also differ by <2% (Footitt et al., 2014).

### 3.3 The wPig genome contains three divergent types of Cl loci

We identified Type 1, 3, and 4 *cifs* in the wPig genome (Martinez et al., 2020). This specific complement of *cifs* is not found in any other published *Wolbachia* genomes, but close relatives to each wPig *cif* Type are. For instance, the wPig Type 1 genes are 99% identical to those in the genome of the *Wolbachia* infecting the gall-inducing wasp *Diplolepis spinosa* (Cynipidae), but less than 90% similar to any others (Martinez et al., 2020). The Type 3 wPig genes are 99% identical to those in the genome of the *Wolbachia* infecting *D. spinosa*, the Staphylinid beetle *Diploeciton nevermanni*, and the water strider *Gerris buenoi*. The wPig Type 4 genes are 99% identical to those in *Wolbachia* infecting *Nomada* bees (wNLeu, wNFla, and wNPa), but less than 95% identical to other Type 4 *cifs*. The *Wolbachia* infecting *D. spinosa* does not have Type 4 *cifs*, distinguishing it from wPig. None of the wPig *cifs* are truncated relative to copies with 99% identity. Additional sequencing is required to make more detailed *cif* comparisons.

### 3.4 Pervasive wPig frequency variation

wPig varied in frequency in several ways. First, frequency varied spatially among all samples (FET, \( p = 0.001 \)) (Table 1), among sites in 2019 (FET, \( p < 0.0001 \)), and 2020 (FET, \( p = 0.033 \)). This variation occurred over a geographic radius of only 20 km in 2019 and 70 km in 2020 (Figure 3). Second, frequency varied across all samples between 2019 (\( p = 0.88 \pm 0.02, N = 169 \)) and 2020 (\( p = 0.95 \pm 0.07; N = 317 \) (FET, \( p = 0.003 \)). For the four sites we sampled in both years, frequencies were only significantly different between 2019 (\( p = 0.73 \pm 0.06; N = 37 \)) and 2020 (\( p = 1.0 \pm 0.01; N = 40 \)) in New Vineyard (FET, \( p < 0.001 \)). Third, across all samples wPig frequency was higher in females (\( p = 0.95 \pm 0.07 \); \( N = 332 \)) than males (\( p = 0.86 \pm 0.09; N = 154 \) (FET, \( p = 0.001 \)). However, this was driven mostly by a paucity of infected males in New Vineyard (males: \( p = 0.69 \pm 0.03, N = 32 \); females: \( p = 1.0 \pm 0.01, N = 45 \) (FET, \( p < 0.0001 \)), with no differences in wPig frequency between males and females in other populations. wPig frequency in males was relatively low in 2019 (\( p = 0.17 \pm 0.03, N = 12 \)), but fixed in 2020 (\( p = 1.0 \pm 0.01, N = 20 \)). We interpret these results as pervasive spatial, and rare temporal and sex-specific, variation in wPig frequency.
4 | DISCUSSION

Our results suggest that wPig is a group-B Wolbachia acquired after the initial divergence of *P. ignipectus* from *P. bicincta*. Analysis of Wolbachia and mtDNA haplotypes indicates that wPig has no apparent effect on the *P. ignipectus* morphological contact zone in Maine. Across all samples, wPig occurs at very high frequencies, consistent with our discovery of three divergent sets of CI loci in the wPig genome. Finally, we document pervasive spatial, and rare temporal, wPig frequency variation. We discuss this in more detail below.

