The impacts of cytoplasmic incompatibility factor (cifA and cifB) genetic variation on phenotypes

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Abstract

Wolbachia are maternally transmitted, intracellular bacteria that can often selfishly spread through arthropod populations via cytoplasmic incompatibility (CI). CI manifests as embryonic death when males expressing prophage WO genes cifA and cifB mate with uninfected females or females harboring an incompatible Wolbachia strain. Females with a compatible cifA-expressing strain rescue CI. Thus, cif-mediated CI confers a relative fitness advantage to females transmitting Wolbachia. However, whether cif sequence variation underpins incompatibilities between Wolbachia strains and variation in CI penetrance remains unknown. Here, we engineer Drosophila melanogaster to transgenically express cognate and non-cognate cif homologs and assess their CI and rescue capability. Cognate expression revealed that cifA;B native to D. melanogaster causes strong CI, and cognate cifA;B homologs from two other Drosophila-associated Wolbachia cause weak transgenic CI, including the first demonstration of phylogenetic type 2 cifA;B CI. Intriguingly, non-cognate expression of cifA and cifB alleles from different strains revealed that cifA homologs generally contribute to strong transgenic CI and interchangeable rescue despite their evolutionary divergence, and cifB genetic divergence contributes to weak or no transgenic CI. Finally, we find that a type 1 cifA can rescue CI caused by a genetically divergent type 2 cifA;B in a manner consistent with unidirectional incompatibility. By genetically dissecting individual CI functions for type 1 and 2 cifA and cifB, this work illuminates new relationships between cif genotype and CI phenotype. We discuss the relevance of these findings to CI’s genetic basis, phenotypic variation patterns, and mechanism.

Keywords: cytoplasmic incompatibility; Wolbachia; phage WO; Drosophila melanogaster; Two-by-one model

Introduction

Wolbachia are intracellular bacteria that occur in 40–65% of arthropod species (Hilgenboecker et al. 2008; Zug and Hammerstein 2012; Weinert et al. 2015; Charlesworth et al. 2019). While often horizontally transmitted between species (Boyle et al. 1993; Huigens et al. 2004; Gerth et al. 2013; Tolley et al. 2019; Scholz et al. 2020), vertical transmission from mother to offspring predominates within species (Turelli and Hoffmann 1991; Narita et al. 2009). Wolbachia can frequently increase their rate of spread in host populations through the matriline by causing cytoplasmic incompatibility (CI). CI results in embryonic death of uninfected embryos after fertilization by Wolbachia-modified sperm (Yen and Barr 1973; Shropshire et al. 2020b). Embryos with compatible Wolbachia are rescued from CI-induced lethality, yielding a relative fitness advantage to Wolbachia-infected females that transmit the bacteria to their offspring (Hoffmann et al. 1990; Turelli 1994; Turelli and Hoffmann 1995). CI frequently manifests between arthropods infected with different Wolbachia strains. Strains may be reciprocally incompatible (bidirectional CI), or only one of the two strains can rescue the other’s sperm modification (unidirectional CI). CI-inducing Wolbachia have garnered attention for their role in suppressing vector populations (Lees et al. 2015; Nikolouli et al. 2018; Crawford et al. 2020), curbing the transmission of pathogenic RNA viruses (O’Neill 2018; Moretti et al. 2018; Gong et al. 2020), and reproductive isolation and incipient speciation (Bordenstein et al. 2001; Jaenike et al. 2006; Brucker and Bordenstein 2012; Shropshire and Bordenstein 2016).

Two adjacent genes in the eukaryotic association module of Wolbachia’s prophage WO cause CI when expressed in males (cifA and cifB) (Bordenstein and Bordenstein 2016; Beckmann et al. 2017; LePage et al. 2017; Chen et al. 2019; Shropshire and Bordenstein 2019), and one of the same genes rescues CI when expressed in females (cifA) (Shropshire et al. 2018; Chen et al. 2019; Shropshire and Bordenstein 2019). These results established the Two-by-One genetic model of CI (Figure 1A) (Shropshire and Bordenstein 2019), but its generality across cif homologs remains to be evaluated. Singly expressing a small set of cifA variants that have only annotated domains or cifB variants that exhibit in vitro deubiquitilase and nuclease activities also does not cause rescuable embryonic death (Beckmann et al. 2017; LePage et al. 2017; Chen et al. 2019; Shropshire and Bordenstein 2019). Cif proteins segregate into at least five

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phylogenetic clades (types 1–5) (LePage et al. 2017; Lindsey et al. 2018; Bing et al. 2020; Martinez et al. 2020), and distant Cif-like homologs are found in Orientia and Rickettsia bacteria, which are not known to cause CI (Gillespie et al. 2018). To date, the genetic basis for CI (Figure 1A) has been tested using cif transgenes from the types 1 and 4 clades in wMel Wolbachia of Drosophila melanogaster and wPip Wolbachia of Culex pipiens mosquitoes (Beckmann et al. 2017; Chen et al. 2019; Shropshire and Bordenstein 2019). Thus, a considerable amount of phylogenetic diversity remains untested.

Moreover, while the genetic basis of CI between infected and uninfected insects is resolved for some strains, the genetic basis of unidirectional or bidirectional CI between insects harboring different Wolbachia strains remains largely unknown. Phylogenetic and sequence analyses of cif genes from incompatibility Wolbachia strains in Drosophila or Culex reveal that incompatibility Wolbachia strains differ in genetic relationship and/or copy number (Bonneau et al. 2018, 2019; LePage et al. 2017), supporting cif variation as the basis of strain incompatibilities. Moreover, since cifA is involved in both CI induction and rescue, a single-step evolutionary model for bidirectional CI has been proposed where a single mutation in cif leads to incompatibility between the ancestral and derived variants while retaining compatibility with the emergent variant and requiring cifB only as an accessory function (Shropshire et al. 2018). However, these hypotheses have not been empirically tested.

In this study, we first test cif homologs from wMel of D. melanogaster, wRec of D. recens, and wRi of D. simulans for CI and rescue when transgenically expressed in uninfected D. melanogaster. We previously determined that wMel genes adhere to the Two-by-One model (Shropshire and Bordenstein 2019), but the genetic bases of wRec and wRi CI remain unknown. wRec and wRi are strong CI inducers that cause high degrees of embryonic death (Turelli and Hoffmann 1991; Werren and Jaenike 1995; Shoemaker et al. 1999). Both encode phylogenetic type 1 cif genes similar to wMel, and wRi also encodes a type 2 cif pair that is highly diverged from wMel (Figure 1B) (LePage et al. 2017). Like wMel, we predict that wRec and wRi CI have a Two-by-One genetic basis. This is the first time type 2 cif genes have been genetically interrogated.

