Cytoplasmic incompatibility between Old and New World populations of a tramp ant

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Contributions

CÜ performed the crosses and qPCR
ES analyzed and visualized the data
AMM assembled *Wolbachia* genomes, performed comparative genomic analyses, calculated the phylogeny and annotated CI genes
LVF conducted FISH analyses
BS revised the taxonomic status of the populations
JH acquired funding and reviewed and edited the manuscript
JO conceived and supervised the study
JO, ES and CÜ wrote the original draft of the manuscript
All authors revised and approved the final version of the manuscript

Abstract

As we enter the Anthropocene, the evolutionary dynamics of species will change drastically, and as yet unpredictably, due to human activity. Already today, increases in global human traffic have resulted in the rapid spread of species to new areas, leading to the formation of geographically isolated populations. These go on to evolve in allopatry, which can lead to reproductive isolation, and potentially, the formation of new species. Surprisingly, little is known about such eco-evolutionary processes in ants, even though numerous invasive ant species are globally distributed in geographically isolated populations. Here, we describe the first case of cytoplasmic incompatibility (CI) between populations of a cosmotropic distributed tramp ant with Asian roots, *Cardiocondyla obscurior*, which has acquired a novel *Wolbachia* strain in the New World. Our study uncovers the first symbiont-induced mechanism of reproductive isolation in ants, providing a novel perspective on the biology of globally distributed ants.

Introduction

Ants are the most abundant group of insects on earth, and numerous ant species are classified as highly invasive on a global scale. Their distribution is, on the one hand, strongly facilitated by humans. This is evidenced, for instance, by the rapid worldwide spread of the Argentine ant and the fire ant from their origin in South America [1,2]. On the other hand, the particular biology of ants, characterized by reproductive division of labor between highly fecund queens and sterile workers, allows the establishment of large populations from just one founding propagule. Introduced populations thus go through a genetic bottleneck, reducing diversity within the population while at the same time increasing diversity between populations [3]. Such populations then evolve in allopatry, and differences caused by genetic drift may be further amplified over time by selection, eventually leading to reproductive isolation. Reproductive isolation, in turn, together with the evolution of mating systems, underlies the formation of new species and, hence, the vast diversity of life. By allowing ants (and other organisms) to establish allopatric populations around the world, humans have inadvertently created a valuable scenario for studying species evolution in real time.

Reproductive isolation between populations that live in allopatry can evolve in the form of post-mating mechanisms of reproductive isolation (PoMRI). Importantly, PoMRI can result from Bateson–Dobzhansky–Muller (BDM) incompatibilities, in which two parental loci that have diverged cause hybrid sterility or inviability. Extending such negative epistatic interactions to the host's microbiome adds three mechanisms that can reduce hybrid fitness: hybrid susceptibility (i.e. inferior immune systems and higher pathogen loads in hybrids), hybrid autoimmunity (i.e. upregulation of immune functions in hybrids because of negative epistasis), and cytoplasmic incompatibility (CI, i.e. hybrid embryo death caused by incompatibilities between members of the parental microbiomes) [4]. PoMRI mechanisms can operate at any developmental stage and can directly affect the fitness of both hybrid offspring and mated parental females. The best-known cases of PoMRI caused by bacteria involve *Wolbachia*, the enigmatic evolutionary omnia rotundiora comprising endosymbionts and endoparasites [5], which infects around 40% of the world's arthropod species [6], with some estimates reaching 66% [7]. *Wolbachia* is famous for manipulating maternal transmission routes, and *Wolbachia* strains capable of reproductive manipulation can sweep through a population by inducing CI, or by favoring vertical maternal

transmission via interference with the host's development, resulting in parthenogenesis, feminization, or male death [8].

Although numerous ant species are highly invasive and form allopatric populations on a global scale, nothing is known about PoMRI mechanisms involving endosymbiotic bacteria in these ecologically dominant insects. In particular, the functional role of *Wolbachia* in ant biology has thus far remained elusive, in spite of its omnipresence [9]. The globally distributed tramp ant *Cardiocondyla obscurior* has spread from its putative origin in Southeast Asia and today occurs in fruit tree plantations, city parks and other disturbed habitats in the tropics and sub tropics. In addition to *Wolbachia* populations of *C. obscurior* carry a main bacterial endosymbiont, *Cand.* Westeberhardia, which exhibits characteristics of an obligate symbiont [10]. Populations of *C. obscurior* from Brazil (BR) and Japan (JP) show remarkable genomic heterogeneity and differ in a number of phenotypic traits [11]; nevertheless, mating between queens and males from the two populations readily takes place in the laboratory. However, a phenotype indicative of PoMRI was found in hybrid crosses between BR and JP populations, with BR queens mated to JP males exhibiting shorter lifespans and lower fecundity than BR queens mated to BR males [12]. At the time, this was attributed to BDM incompatibility of fast evolving sexual traits such as seminal fluid proteins, as suggested by an up-regulation of genes involved with immune response in outbred BR queens [12].