4.1 | Wolbachia acquisition in spittlebugs

In contrast to very high wPig frequencies in *P. ignipectus*, we found no evidence of Wolbachia in our sample of 100 *P. bicincta*. A prior report of one infected *P. bicincta* sample indicates that Wolbachia could infect this species (Anderson et al., 2019). If so, it must be at very low frequencies, given our credible interval here ($p = 0.0 [0.0, 0.04]$; $N = 100$). Mathematical models predict that intense CI drives Wolbachia to high frequencies, balanced by imperfect maternal transmission (Hoffmann et al., 1990; Turelli & Hoffmann, 1995); conversely, Wolbachia that do not cause strong CI tend to occur at much lower frequencies (Cooper et al., 2017; Hague, Mavengere, et al., 2020; Hamm et al., 2014; Kriesner et al., 2016). While crossing to test for CI in the laboratory is not currently feasible in this system, the presence of three sets of CI loci in the wPig genome, combined with its very high frequencies, suggests that wPig causes intense CI.

How did *P. ignipectus* acquire wPig? There are three possibilities: cladogenic transmission from its most recent common ancestor with its sister species, presumably *P. bicincta* or a close relative; by introgression from *P. bicincta* or another close relative; or by horizontal transmission (O'Neill et al., 1992). Given that we find no evidence for a high frequency Wolbachia in *P. bicincta*, cladogenic acquisition seems implausible, although we cannot fully rule it out. Without more extensive analysis of close relatives, we also cannot rule out introgression. However, opportunities for introgression with species other than *P. bicincta* have likely been limited. Other species of the genus *Prosapia* or family Cercopidae occur no further north than the US–Mexico border region, about 1,400 km from the nearest *P. ignipectus* populations and 3,000 km from the populations studied here.

Overall, the limited data are consistent with relatively recent noncladogenic transmission, a process that seems to be common among *Drosophila* species (Turelli et al., 2018). It may also be common among spittlebugs. This would be in stark contrast to obligate transovarial endosymbionts associated with amino acid nutrition in spittlebugs and other hemipterans (Koga et al., 2013).

In addition to the thrip-related *Wolbachia* found in *P. spumarius* in this study, Nakabachi et al. (2020) report that two spittlebug species, *Aphrophora quadrinodata* Say and *Philaeus maghresignus* Drosopoulous & Remane (both *Aphrophoridae*), harbor Wolbachia with 16S rRNA sequence that is identical to Wolbachia in two psyllid species, two whiteflies, an aphid, a planthopper, two leafhoppers, two grasshoppers, a mosquito, and a weevil. Likewise, Lis et al. (2015) report that Wolbachia they studied in *P. spumarius* is closely related to strains in vespids, drosophilids, whiteflies, chrysomelid beetles, and weevils based on five MLST loci. Kapantaidaki et al. (2021) also report *Wolbachia* infections at low levels in *P. spumarius*, as well as higher frequencies in *Neopilina campestris* (Fallén) (*Aphrophoridae*). Based on five MLST loci, their *N. campestris* strain is closely related to *Wolbachia* found in a leafhopper (Hemiptera) and cluster with *Wolbachia* from a planthopper, a scale insect and a psyllid (all Hemiptera), as well as two chrysomelid beetles, two butterflies, a parasitic wasp, and a mosquito. Koga et al. (2013, Table S2) report the presence of *Wolbachia* in the spittlebug *Cosmoscarta heros* (F.) (*Cercopidae*), in addition to *A. quadrinodata* and *P. maghresignus*.

In contrast, five specimens of *Poophilus costalis* (Walker) (*Aphrophoridae*) (Wiwatanaratananabutra, 2015), six specimens of *Philaeus tesselatus* Melichar (Lis et al., 2015), 37 specimens of *Philaeus signatus* Melichar (Kapantaidaki et al., 2021; Lis et al., 2015), and single specimens of *Philaeus arsali* Abdul-Nour & Lahoud, *Philaeus loukasi* Drosopoulous & Asche, and *Philaeus tarifa* Remane