Next, we test the crossing relationships between divergent cif homologs to investigate the basis of interstrain incompatibilities. wRi and wMel Wolbachia are unidirectionally incompatible in a common D. simulans host background. In other words, wRi can rescue wMel-induced CI, but wMel cannot rescue wRi-induced CI (Figure 1C) (Poinsot et al. 1998). Thus, we hypothesize that wRi can rescue wMel-induced CI because it has type 1 cif genes comparable to wMel, and wMel cannot rescue wRi because it does not have genes capable of rescuing wRi’s type 2 genes (Figure 1D) (LePage et al. 2017). Moreover, since wRec’s type 1 cif genes are closely related to wMel genes, we predict them to be compatible upon transgenic expression (Figure 1C and D). We discuss our

Figure 1 Two-by-One model, Cif phylogeny, and predicted relationships between wMel, wRec, and wRi strains and cif gene variants. (A) The Two-by-One genetic basis of CI: cifA,B causes CI that can be rescued by females expressing cifA (Shropshire and Bordenstein 2019). (B) Schematic representation of the evolutionary relationships between CifA and CifB proteins from wMel, wRec, and wRi (LePage et al. 2017). (C) Incompatibilities between wMel, wRec, and wRi Wolbachia strains. Unidirectional CI between wMel and wRi is based on crossing experiments after the transinfection of wMel into D. simulans (Poinsot et al. 1998). Compatibility between wMel and wRec is based on the prediction that strains with closely related cif gene sequences are compatible. (D) Predicted incompatibility relationships between cif homologs from each of the three strains, based on sequence relationship. Lines between strains/gene(s) indicate compatibility relationships. If the line ends in an arrowhead, then the strain/gene(s) at the beginning of the arrow can rescue CI caused by the strain/gene(s) the arrow points toward. If the line ends in a circle, then rescue is not expected. Skull art is modified from vecteezy.com with permissions.
results in the context of the genetic basis of CI in these strains, the causes of CI strength variation and strain incompatibilities, and CI’s molecular basis.

Materials and Methods
Fly lines and maintenance
The following Upstream Activation Sequence (UAS) transgenic constructs were generated for this study: cif\textsubscript{A\textsubscript{wMel}[T1]}, cif\textsubscript{B\textsubscript{wMel}[T1]}, cif\textsubscript{A\textsubscript{wRi}[T1]}, cif\textsubscript{B\textsubscript{wRi}[T1,N]}, cif\textsubscript{B\textsubscript{wRi}[T1,g]}, cif\textsubscript{B\textsubscript{wRi}[T2,g]}, cif\textsubscript{B\textsubscript{wRi}[T2]}, and cif\textsubscript{B\textsubscript{wRi}[T2,R]}. Each transgene was codon-optimized for expression in D. melanogaster and synthesized by GenScript (Hong Kong, China). Valine start codons were replaced with methionine. Wild-type and codon-optimized gene sequences are reported in Supplementary Table S2. Each gene was cloned into the pTiTGER plasmid at GenScript. pTiTGER is a pUASp-based vector designed for germline expression and was previously used to generate cif\textsubscript{A\textsubscript{wMel}[T1]} and cif\textsubscript{B\textsubscript{wMel}[T1]} transgenes also used in this study (LePage et al. 2017). pTiTGER enables phiC31 integration into the D. melanogaster genome, contains a UAS promoter region intended for GAL4/UAS expression, and has a red-eye marker for screening. D. melanogaster embryo injections were conducted by Best Gene (Chino Hills, California) using phiC31 integrase to place cif\textsubscript{A} and cif\textsubscript{B} homologs into the Attp40 (chromosome 2) and Attp2 (chromosome 3) insert sites, respectively. Transformants were screened via eye color, and homozygous transgenic lines were generated for all lines. All lines were negative for Wolbachia based on PCR using Wolb\textsubscript{F} and Wolb\textsubscript{R3} primers (Casiraghi et al. 2005). Dual expressing UAS transgenic lines were generated via standard genetic crossing schemes.

In addition, the following D. melanogaster stocks were used in this study: infected and uninfected y\textsuperscript{w}\textsuperscript{+} (BDSC 1495), uninfected nos-GAL4-VP16 (BDSC 4937), and uninfected UAS transgenic lines homozygous for cif\textsubscript{A\textsubscript{wMel}[T1]}, cif\textsubscript{B\textsubscript{wMel}[T1]}, and cif\textsubscript{A,B\textsubscript{wMel}[T1]} (LePage et al. 2017). Genotypes and infection states were regularly confirmed for transgene expressing fly lines using primers listed in Supplementary Table S3. D. melanogaster stocks were maintained at 12:12 light:dark at 25°C on 50 ml of a standard media. Stocks for virgin collections were stored at 18°C overnight to slow eclosion rate, and virgin flies were kept at room temperature.

Hatch rate assays
To test for CI, hatch rate assays were conducted as previously described (LePage et al. 2017; Shropshire et al. 2018). Briefly, virgin nos-GAL4-VP16 adult females were aged 9–11 days post-eclosion, to control for the paternal grandmother age effect (Layton et al. 2019), and mated with UAS transgenic or y\textsuperscript{w}\textsuperscript{+} males. GAL4-UAS males and females were paired in 8-oz round bottom Drosophila bottles (Genesee Scientific) affixed with a grape-juice agar plate smeared with yeast affixed to the opening with tape. To control the impact of male age and the younger brother effect on CI level (Reynolds and Hoffmann 2002; Yarmada et al. 2007), only young early emerging males (0–48 h) were used in crossings. Conversely, 5–7-day-old females were used since they are highly fecund. The flies and bottles were stored at 25°C for 24 h at which time the plates were replaced with freshly smeared plates and again stored for 24 h. Plates were then removed, and the number of embryos on each plate was counted and stored at 25°C. After 30 h, the remaining unhatched embryos were counted. The percentage of embryos that hatched into larvae was calculated by dividing the number of hatched embryos by the initial embryo count and multiplying by 100.

Embryonic cytology
Flies were collected, aged, and crossed as described for hatch rate assays. However, 60 females and 12 males were included in each bottle with a grape-juice agar plate attached. Flies were siblings of those in hatch rate assays. Embryos laid in the first 24 h were discarded due to low egg-laying. During the second day, embryos were aged 1–2 h and then dechorionated, washed, and fixed in methanol as previously described (LePage et al. 2017, Shropshire et al. 2018). Embryos were stained with propidium iodide and imaged (LePage et al. 2017, Shropshire et al. 2018). Scoring of cytological defects was conducted using previously defined characteristics (LePage et al. 2017).

Sequence analyses
Sequence similarity between Cif proteins was determined using pairwise MUSCLE alignments of protein sequences using default settings. Glimmer 3 was used to identify open reading frames in cif\textsubscript{B\textsubscript{wRi}[T1]} after the early stop codon that truncates the gene. These analyses were conducted in Geneious Prime.