Here, we show that the negative effect on queen fitness is in fact caused by incompatible strains of *Wolbachia* harbored by the two populations. By reducing *Wolbachia* levels in males using antibiotic treatment, we demonstrate that queen fecundity can be rescued. Genomic analyses of the *Wolbachia* strains isolated from the two populations reveal that both strains belong to *Wolbachia* supergroup A, which typically contains strains capable of reproductive manipulation. Surprisingly, CI is only induced by one of the two strains, and this may be linked to functional differences in CI-associated loci. By discovering the first case of CI between two ant populations, our study provides the first description of functional *Wolbachia* infection dynamics in ants and presents a novel perspective on the biology of globally distributed ants.

Results

Unidirectional hybrid incompatibility

We verified the results from [12] by crossing queens and males from laboratory colonies of BR and JP populations collected in 2009 and 2010, respectively [11]. We conducted this experiment once in 2015 with the same outcome (Figure S1, Supplement Methods) and again in 2017/18, the results of which are presented here. As expected, compared to all other crosses the combination BR x JP (queen x male) resulted in lower fecundity as estimated by mean weekly egg number over the first six weeks after initiation of egg laying, regardless of whether the male was infected with *Cand.* Westeberhardia or not (Figure 1, Kruskal Wallis rank sum test, X^2= 78.04, df=6, p<0.001, see Table S1 for Bonferroni-Holm corrected pairwise p-values). Similar results were obtained when maximum weekly egg numbers were compared (Figure S2, Kruskal Wallis rank sum test, X^2=81.10, df=6, p<0.001, see Table S2 for Bonferroni-Holm corrected pairwise p-values). Strikingly, the effect was even stronger when using males naturally uninfected with *Cand.*

Westeberhardia, ruling out the possibility that this endosymbiont plays a role in the induction of reproductive isolation.

We monitored a subset of inter-population crosses for an additional 6 weeks (12 weeks in total). Of the 53 BR x JP colonies that were monitored for 12 weeks, nine failed to produce diploid offspring (queen/worker pupae), and eight produced no pupae at all. We dissected the spermathecae of five of the nine male-only producing queens, and all contained sperm. All of the JP x BR colonies that were monitored for 12 weeks produced pupae, and they produced more pupae than BR x JP colonies, irrespective of whether males were infected with *Cand*. Westeberhardia (Figure 2A; Kruskal-Wallis rank sum test, X^2=39.80, df=4, p<0.001, see Table S3 for Bonferroni-Holm corrected pairwise p-values). Furthermore, sex ratios of produced pupae were more male-biased in BR x JP colonies compared to JP x BR colonies (Figure 2B; binomial GLM: $T_{4,78}$=38.59, p<0.001, see Table S4 for Bonferroni-Holm corrected pairwise p-values), an effect caused entirely by the lack of developing females (Figure S3; Kruskal Wallis rank sum test, X^2=5.61, df=4, p=0.23). Together, these data show consistent incompatibility between BR queens and JP males.

Cytoplasmic incompatibility

Cytoplasmic incompatibility (CI) occurs when an endosymbiont favors its transmission by making infected males incompatible with uninfected females, while infected females can mate with both infected and uninfected males. A necessary test to verify CI is 'curing' the host of the putative CI-causing bacteria using antibiotic treatment, which should result in the 'rescue' of the wild-type phenotype.

We fed JP^{rif+} colonies the antibiotic rifampicin diluted in honey every other week for 10 weeks, which resulted in the complete loss of the main endosymbiont *Cand.* Westeberhardia (Figure S4; Mann-Whitney-U-Test, W=34, df=1, p<0.001) and a significant reduction of *Wolbachia* levels in workers (Figure S4; Mann-Whitney-U-Test, W=95, df=1, p<0.01). Treating colonies with the antibiotic tetracycline did not have a negative effect on *Wolbachia* (Figure S5; Mann-Whitney-U-Test, X^2=24, df=1, p=0.052). While *Cand.* Westeberhardia is permanently eradicated in subsequent generations the negative effect of rifampicin on *Wolbachia* is transient, with levels returning to normal 6 months after termination of treatment (Figure S6; *Cand.* Westeberhardia: Mann-Whitney-U-Test, W=64, df=1, p<0.001; *Wolbachia*: Mann-Whitney-U-Test, W=45, df=1, p=0.19). Five of 28 rifampicin-treated colonies succumbed to the treatment and died during the 10-week period, while all 28 control colonies survived.