| Site            | GPS coordinates            | N | Infected | $p$ [Confidence Interval] |
|-----------------|----------------------------|---|----------|---------------------------|
| Carthage        | 44 36 44N, 70 28 10W       | 116| 98       | 0.84 [0.77, 0.91]         |
| New Portland    | 44 52 17N, 70 07 00W       | 72 | 68       | 0.94 [0.86, 0.98]         |
| New Vineyard    | 44 45 14N, 70 08 01W       | 77 | 67       | 0.87 [0.77, 0.94]         |
| Strong          | 44 47 08N, 70 13 42W       | 69 | 68       | 0.99 [0.92, 1.0]          |
| Silver Lake     | 43 53 01N, 71 10 41W       | 20 | 19       | 0.95 [0.75, 1.0]          |
| Dixfield        | 44 34 10N, 70 27 21W       | 41 | 41       | 1.0 [0.91, 1.0]           |
| Weld            | 44 41 27N, 70 25 30W       | 33 | 32       | 0.97 [0.84, 1.0]          |
| Wilton          | 44 37 58N, 70 18 10W       | 26 | 26       | 1.0 [0.87, 1.0]           |
| Wonalancet      | 43 54 38N, 71 21 29W       | 32 | 31       | 0.97 [0.84, 1.0]          |

Note: Sample sizes (N), infection frequencies ($p$), and exact 95% binomial confidence intervals for each site.

### TABLE 1 wPig infection frequencies in *Prosapia ignipectus* at each sampled site across both years
& Drosopoulos (Lis et al., 2015) were not infected. Based on limited sequence data, the emerging pattern suggests that Wolbachia infection is widespread, but far from ubiquitous among spittlebugs, and that when it does occur, it often involves Wolbachia strains similar to those infecting distantly related insects. Whole Wolbachia and host genomic data are sorely needed to test our hypothesis that horizontal Wolbachia acquisition might be common in spittlebugs.

4.2 Little contribution of wPig to the P. ignipectus morphological contact zone

We find no evidence for differentiation in wPig or mtDNA haplotypes among P. ignipectus color forms. This includes the monomorphic black (New Vineyard) and lined (New Portland) populations that are separated by only 10 km in Maine, with no obvious barriers to dispersal or reproduction (Thompson & Carvalho, 2016). We also found no variation in wPig or mtDNA haplotypes between black and lined individuals in the polymorphic Strong population. wPig frequency also did not vary between color forms. These data indicate that wPig is unlikely to significantly contribute to the maintenance of the P. ignipectus morphological contact zone.

How common are Wolbachia effects on host RI? Obligate Wolbachia infections in co-occurring D. paulistorum semispecies contribute to assortative mating and generate hybrid inviability and male sterility (Miller et al., 2010). Wolbachia also contribute to reinforcement between Wolbachia-infected D. recens and uninfected D. subquinaria (Jaenike et al., 2006; Shoemaker et al., 1999). In contrast, Wolbachia do not contribute to premating, gametic, or postzygotic RI among the three D. yakuba-clade host species (Cooper et al., 2017). While the crossing schemes used in these Drosophila studies to dissect Wolbachia contributions to RI are not feasible in P. ignipectus and many other systems, our genetic data here lend support to our prior conjecture that Wolbachia contributions to RI observed in some Drosophila may be the exception rather than the rule (Cooper et al., 2017; Turelli et al., 2014).

4.3 Pervasive wPig frequency variation

Mathematical models indicate that imperfect maternal transmission, Wolbachia fitness effects, and the severity of CI govern Wolbachia frequencies in host populations. Wolbachia that cause intense CI tend to occur at high and stable frequencies, balanced by imperfect maternal transmission (Barton & Turelli, 2011; Carrington et al., 2011; Hoffmann et al., 1990; Kriesner et al., 2013; Turelli & Hoffmann, 1991, 1995), while Wolbachia that cause weak or no CI tend to persist at intermediate, often variable frequencies (Cooper et al., 2017; Hague, Mavengere, et al., 2020; Hamm et al., 2014; Kriesner et al., 2016). Accumulating evidence for variable infection frequencies (Cooper et al., 2017; Hamm et al., 2014; Hughes, Allsopp, et al., 2011; Hughes, Ren, et al., 2011; Kriesner et al., 2016; Lis et al., 2015; Schuler et al., 2016), including our discovery here, highlights that infection frequencies are not static, even for high-frequency variants.