Statistical analyses
All statistical analyses were conducted in GraphPad Prism 8. Hatch rate statistical comparisons were made using Kruskal–Wallis followed by a Dunn’s multiple comparison test. Samples with fewer than 25 embryos laid were removed from hatch rate analyses as previously described (LePage et al. 2017). Hatch rates in main text figures display the combination of two replicate experiments, which were analyzed simultaneously, and those in the supplement display only single experiments (N = 8–58 per cross after exclusion). Replicate data were statistically comparable in all cases. Cytological abnormalities were compared using a pairwise Fisher’s exact test followed by a Bonferroni–Dunn correction test (N = 43–167 embryos per cross). Figure esthetics were edited in Affinity Designer 1.7 (Serif Europe, Nottingham, UK). All P-values are reported in Supplementary Table S1, and the exact sample sizing information is available in Supplementary File S1.

Data availability
All data are made publicly available in the supplement of this manuscript. Fly lines not otherwise available in the Bloomington Drosophila Stock Center are available upon request.

Supplemental material is available at figshare DOI: https://doi.org/10.25386/genetics.13215503.
will hereafter be referred to as the “compatible control”. All protein annotations are derived from prior works (Lindsey et al. 2018). Substitutions inside schematics represent sequence identity relative to CifA<sub>wMel[T1]</sub>. Substitutions marked with a circle above the protein schema are shared between CifA<sub>wRec[T1]</sub> and CifB<sub>wRec[T1]</sub>. “R1032*” represents an arginine to stop codon mutation. Hatch rate analyses testing (B) cifA<sub>wRec[T1]</sub>, cifB<sub>wRec[T1]</sub>, and cifA<sub>B</sub><sub>wRec[T1]</sub> for CI and rescue (N = 12–51 where each dot represents a clutch of embryos from a single mating pair), (C) cifA<sub>wRec[T1]</sub>cifB<sub>wRec[T1]</sub> for CI (N = 36–55), and (D) cifA<sub>wRec[T1]</sub>cifB<sub>wMel[T1]</sub> for CI (N = 27–58). Horizontal bars represent median embryonic hatching from single pair matings. Genotypes for each cross are illustrated below the bars where the genes expressed in each sex are represented by colored circles. Blue circles represent cifA<sub>wMel[T1]</sub> genes, and green circles represent cifB<sub>wRec[T1]</sub> genes. Each hatch rate contains the combined data of two replicate experiments, each containing all crosses shown. Asterisks above bars represent significant differences relative to a control transgenic rescue cross (denoted Ctrl) with α = 0.05. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Exact P-values are provided in Supplementary Table S1.

**Figure 2** Cif<sub>wRec[T1]</sub> protein similarity and results of transgenic crosses including Cif<sub>wRec[T1]</sub> proteins. (A) Protein architecture of Cif<sub>wMel[T1]</sub> and Cif<sub>wRec[T1]</sub> (Lindsey et al. 2018). Substitutions inside schematics represent sequence identity relative to CifA<sub>wMel[T1]</sub>. Substitutions marked with a circle above the protein schema are shared between CifA<sub>wRec[T1]</sub> and CifB<sub>wRec[T1]</sub>. “R1032*” represents an arginine to stop codon mutation. Hatch rate analyses testing (B) cifA<sub>wRec[T1]</sub>, cifB<sub>wRec[T1]</sub>, and cifA<sub>B</sub><sub>wRec[T1]</sub> for CI and rescue (N = 12–51 where each dot represents a clutch of embryos from a single mating pair), (C) cifA<sub>wRec[T1]</sub>cifB<sub>wRec[T1]</sub> for CI (N = 36–55), and (D) cifA<sub>wRec[T1]</sub>cifB<sub>wMel[T1]</sub> for CI (N = 27–58). Horizontal bars represent median embryonic hatching from single pair matings. Genotypes for each cross are illustrated below the bars where the genes expressed in each sex are represented by colored circles. Blue circles represent cifA<sub>wMel[T1]</sub> genes, and green circles represent cifB<sub>wRec[T1]</sub> genes. Each hatch rate contains the combined data of two replicate experiments, each containing all crosses shown. Asterisks above bars represent significant differences relative to a control transgenic rescue cross (denoted Ctrl) with α = 0.05. *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001. Exact P-values are provided in Supplementary Table S1.

**Do phylogenetic type 1 cif genes from wRec transgenically induce and rescue CI?**

Relative to CifA<sub>wMel[T1]</sub> proteins, CifA<sub>wRec[T1]</sub> has two amino acid substitutions in unannotated regions: one prior to CifA’s putative DUF3243 and another after the annotated STE domain (Figure 2A). CifB<sub>wRec[T1]</sub> has 13 amino acid changes that include a seven amino acid extension on the N-terminus, four substitutions in the N-terminal unannotated region, a single substitution in the first putative PD-(D/E)XK-like nuclease domain (hereafter PDDEXXK), and a stop codon that truncates amino acids at residues 1032–1173 on the C-terminus of the protein (Figure 2A). wRec causes strong CI in D. rezens (Shoemaker et al. 1999; Werren and Jaenike 1995), the wRec genome lacks other cif genes (Metcalf et al. 2014), and these variants are highly similar to cif<sub>wMel[T1]</sub> genes (Figure 2A). Thus, we predicted that cifA<sub>B</sub><sub>wRec[T1]</sub> expression in uninfected males will cause CI, transgenic cifA<sub>wRec[T1]</sub> expression in uninfected females will rescue CI, and CI induced by cifA<sub>B</sub><sub>wRec[T1]</sub> transgenes will be compatible with cifA<sub>wMel[T1]</sub> transgenes.