Mating JP^{rif+} males collected shortly after ceasing antibiotic feeding with BR queens resulted in significantly rescued fecundity, although mean and maximum weekly egg numbers did not quite reach levels of BR and JP inbred queens (Figure 1, Table S1). This rescue effect was evident regardless of whether JP^{rif+} males came from *Cand.* Westeberhardia infected or uninfected colonies. Rescued BR x JP^{rif+} colonies also produced more pupae over a 12-week period than their non-rescued BR x JP counterparts (Figure 2A), and sex ratios among pupae were as female-biased as in JP x BR crosses (Figure 2B). Strongly reduced *Wolbachia* levels in JP^{rif+} males (Figure 3, Mann-Whitney-U-Test, W=163, df=1, p<0.001) support the role of *Wolbachia* in inducing hybrid incompatibility.

We confirmed unidirectional CI with males from laboratory colonies of a second Asian population collected in Taiwan (TW), which is infected with a *Wolbachia* strain that amplifies a *wsp* sequence (coding for the hypervariable *Wolbachia surface protein*) identical to those from JP samples (Figure S7). We set up crosses with queens from BR (BR x TW) and JP (JP x TW), and the reciprocal combination (TW x JP). Again, only the combination BR x TW resulted in reduced fecundity, both when mean weekly egg numbers (Figure S8; Kruskal Wallis rank sum test, X^2=9.96, df=2, p<0.01, see Table S5 for Bonferroni-Holm corrected pairwise p-values) and maximum weekly egg numbers over six weeks (Figure S8; Kruskal Wallis rank sum test, X^2=10.19, df=2, p<0.01, see Table S6 for Bonferroni-Holm corrected pairwise p-values) were compared.

Wolbachia genomics and phylogeny

We assembled the *Wolbachia* genomes from BR (*w*Cobs-BR) and JP (*w*Cobs-JP) populations of *C. obscurior* using Illumina HiSeq2000 100-bp reads of 200 and 500-bp insert paired-end libraries [11]. The genomes of both *Wolbachia* were assembled to 148 (BR) and 182 (JP) scaffolds (Table 1). Both strains showed similar genome sizes and G+C contents, with an estimated 1,013 (BR) and 1,058 (JP) predicted protein-coding genes. An analysis on shared genes revealed that both strains share a core of 907 proteins, with 85 unique to *w*Cobs-BR and 109 unique to *w*Cobs-JP. According to Bayesian phylogenetic analyses of ribosomal proteins from 23 *Wolbachia* strains, both *Wolbachia* strains belong to supergroup A, which together with supergroup B contains strains capable of reproductive manipulation. *w*Cobs-JP was found to be more closely related to *w*Ha and *w*CauA strains, while *w*Cobs-BR was phylogenetically closer to *w*Au and *w*Mel (Figure 4A). A synteny analysis revealed that both *w*Cobs-JP and *w*Cobs-BR strains keep general synteny, but with several rearrangements (Figure 4B). This is consistent with previous observations of group A *Wolbachia* strains [13].

Wolbachia phenology

To compare infection titers between populations we performed qPCR of the *Wolbachia* gene *Cytochrome c oxidase subunit 1* (*coxA*) against the ant housekeeping gene *elongation factor 1-alpha 1* (*EF1*). JP queens had higher relative *w*Cobs-JP titers than BR queens infected with *w*Cobs-BR (Figure S9, Mann-Whitney-U-Test, W=23, df=1, p=0.043). Likewise, JP workers showed higher infection titers with *w*Cobs–JP compared to BR workers infected with *w*Cobs-BR (Figure S9, Mann-Whitney-U-Test, W=16, df=1, p=0.017).

To test for morph-bias in infection we compared *coxA* expression in adult queens, workers, winged males and wingless males using qPCR. *coxA* levels did not differ between sexes and were consistently higher in winged morphs (queens, winged males) compared to wingless morphs (workers, wingless males) (Figure S10, Kruskal Wallis rank sum test, X^2=19.53, df=3, p<0.001).

To measure the potential cost of *Wolbachia* infection and to test for a selective loss in workers, we compared infection levels of workers and queens over time. In contrast to *Cand.* Westeberhardia [10], *Wolbachia* levels quickly reached a plateau in both queens and workers. There was no indication that individuals lose *Wolbachia* over the first few weeks