With the exception of model systems like wRi in D. simulans, few estimates of the key parameters required to approximate population frequency dynamics and equilibria of Wolbachia exist (Carrington et al., 2011; Turelli & Hoffmann, 1995). wMel-like Wolbachia frequencies in the D. yakuba clade vary through space and time in west Africa (Cooper et al., 2017), due in part to effects of cold temperatures on wYak titer (Hague, Mavengere, et al., 2020). CI strength also varies in the D. yakuba clade, which may influence infection frequencies (Cooper et al., 2017; Hague, Caldwell, et al., 2020). wMel frequencies vary with latitude in D. melanogaster populations, potentially due to wMel fitness costs in the cold (Kriesner et al., 2016). Interestingly, hot temperatures reduce wMel CI strength and transmission in transfected Aedes aegypti used for biocoroll of human disease (Ross et al., 2017, 2020), suggesting that temperature may generally influence key parameters underlying Wolbachia infection frequencies.

What underlies variable wPig frequencies in nature? High wPig frequencies and the presence of three divergent sets of cifs suggest, but do not confirm, that wPig causes strong CI. It seems plausible that some or all of these loci were horizontally acquired (Cooper et al., 2019), but additional sequence data are required to test this. We hypothesize that variable wPig transmission rates contribute to the frequency variation we observe, potentially due to environmental effects on titer, as observed for wYak (Hague, Mavengere, et al., 2020). Temporal variation in transmission was also observed for wRi between two samples of D. simulans collected from Ivanhoe, California, in April and November of 1993 (Carrington et al., 2011; Turelli & Hoffmann, 1995), although the relative stability of wRi frequencies in global D. simulans populations suggests that its transmission persists across a range of environmental conditions. Additional analyses of Wolbachia titer and transmission in the field, and across environmental contexts, are needed to better understand the causes of Wolbachia frequency variation. Comparing the titer and transmission of Wolbachia that occur at different frequencies in nature—for example, those that do and do not cause intense CI—would be particularly useful.

ACKNOWLEDGMENTS

We thank M. Thorne and A.G. Dale for Prosapia bicincta collections and D. McVicar and F. Selchin for access to the Wonaloncet site. Michael Turelli provided comments that greatly improved an earlier draft. We also thank M. Hague, D. Shropshire, and K. Van Vaerenbergh for very helpful comments. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health (NIH) under award number R35GM124701 to B.S.C. and by the University of Montana Genomics Core.

CONFLICT OF INTEREST

We declare no conflicts of interests.
AUTHOR CONTRIBUTIONS

Timothy B. Wheeler: Data curation (supporting); Investigation (lead); Validation (supporting); Visualization (lead); Writing-original draft (supporting); Writing-review & editing (supporting).

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William R. Conner: Data curation (supporting); Formal analysis (equal); Investigation (equal); Writing-original draft (supporting); Writing-review & editing (supporting).

Brandon Cooper: Conceptualization (equal); Data curation (supporting); Formal analysis (equal); Funding acquisition (lead); Investigation (equal); Methodology (equal); Project administration (supporting); Resources (equal); Supervision (lead); Validation (equal); Visualization (supporting); Writing-original draft (lead); Writing-review & editing (equal).

DATA AVAILABILITY STATEMENT

The Wolbachia infection frequency data, assemblies, and scripts are archived on DRYAD (https://doi.org/10.25338/b8ms7n). The base-called Nanopore reads (SRR14328167), Wolbachia MLST sequences (MZ291996–MZ292028), and mitochondrial DNA sequences (MZ254780–MZ254784) are available a NCBI.

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**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Wheeler, T. B., Thompson V., Conner W. R., & Cooper B. S. (2021). *Wolbachia* in the spittlebug *Prosapia ignipectus*: Variable infection frequencies, but no apparent effect on host reproductive isolation. *Ecology and Evolution, 11*, 10054–10065. https://doi.org/10.1002/ece3.7782