Consistent with prior reports in D. melanogaster, cifA<sub>B</sub><sub>wMel[T1]</sub> males induce strong CI that is rescued by the compatible control cross with cifA<sub>wMel[T1]</sub> females (Figure 2B) (Shropshire and Bordenstein 2019). cifA<sub>B</sub><sub>wRec[T1]</sub> males also cause a small but statistically significant reduction in hatching (Mdn = 75.4% hatching; P < 0.0001; Figure 2B) that is rescued by cifA<sub>wRec[T1]</sub> females but not by cifA<sub>B</sub><sub>wRec[T1]</sub> females (Mdn = 79.6% hatching; P = 0.0054). Results therefore suggest that cifA<sub>wRec[T1]</sub> is a rescue gene, weak cifA<sub>B</sub><sub>wRec[T1]</sub> CI is rescuable, and cifB<sub>wRec[T1]</sub> may reduce cifA<sub>wRec[T1]</sub> rescue capacity in embryos. Since neither cifA<sub>wMel[T1]</sub> nor cifB<sub>wMel[T1]</sub> induces CI alone (LePage et al. 2017; Shropshire and Bordenstein 2019), we predicted neither cifA<sub>wRec[T1]</sub> nor cifB<sub>wRec[T1]</sub> would reduce hatching. Indeed, cifA<sub>wRec[T1]</sub> males did not reduce hatching (P > 0.99). However, cifB<sub>wRec[T1]</sub> males caused complete embryonic death (Mdn = 0% hatching; P < 0.0001; Figure 2B) that was not rescued by cifA<sub>wRec[T1]</sub> (Mdn = 0% hatching), cifA<sub>B</sub><sub>wRec[T1]</sub> (Mdn = 0% hatching), cifB<sub>wRec[T1]</sub> (Mdn = 0% hatching), or wMel-infected (Mdn = 0% hatching) females (Figure 2B and...
Supplementary Figure S1). Embryos fertilized by cifBwRi[T1] males had an unusually high percentage of early mitotic failures and single puncta indicative of unfertilized embryos or embryos undergoing mitotic failure in the first division (Supplementary Figure S2). However, unlike cifA;BwMel[T1] males, there were no later stage regional mitotic failures or chromatin bridging phenotypes, and the cifBwRi[T1] defects were unrescuable (Supplementary Figure S2) (LePage et al. 2017). Taken together, these results indicate that cifBwRi[T1] alone causes an atypical embryonic lethality relative to cifA;BwMel[T1]-induced CI.

Next, we tested crossing relationships between cifAwMel[T1] and cifAwRi[T1] transgenic males and females. Weak cifA;BwMel[T1]-induced CI was reduced when mated with cifAwMel[T1] females (P > 0.99) relative to the compatible control. Similarly, cifA;BwMel[T1] CI was reduced when mated with cifAwRi[T1] (P > 0.99) or cifA;BwRi[T1] (P = 0.10) females (Figure 2B). However, cifA;BwRi[T1] females only partially rescue cifA;BwMel[T1] CI, and since cifA;BwRi[T1] females do not rescue cifA;BwRi[T1] CI (Figure 2B), a firm conclusion cannot be made on whether cifA;BwRi[T1] females can rescue cifA;BwMel[T1] CI. Together, these data indicate that cifA;BwRi[T1] and cifA;BwWit[T1], which differ by two amino acid substitutions in the putative DUF3243 and STE domains (Figure 2A), rescue the other strain’s transgenic CI. This is perhaps unsurprising since prior mutagenesis assays suggest conserved sites in DUF3243 and STE domains are not related to rescue (Shropshire et al. 2020a).

Finally, since cifA;BwRi[T1] males induce weak CI relative to cifA;BwMel[T1] males, we hypothesized that cifAwRi[T1] or cifAwRi[T1] sequence variation underpins CI level variation. We tested this hypothesis by engineering and expressing non-cognate combinations of cifAwRi[T1] and cifAwRi[T1] transgenes. We report that cifAwMel[T1];cifAwRi[T1] males cause a weak but statistically significant reduction in hatchability relative to the compatible control (Mdn = 77.6% hatching; P = 0.0008; Figure 2C), and this hatch rate reduction was comparable to that of cognate cifA;BwRi[T1] (Mdn = 75.4% hatching; Figure 2B) and likewise rescued when crossed to cifA;BwMel[T1] (P > 0.99) or cifA;BwRi[T1] (P > 0.99) females (Figure 2C). In contrast, cifA;BwRi[T1];cifA;BwWit[T1] males caused strong CI (Mdn = 0% hatching; P = 0.0001) that was also rescued by cifA;BwRi[T1] (Mdn = 97.1% hatching; P > 0.99) or cifA;BwWit[T1] (Mdn = 95.9% hatching; P > 0.99) females (Figure 2D). These data demonstrate that the two closely related cifA sequences are interchangeable and fully capable of CI and rescue and that sequence variation in cifB is crucially responsible for weak cifA;BwRi[T1] transgenic CI in D. melanogaster.

Do phylogenetic type 1 cif genes from wRi transgenically induce and rescue CI?

wRi has three cif gene pairs: two identical type 1 pairs and one type 2 pair (LePage et al. 2017; Lindsey et al. 2018). We first focus attention on the cifAwRi[T1] genes, their protein sequence differences, and CI phenotype variation. Relative to CifAwMel[T1], CifAwRi[T1] protein has five amino acid substitutions in unannotated regions flanking the predicted domains (Figure 3A). One of these CifA substitutions is also present in CifAwRi[T1]; CifAwRi[T1] has an in-frame stop codon introduced at residue 213 in the 1173-amino-acid-long protein (Figure 3A), and Glimmer 3 predicted that another protein coding sequence begins at residue 229 in the protein. Thus, cifAwRi[T1] may yield two proteins: an N-terminal 212 amino acid protein and a C-terminal 945 amino acid protein. We refer to the gene sequences yielding the N-terminal and C-terminal peptides as cifBuRi[T1,N] and cifBuRi[T1,C], respectively. Relative to CifBuWit[T1], CifBuRi[T1,N] has two amino acid substitutions, a seven amino acid N-terminal extension, and an early stop codon. In this region, CifBuRi[T1] has the same sequence variations, excluding the early stop codon in addition to one extra substitution. CifBuRi[T1] has three substitutions relative to CifBuMel[T1]—the first in the PDDEXX domain, a valine to methionine substitution marking the translation start site, and one in the unannotated region prior to the first PDDEXX domain (Figure 3A). In this C-terminal region, CifBuWit[T1] shares all but the valine to methionine substitution. To investigate the genetic basis of wRi CI, we generated cifAwRi[T1]; cifBuRi[T1,N], and cifBuRi[T1,C] transgenes. We additionally created a polycistronic cifBuRi[T1] transgene that expressed both the N-terminal and C-terminal peptides from a single transcript using a T2A sequence between the two proteins (Donnelly et al. 2001a,b). We refer to this polycistronic transgenic construct as cifBuRi[T1,poly].

We first tested cifA;BwRi[T1,poly] males for their ability to induce CI and found that they did not reduce hatching (P = 0.55; Figure 3B). Males dually expressing cifAwRi[T1] with either cifBuRi[T1,N] (P = 0.55; Supplementary Figure S3A) or cifBuRi[T1,C] (P = 0.32; Supplementary Figure S4A) also failed to reduce hatching, suggesting that dual expression of cifBuRi[T1] transgenes cannot recapitulate CI. In addition, singly expressing cifAwRi[T1] (P > 0.99) or cifBuRi[T1,poly] (P > 0.99) males does not cause CI (Figure 3B). Next, we test if cifBuWit[T1] genes can rescue strong cifAwRi[T1] CI. We crossed cifA;BwMel[T1] males with cifAwRi[T1] (P > 0.99) and cifA;BwRi[T1,poly] (P > 0.99) females, both of which yielded hatching levels comparable to cifAwMel[T1] rescue (Figure 3B). These results indicate that cifAwRi[T1] is a rescue gene, and cifBuRi[T1] transgenes do not cause CI when singly or dually expressed as cognate partners in D. melanogaster.