234 of their lives, which typically last for up to three (workers) or six months (queens) (Figure
235 S11, workers: ANOVA, $F_{4,26}$= 2.69, p=0.059; queens: ANOVA, $F_{5,50}$= 15.95, p<0.001).
236
237 CI mechanism
238 CI usually results in the failure to produce viable zygotes but how CI operates functionally
239 is still not clear. The two championed models are based on 1) a sperm modification step
240 during spermatogenesis which is reversed by a rescue factor or 2) the transfer of a toxic
241 product via the sperm and subsequent binding to a female-derived antidote [14-17]. The
242 current genetic view for CI in *Drosophila* favors a two-by-one model [17], involving two
243 adjacent genes (coined *cifA* and *cifB*) located in a region of prophage origin in *w*Mel [16-21],
244 which contains several genes with eukaryote function [22]. Expression of both *cifA* and *cifB* in
245 males is required to cause CI, while expression of *cifA* rescues embryo mortality in females
246 [21]. Phylogenetic analyses of the Cif proteins have revealed at least 5 distinct phylogenetic
247 clades referred to as Types 1-5, respectively [18,23,24].
248
249 We found homologues of *cifA* and *cifB* in *Wolbachia* strains from BR and JP. In the latter,
250 we found two additional homologues. Through Bayesian phylogenetic inference, and
251 following the classification scheme of [23], we found that all copies of *cifA* and *cifB* belong to
252 Type I (Figure 5A, B). The *cifA* and *cifB* genes found in NODE_002 of *w*Cobs-JP are
253 closely related to those found in *w*Cobs-BR and in the cryptic phage of the strain *w*Sol
254 isolated from the fig wasp *Ceratosolen solmsi* (Figure 5A, B). The *cifA* and *cifB* genes
255 found in NODE_063 of *w*Cobs-JP show a more distant relationship to their homologues in
256 *w*Cobs-BR. Following [23], we performed a protein remote homology search with the
257 webserver of HHpred's v3.2.0 webserver [25]. While the *cifA* gene of *w*Cobs-BR contained a
258 DUF249 domain, neither *cifA* homologues in *w*Cobs-JP showed homology to known
259 domains (Figure 5C). In contrast, putative domains were found in all *cifB* genes. These
260 shared a putative nuclease domain (PD-(D/E)XK nuclease/DpnII-MboI) found in other type
261 I *cifB* genes. The *cifB* homologue found in NODE_063 in *w*Cobs-JP shared most domains
262 with the *cifB* homologue in *w*Cobs-BR, with an additional peptidase domain
263 (Peptidase_C58; PF03543).
264
265 *Wolbachia* in males
266 As mentioned above, *Wolbachia*-induced CI has often been linked to sperm modification
267 during spermatogenesis [26] or the transfer of a CI toxin along with or in the sperm. The
268 presence of *Wolbachia* in the testes is thus expected, although their specific localization in
269 spermatocytes or spermatids might not be a prerequisite for inducing CI, as has been
270 observed in the wasp *Nasonia vitripennis* [27]. We used fluorescence in situ hybridization to
271 localize *Wolbachia* in the abdomens of male *C. obscurior*, confirming the presence of the
272 bacteria throughout different tissues including the testes in both JP (Fig. 6A-C) and BR
273 (Figure 6D) individuals. *Wolbachia* was also observed co-infecting bacteriocytes
274 dominated by *Cand.* Westeberhardia, yet in significantly lower densities (Figure 6C).
275
276 Discussion
277 Insect-*Wolbachia* interactions can range from beneficial to detrimental and from
278 facultative associations to rare cases of ultimate mutualisms where hosts have higher
279 fitness with *Wolbachia* than without [5]. This prevalence and diversity of *Wolbachia*
280 associations predictably also occurs in ants; however, our knowledge of ant-*Wolbachia*

interactions is limited [9]. In the few ant-*Wolbachia* associations studied so far, infection is facultative (e.g. [28-30]), indicating ongoing arms races between hosts and symbionts [31]. One particularly obvious question to ask is whether *Wolbachia* has the power to control sex allocation, a trait shaped by kin selection in social Hymenoptera [32]. In this context, facultative *Wolbachia* infections have been reported to cause transient changes in sex allocation [29], or to have no effect [33]. Similarly contrasting results have been obtained regarding effects on colony productivity [29,34]. It has furthermore been speculated that *Wolbachia* can bias caste ratios towards the production of queens [28,35], but current data are scarce and provide little support for this hypothesis [9]. By providing the first functional description of an ant-*Wolbachia* association involving two strains of *Wolbachia*, our study lays the groundwork for in-depth investigations into how *Wolbachia* affect ant reproductive biology and fitness.

Wolbachia clearly play a significant role in the biology of *C. obscurior*. First, all screened individuals were infected regardless of population, caste or sex. *Wolbachia* titers were higher in queens and winged males compared to workers and wingless males, a difference that is likely associated with the relative amounts of reproductive tissue as *C. obscurior* workers lack ovaries and winged males have larger testes than wingless males [36]. Second, rifampicin treatment merely reduced *Wolbachia* titers, and this effect was reversible. This was not the case for *Cand.* Westeberhardia, which was permanently cleared by rifampicin treatment. Third, *Wolbachia* titers did not decline with age in workers. *Wolbachia* infection can be costly and selection may thus favor reduced infection loads in sterile workers, as these do not contribute to vertical transmission [28,35]. Such a mechanism appears to be acting on *Cand.* Westeberhardia, where titers in workers (but not queens) decreased significantly with age, indicating that while the host may benefit from infection during development or in the adult egg-laying queen stage, the symbiont is not required [10]. Consistently high titers in workers indicate that *Wolbachia* do not have deleterious effects on worker performance in *C. obscurior*, unlike *Wolbachia* in *Acromyrmex* ants [37]. To what degree the population-specific strains differ in their effect on host phenotype remains unclear. Higher *Wolbachia* titers in JP individuals suggest that the *w*Cobs-JP strain may be better integrated into the host's biology. Alternatively, these differences may be driven by population-specific competition with *Cand.* Westeberhardia over limited resources.