To further evaluate if cifBuRi[T1] transgenes are capable of CI and whether variation in cifA or cifB may underpin the lack of CI above, we engineered and dually expressed non-cognate pairs of cifBuRi[T1] genes with cifBuMel[T1] genes. cifAwMel[T1];cifBuRi[T1,poly] males did not yield a reduction in hatching compared to the compatible cross (P > 0.99; Figure 3C). Similarly, males dually expressing cifAwMel[T1] and either cifBuRi[T1,N] (P > 0.99; Supplementary Figure S3B) or cifBuRi[T1,C] (P > 0.99; Supplementary Figure S4B) did not reduce hatching. However, cifAwRi[T1];cifBuWit[T1] males caused near-complete embryonic death (Mdn = 0% hatching; P < 0.0001) that could be rescued by cifAwRi[T1] and cifBuWit[T1] females (Figure 3D). These findings suggest that cifBuWit[T1] contributes to both rescue and CI induction, but cifBuRi[T1] transgenes fail to contribute to CI.

Do the phylogenetic type 2 cif genes from wRi transgenically induce and rescue CI?

Pairwise alignments of CifAwMel[T1] and CifAwRi[T1] proteins (488 and 1239 amino acids for CifA and CifB, respectively) reveal major divergence. First, CifAwMel[T1] and CifAwRi[T1] differ by 267 sites (45.3% identical sites), with 221 amino acid substitutions and 46 gap sites in the alignment (Figure 4A and Supplementary Figure S5). CifAwRi[T1] has substitutions in all six of the sites that vary in CifAwRi[T1] and CifAwWit[T1], and two of the CifAwRi[T1] substitutions are shared with both proteins, and a third is shared with CifAwRi[T1]. Second, CifBuMel[T1] and CifBuRi[T1] differ by 991 sites (20% identical sites), with 433 substitutions and 558 gap sites in the alignment (Figure 4A and Supplementary Figure S5). In addition, CifBuRi[T1] has substitutions in four of the six sites that vary in CifAwRi[T1] and CifBuWit[T1], but the specific amino acids are unique to CifBuRi[T1] (Supplementary Figure S5). Moreover, while the sequence lengths of the two CifA variants are comparable, CifBuRi[T1] does not have the C-terminal Upl1 domain that, for other distant type 1 Cif variants, acts in vitro as a deubiquitinase (Beckmann et al. 2017). It also has
an eight-amino-acid N-terminal extension (Supplementary Figure S5), of which four amino acids are shared in the N-terminal extensions of CifB<sub>w</sub><sup>r</sup><sub>T1</sub> and CifB<sub>w</sub><sup>r</sup><sub>Ri</sub><sup>T1</sup>. First, we test if cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> transgenes cause and rescue CI in <i>D. melanogaster</i>. cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males caused a weak but statistically significant hatch rate reduction (Mdn = 84.4% hatching; <i>P</i> = 0.01; Figure 4B) that was rescued upon crossing with cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> females (<i>P</i> > 0.99; Figure 4B). Similar to results with cifA;B<sub>w</sub><sup>Rec</sup><sub>T1</sub> females above (Figure 2B), crossing cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males with cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> females only slightly improved hatching such that it was no longer statistically different from the compatible control (Mdn = 86.9% hatching; <i>P</i> = 0.15); however, the median hatch rate was comparable when cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males were mated to uninfected females (Mdn = 84.4% hatching; Figure 4B). Thus, similar to cifB<sub>w</sub><sup>Rec</sup><sub>T1</sub>, it can be concluded that cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males are rescue-capable yet, but cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> females clearly rescue cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> CI as expected under the Two-by-One Model. In parallel, we showed that neither cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> (<i>P</i> = 0.84) nor cifB<sub>w</sub><sup>Ri</sup><sub>T2</sub> (<i>P</i> = 0.13) males alone reduce hatching, as expected (Figure 4B). These data suggest that cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males can cause very weak CI that can be rescued by cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> females.

Next, we aimed to determine if the considerable intertype divergence between cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> and cifA<sub>w</sub><sup>Mel</sup><sub>T1</sub> underpins incompatibility between the strains (Figures 1C and 3A). Embryo death was observed when cifA;B<sub>w</sub><sup>Mel</sup><sub>T1</sub> males mated with cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> (Mdn = 0%; <i>P</i> < 0.0001) or cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> females (Figure 4B), suggesting incompatibility between the gene variants. Reciprocally, embryonic hatching increased to compatible levels when cifA;B<sub>w</sub><sup>Ri</sup><sub>T2</sub> males mated with cifA<sub>w</sub><sup>Mel</sup><sub>T1</sub> females (<i>P</i> > 0.99) (Figure 4B). Together, these data suggest unidirectional CI between cifA<sub>w</sub><sup>Mel</sup><sub>T1</sub> and cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> such that cifA<sub>w</sub><sup>Mel</sup><sub>T1</sub> can rescue CI caused by both variants, but cifA<sub>w</sub><sup>Ri</sup><sub>T2</sub> can only rescue its own lethality. This is the first empirical finding that type 1 and 2 cif genes are partially compatible and thus likely share similar CI mechanisms.

Finally, as previously done for cifB<sub>w</sub><sup>Rec</sup><sub>T1</sub> and cifB<sub>w</sub><sup>Ri</sup><sub>T1</sub>, we combinatorially tested if non-cognate expression of cifB<sub>w</sub><sup>Ri</sup><sub>T2</sub> and cifB<sub>w</sub><sup>Mel</sup><sub>T1</sub>
genes underpins the genetic basis of CI level variation. First, cif\textsubscript{AwMel[T1]}; cif\textsubscript{BwRi[T2]} males yielded a small but significant hatch rate reduction (Mdn = 92.0% hatching; \( P = 0.01 \), relative to the compatible control. Second, cif\textsubscript{AwRi[T2]} (\( P > 0.99 \)) and cif\textsubscript{AwMel[T1]} (\( P = 0.40 \)) females rescued the weak hatch rate reduction (Figure 4C). Finally, cif\textsubscript{AwRi[T2]}; cif\textsubscript{BwMel[T1]} males had a similar, but statistically insignificant, impact on hatching (\( P = 0.07 \)) relative to cif\textsubscript{AwMel[T1]}; cif\textsubscript{BwRi[T2]} males (Figure 4D). Thus, dual expression of both non-cognate pairs yields a small reduction in hatching, and weak cif\textsubscript{AwMel[T1]}; cif\textsubscript{BwRi[T2]} CI was rescuable. Contrary to non-cognate expression of cif\textsubscript{AwRec[T1]} or cif\textsubscript{AwRi[T1]} with cif\textsubscript{BwMel[T1]}, neither non-cognate pairing of cif\textsubscript{AwRi[T2]} and cif\textsubscript{BwRec[T1]} yielded strong CI. These data again suggest that divergent cif types can work together to cause a weak CI-like phenotype.