Sex allocation in *C. obscurior* is female-biased [38] and queens presumably have full control over male production, as investment into males increases in the presence of competing queens [39-41], Accordingly, we found no indication that *Wolbachia* influences sex allocation. Queens from non-CI (JP x BR) crosses produced few males in the first 12 weeks of their lives. This may be a consequence of the short observation period, equaling ~50% of the average queen lifespan [38]. The sex ratios produced by CI (BR x JP) crosses showed a bimodal distribution, with several queens matching sex ratios produced by non-CI queens (albeit with lower overall productivity), while others produced only males. On average, however, CI queens did not produce more males than non-CI queens. There was also no indication that *Wolbachia* altered caste ratios towards production of queens (data not shown). Concordantly, a previous study comparing investment into sexuals and workers did not find differences between the BR and JP populations [38]. Testing directly for a caste-biasing effect of *Wolbachia* requires complete elimination of the bacterium. While this

328 cannot be done without also killing the ant host, preliminary trials indicate that cross-
329 infection of the two *Wolbachia* strains is possible. In the future, such manipulations may
330 help clarify whether *Wolbachia* have caste-manipulating power in *C. obscurior*.
331
332 Global exchange of stowaway ants leading to secondary contact between allopatric
333 populations is common (e.g. in the tropical fire ant *Solenopsis geminata* [42], and the little
334 fire ant *Wasmannia auropunctata* [43]) and thus the presented scenario, albeit conceived in
335 the laboratory, is not an unrealistic one. A previous study emphasized the role of BDM
336 incompatibility between allopatric *C. obscurior* populations in causing a CI-like phenotype
337 [12]. *Wolbachia*-induced incompatibilities were ruled out because of identity of a partial
338 sequence of *wsp* extracted from seven individuals (three from BR colonies collected in
339 2004, four from JP colonies collected in 2005) using the wsp81F/wsp691R primer
340 combination [44]. Gene expression comparisons of allopatrically and sympatrically mated BR
341 queens pointed towards elevated immune responses and other changes that mimicked a
342 CI-like phenotype. In *Drosophila*, *Wolbachia* can manipulate expression of key regulatory
343 genes during spermatogenesis, which leads to embryonic lethality and a CI-like
344 phenotype [45]. Thus, what appeared to be BDM incompatibility may have been caused by
345 *Wolbachia* inducing a CI-like phenotype via interference with queen gene expression. The
346 alternative and more parsimonious explanation for the CI-like phenotype based on the
347 results from the present study is a classic unidirectional *Wolbachia*-driven CI effect
348 involving incompatible *Wolbachia* strains. This also explains why JP x BR crosses did not
349 elicit a CI phenotype. The overexpression of immunity-related genes in allopatrically
350 mated BR queens detected by Schrempf et al. 2015 may simply reflect physiological
351 responses to aborted oogenesis, as opposed to responses to ejaculate quality, or to
352 injuries caused by non-matching male genitalia. Furthermore, we recently found that some
353 BR colonies were infected with an additional *Wolbachia* strain displaying partial *wsp*
354 sequences identical to those of *w*Cobs-JP (data not shown), indicating horizontal
355 transfection in our rearing facilities, especially in older laboratory colonies.
356
357 The mechanism of CI in *C. obscurior* appears to align along the *Wolbachia cif* gene axis
358 described in *Drosophila* fruit flies and *Culex* mosquitoes [15,17,19,23,46]. This genetic region,
359 which is of prophage origin and capable of manipulating the eukaryote host [18], showcases
360 the importance of using a hologenomic approach to study evolution [47]. Similar prophage-
361 derived regions containing pairs of homolog *cif*/*cif*-like genes have since been described
362 in a number of *Wolbachia* strains [23,24]. Surprisingly, the homologs of *cifB* in *w*Pip and *w*Mel
363 have different biochemical functions: both putatively contain ankyrin-like repeats for
364 protein-protein interaction with eukaryotic DNA [46] but $cifB_{wMel}$ is not a putative
365 deubiquitinase [16], as postulated for $cifB_{wPip}$ [19]. Both *Wolbachia* strains isolated from *C.*
366 *obscurior* have homologs of *cifA* and *cifB* but only the *w*Cobs-JP strain has an additional
367 *cifA*/*B* pair, pointing towards a role for the latter pair in inducing reproductive isolation.
368 Whether the second *cifA*/*B* containing region was lost in *w*Cobs-BR, or whether it stems
369 from a duplication event and subsequent divergence in *w*Cobs-JP as has been described
370 for the *w*AlbB strain of *Aedes albopictus* [48], remains to be shown. Alternatively, functional
371 differences in *cifA*/*B* homologs between *w*Cobs-JP and *w*Cobs-BR, or an interaction of the
372 two *cif* gene products in JP males could be causing CI. Irrespective of the exact CI
373 mechanism, we do not know why a mere reduction of *Wolbachia* titers in males was
374 sufficient to rescue queen fecundity. This requires studying the effects of *Wolbachia* and