**Discussion**

The Two-by-One genetic model of CI states that cif\textsubscript{A,B} males cause CI, and cif\textsubscript{A} females rescue that CI (Shropshire and Bordenstein 2019). However, it remains unknown if this model can be widely generalized across cif variants. Likewise, it is unknown whether cif variation alone explains incompatibilities between Wolbachia strains and CI level variation. Here, we use transgenic tools in D. melanogaster to test if cif homologs from \( \text{wRec} \) and \( \text{wRi} \) contribute to CI and rescue, whether cif genetic variation relates to strain incompatibility (Charlat et al. 2001; LePage et al. 2017; Shropshire et al. 2018; Bonneau et al. 2018, 2019), and if cif sequence variation determines transgenic CI levels.

We report four key findings (Figure 5): (i) Evidence is consistent with a Two-by-One genetic basis for rescuable CI, but only weak CI is caused by cif\textsubscript{AwRec[T1]} and cif\textsubscript{AwRi[T2]} homologs (Figure 5A). (ii) Both type 1 cif\textsubscript{A} homologs rescue strong cif\textsubscript{BwMel[T1]} CI (Figure 5B), supporting the hypothesis that closely related cif genes are compatible (Charlat et al. 2001; LePage et al. 2017; Shropshire et al. 2018; Bonneau et al. 2018, 2019), and if cif sequence variation determines transgenic CI levels.

We report four key findings (Figure 5): (i) Evidence is consistent with a Two-by-One genetic basis for rescuable CI, but only weak CI is caused by cif\textsubscript{AwRec[T1]} and cif\textsubscript{AwRi[T2]} homologs (Figure 5A). (ii) Both type 1 cif\textsubscript{A} homologs rescue strong cif\textsubscript{BwMel[T1]} CI (Figure 5B), supporting the hypothesis that closely related cif genes are compatible (Charlat et al. 2001; LePage et al. 2017; Shropshire et al. 2018; Bonneau et al. 2018). (iii) Type 1 cif\textsubscript{AwRi[T2]} homologs cannot rescue cif\textsubscript{BwMel[T1]} CI, but the type 1 cif\textsubscript{AwMel[T1]} can rescue weak cif\textsubscript{BwRi[T2]} CI (Figure 5C), suggesting that different cif types may mechanistically work together, and genetic distance may contribute to unidirectional CI instead of the simple
Type 1.cifB genetic variants determine CI level variation when paired with cifAwMel[T1] whereas both type 1 cifA homologs contribute to strong CI when paired with cifAwRi[T1] (Figure 5D). We also report two results contrary to our initial predictions: cifBwRec[T1] can rescue type 2 transgenic CI but cifAwRi[T2] fails to rescue type 1 transgenic CI. (D) Dual non-cognate expression of type 1 homologs reveals that cifB homologs cause weak or no CI while cifA homologs can contribute to strong transgenic CI. Non-cognate pairs that cause CI can be rescued by cifA-expressing females. Dual non-cognate expression of type 1 and 2 cif homologs reveals that despite amino acid and domain divergence, they may functionally work together to induce weak or marginal CI. * denotes significant or nearly significant levels of very weak CI. (E) cifAwRec[T1] do not contribute to CI and cifBwRec causes complete embryonic death that cannot be rescued by cifAwRec, cifAwMel, or wMel-infected females.

The genetic basis of wRec (type 1) and wRi (type 2) CI and rescue

wRec and wRi induce strong CI in their native hosts (Turelli and Hoffmann 1991; Warren and Jaenike 1995; Shoemaker et al. 1999), leading to the prediction that their corresponding cif genes could yield strong transgenic CI in D. melanogaster. However, a small but significant and repeatable CI was observed when cifA;BwRel[T1] and cifA;BwRi[T2] were expressed in uninfected D. melanogaster males, and that CI was rescued by females expressing their cognate cifA or cifA;BwMel[T1]. Thus, we conclude that these gene pairs function in accordance with the Two-by-One genetic model of CI (Shropshire and Bordenstein 2019). Moreover, this is the first report of a CI-like phenotype caused by the phylogenetic type 2 cif genes. However, it is important to emphasize that a firm conclusion about the full genetic determinants of CI and rescue for these gene pairs is inhibited by the weakened CI levels. Unlike cifA;BwRec[T1] and cifA;BwRi[T2], dual expression of cifA;BwRec[T1] failed to cause CI. We propose three non-exclusive hypotheses for why weak CI is induced by cifwRec[T1] and cifwRi[T2] genes. These may include additional cif gene copies or other Wolbachia and prophage WO genes.
contains both type 1 and type 2 cif genes (LePage et al. 2017), and all Wolbachia strains known to carry type 2 cif also harbor genes from other cif types (LePage et al. 2017; Lindsey et al. 2018). Thus, co-expression of both cif types may be necessary to cause strong CI, or additional genes predicted to interact with eukaryotes may modulate CI (Wu et al. 2004; Yamada et al. 2011; Bordenstein and Bordenstein 2016). Third, several transinfection and introgression studies show that host genotype affects CI levels (Poinset et al. 1998; Bordenstein et al. 2003). The proximal basis of this affected remains unknown, but it is predicted to be related to Wolbachia titer and cif expression profiles (Shropshire et al. 2020b). For instance, uMel is considered as a weak CI inducer, but strict control of several variables that co-vari with Wolbachia titer and cif expression enables strong CI (Reynolds and Hoffmann, 2002, Yamada et al. 2011, Layton et al. 2019). Moreover, strong uMel transgenic CI is possible (Shropshire et al. 2019), thus suggesting that a weak CI strain can cause strong transgenic CI. However, while titer and cif gene expression likely control CI strength within a system, it is plausible that Cif amino acid sequence also corresponds with a change in efficiency when binding to D. melanogaster targets in a heterologous expression system.

In summary, while these data are currently in line with the Two-by-One generic model of CI, optimization of the transgenic expression system in D. melanogaster (Duffy 2002) will be necessary to confirm that these genes can recapitulate strong CI and rescue. If optimization fails to improve the penetrance, then other proteins may modulate the phenotypic potency of CI and be required for strong CI. Alternatively, homologous proteins may not efficiently bind to targets in other hosts, preventing strong CI under heterologous expression. Notably, since non-cognate expression of cifA homologs with cifA_BwMel[T1] yielded strong CI, it is clear that cif sequence variation is not responsible for weakened CI. This is perhaps unsurprising given that mutagenesis assays of cifA_BwMel[T1] proteins reveal that CI expression is more likely to be impacted by mutations in CifB than in CifA (Shropshire et al. 2020a). Thus, the aforementioned effects of suboptimal expression, need for additional genes, or inefficient binding to D. melanogaster targets could be related to the expression of CifB homologs.

Similarly, cifA_BwRi[T1] males do not cause CI, but notably non-cognate dual expression with cifA_BwMel[T1] genes revealed that cifA_AwRi[T1], but not cifA_BwRi[T1], contributes to strong CI. This result is perhaps expected since cifA_BwRi[T1] has an early in-frame stop codon relative to cifA_BwMel[T1] that contributes to its annotation in the wRi genome as a nonfunctional pseudogene. Despite this, we hypothesized that cifB_wRi[T1] would contribute to CI since wRi’s expression of both type 1 and 2 cif genes aligns with the patterns of unidirectional CI between wRi and uMel (Figure 1D). We provide four hypotheses to explain the absence of CI under cifA_BwRi[T1] expression. First, cifA_BwRi[T1] is a pseudogene and is not capable of contributing to CI. Second, since wRi harbors two identical type 1 gene pairs and a type 2 gene pair (LePage et al. 2017), both type pairs may be required for complete CI expression. Third, the early stop codon in cifB_wRi[T1] may not prevent translation of the full-length protein since some stop codons slow translation instead of halting it (Wangen and Green 2020). Thus, a full-length CifB_wRi[T1] protein may be generated despite the internal stop codon, and we did not test that here. Finally, to co-express the N-terminal and C-terminal CifB_wRi[T1] proteins, we introduced a sequence between the two proteins that causes translational slippage and multi-protein translation from a single transcript (Donnelly et al. 2001a,b). This method yields a C-terminal sequence extension to the first protein that may alter protein function. In summary, these data currently support pseudogenization of the cifB_wRi[T1] gene, but transgenic optimization and co-expression with other cif proteins will be necessary to fully rule out alternative explanations.

Contrary to initial predictions, cifB_wRec[T1] transgenic males cause sperm infertility and/or embryonic death when mated with uninfected females. At its surface, cifB_wRec[T1] alone may be interpreted to cause CI. However, this lethality is not rescued by cifA_wRec[T1], cifA_BwMel[T1], or uMel females, and it associates with unusual cytological defects relative to wMel transgenic CI. As bona fide CI is defined by male embryonic lethality, a standard set of cytological defects, and the ability to rescue them, we do not interpret cifB_wRec[T1] lethality as CI. However, it is plausible that a wRec-infected fly may rescue cifB_wRec[T1] lethality. Since testing this requires difficult transinfection of wRec into D. melanogaster, we did not test this hypothesis. Conversely, cifA_BwRec[T1] males also weakly reduce hatching that is rescuable by cifA_AwRec[T1] and cifA_BwMel[T1]. Thus, these data suggest that while cifB_wRec[T1] alone may cause an unusual lethality, a CI-like phenotype is only achieved when Cifa and CifB proteins are dually expressed in males. We discuss our mechanistic interpretations of the results below (see “CI mechanism” section).

Incompatibility relationships

uMel and uRi Wolbachia are unidirectionally incompatible when uMel Wolbachia are transinfected into D. simulans (Poinset et al. 1998) (Figure 1C). Specifically, uRi rescues uMel CI, but uMel does not rescue uRi CI. We hypothesized that cif sequence and copy number variation controls these incompatibility relationships (LePage et al. 2017). Since uRi has both type 1 and 2 cif genes, we expected cifA_AwRi[T1] to rescue cifA_BwMel[T1]-induced CI because the cifA variants are closely related, and cifA_AwMel[T1] would not rescue cifA_BwRec[T1]-induced CI because cifA_AwMel[T1] is highly divergent from the type 2 gene pair (LePage et al. 2017) (Figure 1D). In addition, wRec and uMel have only type 1 genes with a few amino acid changes, leading to the prediction that they are compatible (Figure 1C and D). We tested three key predictions of this cif genotype—CI phenotype hypothesis: (i) cifA_BwRi[T1] rescues transgenic cifA_AwMel[T1] CI, (ii) cifA_AwRi[T1] cannot rescue transgenic cifA_AwMel[T1] CI, and (iii) cifA_AwMel[T1] cannot rescue transgenic cifA_BwRi[T1] CI.

As predicted, type 1 cifA_BwRec[T1] and cifA_AwRi[T1] can each rescue transgenic cifA_BwMel[T1] CI. In addition, cifA_BwRi[T1] cannot rescue cifA_BwMel[T1] CI, despite being able to rescue cifA_BwRi[T2] CI. These data align with expectations that only closely related cif homologs are compatible (Figure 1D). However, we also hypothesized that cifA_AwMel[T1] does not rescue cifA_BwRi[T2] CI, but rescue occurred at the same levels for both cifA_AwMel[T1] or cifA_AwRi[T2] females, suggesting that both CifA variants were capable of rescuing transgenic cifA_BwRi[T2] CI. These results imply a unidirectional incompatibility between type 1 and type 2 genes where type 1 genes cannot be rescued by type 2 genes, but the reciprocal cross is compatible. Not only are these results contrary to our expectations, but they also fail to sufficiently explain the reported unidirectional CI between uMel and uRi since rescue occurs in the opposite direction than we report here (Poinset et al. 1998). We propose two possible explanations for these results.

First, host genotype may impact incompatibility relationships. Two studies evaluated the CI relationships between uMel and uRi, revealing unidirectional CI when wMel is transinfected into D. simulans (Poinset et al. 1998) and no incompatibility when uMel-infected D. melanogaster is crossed with uRi-infected D. simulans (Gazla and Carracedo 2009). Similarly, two Wolbachia from the Nasonia longicornis parasitoid wasp switched from being...
unidirectionally to bidirectionally incompatible when moved into the same genetic background (Raychoudhury and Werren 2012). Thus, there is support for host control of Wolbachia reproductive parasitism and incompatibility relationships. It is unknown what kind of incompatibility relationships might occur if both \(w\)Mel and \(w\)Ri are in a \(D.\ melanogaster\) host background. However, our transgenic \(c\)if expression data suggest that \(w\)Mel can rescue \(w\)Ri, but not vice versa. Thus, we hypothesize that rescue, in particular, is impacted by host genotype such that \(c\)ifA expressed natively (e.g. \(w\)Mel in \(D.\ melanogaster\) or \(w\)Ri in \(D.\ simulans\)) has expanded rescue capability as compared to expression in introduced strains. This hypothesis can be tested through transinfection of \(w\)Ri into a \(D.\ melanogaster\) background or through transgenic expression of \(c\)if\(_{w\text{Mel}[T1]}\), \(c\)if\(_{w\text{Ri}[T1]}\), and \(c\)if\(_{w\text{Ri}[T2]}\) in \(D.\ simulans\). Second, it remains possible that there are dynamic interactions between Cifs such that multiple phylogenetic types interact with one another to impact the phenotypic output. For instance, since \(w\)Ri naturally maintains both type 1 and 2 \(c\)if genes (LePage et al. 2017; Lindsey et al. 2018), expression of both may be required to induce the reported compatibility relationships between \(w\)Mel and \(w\)Ri (Poinset et al. 1998). This hypothesis can be tested through the dual expression of both types 1 and 2 gene pairs and crossing to \(c\)if\(_{w\text{Mel}}\) expressing flies.