antibiotic treatment on sperm production and development. One possibility is that rifampicin treatment causes ribosomal damage that prevents *Wolbachia* from modifying sperm. At the same time, antibiotic-induced damage to sperm mitochondria [49] may explain the slightly lower productivity of "CI rescue" crosses compared to non-CI crosses. It is also unclear why the CI effect was stronger on egg numbers but not pupae numbers when using males from colonies naturally uninfected with *Cand.* Westeberhardia, but this could point toward interaction effects between the two symbionts.

Our study raises many questions about the evolutionary history of the *C. obscurior-Wolbachia* association. The putative origin of *C. obscurior* in Southeast Asia, together with sequence identity between *Wolbachia* strains isolated from populations found in Japan and Taiwan, suggest that these strains represent the ancestral association. Geographic patterning separating New from Old World *Wolbachia* strains has been observed previously in ants [50], and frequent horizontal transmissions [51,52] can lead to a surprising diversity within species [53]. *C. obscurior* and its sister species *C. wroughtonii* are the only known representatives of the 100+ described species in the genus *Cardiocondyla* (the so-called heart-node ants) that have adopted an arboreal lifestyle, as opposed to nesting in soil and rock crevices. This makes them highly susceptible to horizontal transmission of *Wolbachia* from other plant-associated insects. Although *Wolbachia* from different supergroups may co-exist in a host [13], it is unlikely that two strains from the same supergroup co-exist without eventually recombining and merging. Generally, unidirectional CI should facilitate introgression between populations, while bidirectional CI should promote divergence. This predicts mixed *C. obscurior* genotypes but only the presence of the *w*Cobs-JP strain in secondary contact zones, assuming that hybrids perform as well as parental lineages. How then has the ancestral Asian strain been replaced? *Wolbachia* acquisition and persistence in *C. obscurior* may be driven by selection on the strain that is better adapted to the local environment, perhaps analogous to *Wolbachia*-driven thermal preferences in *Drosophila* flies [54,55]. Resolving how *w*Cobs-BR replaced the putatively ancestral *w*Cobs-JP strain, which holds the power of CI, requires testing how mixed combinations of host and *Wolbachia* perform under varying ecological conditions. Coupled with a global survey of *Wolbachia-C. obscurior* associations, this will help unravel how the symbiosis shapes the worldwide distribution of this tramp ant.

Conclusion

One important consequence of unidirectional reproductive isolation is unequal inheritance, which allows a BDM-causing locus or a reproductive manipulator to quickly spread in secondary contact zones. Such disruptive factors can be more powerful than natural selection on other fitness-relevant traits, and lead to the evolution of PoMRI and hybrid inviability, processes estimated to require several million years in *Drosophila* flies and *Pogonomyrmex* ants [56,57]. Our discovery of reproductive isolation between allopatric ant populations, driven by two naturally occurring, closely related *Wolbachia* strains that differ in their genomic makeup, demonstrates that these evolutionary processes can be observed in real time and lays the groundwork for detailed studies of *Wolbachia*-ant biology. In particular, a system in which CI is accompanied by a CI-like phenotype in host gene expression may prove useful for understanding the function of the *cif* gene family. More generally, hologenomic approaches such as the one presented here will help tackle