**CI mechanism**

The cellular and molecular bases of CifA and CifB in CI remain an active area of investigation. To date, in vitro assays determined that \(c\)if\(_{w\text{Mel}[T1]}\) and \(c\)if\(_{w\text{Ri}[T1]}\) act in part as deubiquitinases, \(c\)if\(_{w\text{Ri}[T4]}\) acts in part as a nuclease, cognate CifA-B pairs of \(w\)Mel and \(w\)Ri can bind, and both CifA and CifB interact with host proteins when transgenically expressed in \(D.\ melanogaster\) (Beckmann et al. 2017, 2019; Chen et al. 2019). There are two mechanistic models for CI that are currently debated: host modification (HM) and toxin antidote (TA) (Hurst 1991; Poinset et al. 2003; Shropshire et al. 2019; Beckmann et al. 2019a,b). HM models posit that CifA-B proteins cause CI by modifying host factors during spermatogenesis, and those modifications are transferred to the embryo. Rescue occurs when CifA in females reverses those effects are not consistent with our expectations for CI induction. We propose two hypotheses for these results. First, CifB may cause CI in the absence of CifA. Singly expressing CifB homologs in yeast causes temperature-sensitive lethality that can be reduced when dually expressed with cognate CifA (Beckmann et al. 2017, 2019a,b; Chen et al. 2019). However, aside from singly expressing \(c\)if\(_{w\text{Ri}[T1]}\) in this study, only \(c\)if\(_{w\text{Ri}[T4]}\) males cause weak embryonic lethality (Chen et al. 2019). However, there is no evidence of paternal transfer of Cif toxin(s), and it remains unclear whether CifA-B binding is related to CI or rescue (Shropshire et al. 2019). Thus, current data are insufficient to support one model over the other. Here, we place three findings above into the context of CI’s mechanistic basis: (i) CifB sequence variation impacts CI level variation, (ii) closely related type 1 CifA can be interchanged for CifB sequence variation, (iii) CifB variation, and (iv) closely related type 1 CifA can be interchanged for CifB sequence variation impacts CI level variation. Sequence variation on CI levels. First, CifB-mediated sperm toxicity. However, it then becomes unclear why embryonic death increases in all other cases of dual transgene expression in insects and why \(c\)if\(_{w\text{Ri}[T1]}\) and \(c\)if\(_{w\text{Ri}[T2]}\) sequence variation may contribute to variation in its tissue localization, subcellular localization, or ability to diffuse between cellular components. CI levels have been correlated with the number of Wolbachia-infected spermatocytes and spermatis during spermatogenesis in \(w\)Ri-infected \(D.\ simulans\) (Clark and Karr 2002; Veneti et al. 2003; Clark et al., 2003), but even uninfected spermatocytes often result in modified sperm that can cause CI (Riparbelli et al. 2007), suggesting that CifA and/or CifB are diffusible between spermatocytes during spermatogenesis. Binding and localization studies would elucidate these hypotheses.

While \(c\)if\(_{w\text{Rec}[T1]}\) and \(c\)if\(_{w\text{Ri}[T1]}\) sequence variation clearly impacts the CI level in transgenic \(D.\ melanogaster\), type 1 \(c\)if\(_{w\text{Rec}[T1]}\) and \(c\)if\(_{w\text{Ri}[T1]}\) homologs were notably interchangeable and contribute to both strong CI and rescue. These data importantly suggest that while \(c\)if\(_{w\text{Rec}[T1]}\) and \(c\)if\(_{w\text{Ri}[T1]}\) sequence variation may be specifically attuned to a distinct host background, transgenic CifA is less subject to variation in host background. For instance, it is plausible that while CifB is interacting with rapidly evolving host targets in an arms race, CifA interacts with a set of conserved targets. One prediction of this hypothesis would be that CifA would be under purifying selection to retain compatibility with conserved hosts. Indeed, comparative sequence analyses reveal not only that type 1 CifAs are under strong purifying selection (Shropshire et al. 2018), but also that CifA sequence length is highly conserved across the phylogenetic types (LePage et al. 2017; Lindsey et al. 2018) and less prone to pseudogene than CifB (Martinez et al. 2020). Thus, a type of HM model could be proposed whereby CifB acts simply as an “accessory” to bind CifA and unlock its access to conserved host processes not otherwise accessible in the absence of CifB. In addition, theory suggests that hosts will evolve resistance to CI while maintaining rescue (Turelli 1994), and many of the same predictions above would also apply in this scenario. For instance, if CifA’s targets in rescue CI are similar, then one would predict the conservation of those targets to maintain rescue, while also maintaining CifA’s ability to contribute to CI. However, variation in CifB’s targets would only inhibit CI induction; thus, selection may favor variation in CifB targets to develop resistance against CI.
and the relationship between CI phenotypes and host genetics. To rescue transgenic variation in novel hypotheses regarding the cif and incompatibilities between Wolbachia type 1 test CI capacity and links between relationships. Moreover, we engineered non-cognate gene sets to the absence of CifA’s C-terminus and the functional role of CifA-B binding. In summary, CifB-induced effects are of interest, but significant caution is warranted as this lethality is not rescuable, which is a requirement for bona fide CI.

Summary

Here, we set out to investigate the hypothesis that cif sequence variation directly relates to CI phenotypic variation by evaluating cognate combinations of the cif genes and their incompatibility relationships. Moreover, we engineered non-cognate gene sets to test CI capacity and links between cif sequence variation and variation in CI level. In summary, we determined for the first time that type 1 cif homologs from uRec and type 2 cif homologs from wRi cause weak CI when transgenically expressed in D. melanogaster. Variation in cifB contributes to CI level variability, divergent cifA fails to rescue transgenic cifA;BwMel[T1] CI, and type 1 cifA homologs are interchangeable for inducing both strong CI and rescue. We discuss these results in the context of CI’s Two-by-One genetic basis in uRec and wRi, incompatibility relationships, and CI mechanism. The work expands upon our understanding of the genetics of CI and incompatibilities between Wolbachia strains, and they establish novel hypotheses regarding the cif mechanism, CI level variation, and the relationship between CI phenotypes and host genetics.

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Conflicts of interest

J.D.S. and S.R.B. are listed as inventors on a pending patent relevant to this work. S.R.B. is a coinventor on two other pending patents related to controlling arthropods.

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