421 the challenges associated with human-induced changes to ecological and evolutionary
422 dynamics.
423
424 Methods
425 *Cardiocondyla obscurior* presumably originates from Southeast Asia and is now
426 distributed around the tropics and subtropics. Queens in *C. obscurior* are produced year-
427 round and have a short generation time of ~120 days (from egg to queen to egg).
428 Colonies usually contain 2-5 queens [11] and a single wingless male that can live up to a few
429 months and continually produces sperm [58]. Such males transfer on average 1000 sperm
430 cells per copulation [59], which is sufficient for a lifelong production of diploid offspring by
431 an inseminated queen. Newly hatched queens can decide to stay in the maternal nest or
432 to leave - alone or with a small group of workers - irrespective of mating status. Like all
433 Hymenoptera, ants exhibit haplodiploid sex determination and virgin females can
434 produce males from haploid, unfertilized eggs. Virgin queens in *C. obscurior* can copulate
435 with their own sons (pers. obs.), enabling the production of workers and queens, similar to
436 a related *Cardiocondyla* species [60]. This biology has facilitated the worldwide spread of
437 several species of the genus [61].
438
439 In this study, we used inbred laboratory 'populations' derived from colonies collected in
440 Bahia, Brazil (BR) in 2009 (Brazilian Ministry of Science and Technology, permits 20324-
441 1/40101-1) and Okinawa, Japan (JP) in 2010. The JP population exhibits a natural
442 polymorphism regarding the presence of the endosymbiont *Candidatus* Westeberhardia
443 cardiocondylae, with some colonies being naturally uninfected (JP_{we-}) [10]. Although the BR
444 and JP populations differ in cuticular hydrocarbon composition, queen size and behavior
445 [11], workers are morphologically indiscernible according to exploratory data analyses by
446 hierarchical and nonhierarcical, vector-based forms of Nest Centroid clustering: NC-Ward,
447 NC-part.hclust, NC-part.kmeans, NC-NMDS-kmeans [62,63]. These analyses considered 15
448 continuous morphometric characters. Specifically, when considering 82 cosmopolitan nest
449 samples of the sister taxa *C. wroughtonii* and *C. obscurior*, all JP and BR samples were
450 allocated to the latter species by all four types of NC-clustering. This result was confirmed
451 with posterior probabilities of $p > 0.98$ if these samples were run as wild-cards in a linear
452 discriminant analysis. Analyses of 32 nest samples representing only the *C. obscurior*
453 cluster could not allocate the JP and BR populations to different intraspecific clusters nor
454 could these analyses demonstrate any intraspecific structuring at all. This suggests that
455 *Wolbachia*-induced reproductive isolation has not yet led to a divergence into
456 morphospecies. In addition to JP and BR colonies, we used colonies collected from a
457 second Asian population in Taipei, Taiwan in 2013 (TW) to confirm the presence of
458 unidirectional CI. For all experiments, colonies were kept in square plaster-bottom nests
459 (100 x 100 x 20 mm, Sarstedt, Germany) and held in climate chambers under a 12h/12h
460 light/dark cycle and a 28°C/22°C temperature cycle. Food (honey and *Drosophila* or
461 pieces of *Periplaneta americana*) and water were provided to stock colonies every 3 days.
462 All animal treatment guidelines applicable to ants under international and German law
463 were followed. Collection of colonies that form the basis of the laboratory population used
464 in this study was permitted by the Brazilian Ministry of Science and Technology (RMX
465 004/02). No other permits were required for this study.
466
467 Antibiotic treatment

468 Rifampicin is a broad-spectrum antibiotic that acts via inhibition of bacterial RNA
469 polymerase [64]. To produce *Wolbachia*-cured males, we split large *Cand*. Westeberhardia-
470 infected (JP) and uninfected (JP_{we-}) stock colonies into two equal halves. Half of these splits
471 were designated as controls (JP: n=13, JP_{we-}-: n=5), while the other half was treated with
472 antibiotics (JP: n=13, JP_{we-}: n=5). We diluted 0.0025 g of solid rifampicin (Sigma-Aldrich,
473 USA) in 500 µl of a 1:1 honey-water solution for a final antibiotic concentration of 0.5%.
474 The antibiotic solution was placed on a shaker for 4 hours to ensure complete mixing, and
475 subsequently covered with aluminum foil and stored at -20°C. Treated colonies were fed
476 with the antibiotic solution twice per week every other week, for a total duration of ten
477 weeks. On the days following treatment, the antibiotic solution was removed from the
478 nest. Between antibiotic feedings (i.e. every other week), colonies were fed with honey
479 and pieces of autoclaved cockroach (to prevent bacterial re-infections) and water ad
480 libitum. Control colonies were fed twice a week with honey and cockroaches and received
481 water ad libitum.
482
483 ***Wolbachia* and *Cand.* Westeberhardia infection**
484 We assessed the presence and titers of bacterial infections using PCR and qPCR,
485 respectively, on DNA extracted from whole bodies (worker pupae & adults, queen pupae
486 & adults, adult males). DNA was extracted from samples using a standard CTAB protocol.
487
488 *Presence of infection*
489 We confirmed infection in a subset of workers from BR and JP colonies. Infection with
490 *Wolbachia* was tested with a PCR assay using primers specific for a fragment of the
491 *Wolbachia surface protein* gene of the BR (105-bp, *wsp_wCobs_BR-for: 5-*
492 *'TAAATCTTGCATCTGTAACATT-3',* *wsp_wCobs_BR-rev: 5'-*
493 *CTGCGGATACTGATACAACTACTG-3'*) and JP (254-bp, *wsp_wCobs_JP-for: 5'-*
494 *CATTTTGACTACTCACAGCGGTTG-3',* *wsp_wCobs_JP-rev:*
495 *5'CTGCGGATACTGATACAACTACTG-3'*) strains. *Cand.* Westeberhardia infection was
496 tested by amplifying a 204-bp fragment of the *ribonucleoside-diphosphate reductase 1*
497 *subunit beta* gene of *Westeberhardia* (*WEOB_403*) (*nrdB_weCobs-for:* 5'-
498 GGAAGGAGTCCTAATGTTGCG-3'; nrdB_*weCobs*-rev: 5'-ACC
499 AGAAATATCTTTTGCACGTT-3'). A 104-bp fragment of the *C. obscurior* gene *elongation*
500 *factor 1-alpha 1* (*Cobs_01649*) (*EF1-for:* 5'-TCACTGGTACCTCGCAAGCCGA-3'; *EF1-rev:*
501 5'-AGCGTGCTCACGAGTTTGTCCG-3') was used as a positive control. In addition,
502 infection was assessed in two adult queens from each of five TW colonies to confirm that
503 this population carries the *w*Cobs-JP strain. Each PCR reaction contained 5 µL Taq
504 polymerase (GoTaq, Promega), 3 µL H_2O, 0.5 µL forward primer (10 µM), 0.5 µL reverse
505 primer (10 µM) and 1 µL DNA. PCRs were run at 94°C for 4 minutes followed by 36 cycles
506 at 94°C for 30s, 57°C for 30s and 72° for 30s, with a final step of 72°C for 10 minutes.
507 Electrophoresis separation of PCR products was performed on a 1.5% TAE-Gel with 0.5 µl
508 of 0.001 % Gel-Red for 45 minutes at 60 V and 65 mA.
509
510 *Population-specific Wolbachia titers*
511 We compared *Wolbachia* infection titers of adult workers and queens from BR (n=9
512 workers, n=10 queens) and JP (n=10 workers, n=9 queens) colonies by amplifying a
513 portion of the *Cytochrome c oxidase subunit 1* gene (*coxA_wCobs-for*: 5'-
514 TTGGTCATCCAGAAGTTTACGT-3', *coxA_wCobs-rev*: 5'-TGAGCCCAAACCATAAAGCC-

515 3′) in qPCR. As a relative standard *elongation factor 1-alpha 1* (*Cobs_01649*) was used.
516 Each qPCR reaction contained 5 µL SYBR (Peqlab), 2 µL H_2O, 1 µL forward primer (2 µM), 1
517 µL reverse primer (2 µM) and 1 µL DNA. Reactions were performed in duplicates and run
518 at 95°C for 3 minutes followed by 40 cycles at 95°C for 5s, 60°C for 20s, and 95° for 10s,
519 and a final melt curve analysis step in which reactions were heated from 65°C to 95°C in
520 5°C steps. Single-amplicon production was confirmed with melt curve analyses and
521 relative *Wolbachia* titers calculated with the $2^{-\Delta CT}$ method [65].
522
523 *Age and morph-specific Wolbachia titers*
524 We quantified *Wolbachia* titers in individual queens and workers from BR colonies by
525 determining relative *coxA_wCobs* copy numbers with qPCR across developmental stages
526 and adult age classes. In workers, titers were measured in pupae (n=4 white pupae, n=5
527 dark pupae) and in adults two days (n=5), 14 days (n=7), and 28 days (n=6) post-hatching.
528 In queens, titers were measured in pupae (n=5 white pupae, n=4 dark pupae), as well as in
529 adults two days (n=7), 14 days (n=7), 28 days (n=10 virgin queens, n=7 mated queens)
530 and 48 days (n=9) post-hatching. In addition, we quantified *coxA_wCobs* copy numbers in
531 adult morphs (n=15 queens, n=12 workers, n=6 winged males, n=7 wingless males). For
532 all reactions, *elongation factor 1-alpha 1* (*Cobs_01649*) was used as a housekeeper. Each
533 qPCR reaction contained 5 µL SYBR (Peqlab), 2 µL H_2O, 1 µL forward primer (2 µM), 1 µL
534 reverse primer (2 µM) and 1 µL DNA. Reactions were performed in triplicates and run as
535 described above. Single-amplicon production was confirmed with melt curve analyses and
536 relative quantities of *Wolbachia* calculated with the $2^{-\Delta CT}$ method.
537
538 *Rifampicin effects on infection titers*
539 We verified the efficacy of rifampicin treatment by measuring *Wolbachia* and
540 *Westeberhardia* titers in dark worker pupae produced by antibiotic-treated (n=21 from 9
541 colonies) and control colonies (n=21 from 8 colonies) using qPCR of *coxA_wCobs and*
542 *nrdB_weCobs*. In addition, we measured the *Wolbachia* titers of JP males used in mating
543 combinations BR x JP and BR x JP^{rif+} (JP: n=15, JP^{rif+}: n=12) with qPCR of a portion of the
544 JP-specific *wsp* gene (*wsp_wCobs_JP*). For all samples, *elongation factor 1-alpha 1*
545 (*Cobs_01649*) was used as a housekeeper. Each qPCR reaction contained 5 µL SYBR
546 (Peqlab), 2 µL H_2O, 1 µL forward primer (2 µM), 1 µL reverse primer (2 µM) and 1 µL DNA.
547 Reactions were performed in duplicates (males) or triplicates (worker pupae) and run as
548 described above (except with an annealing temperature of 57°C for male pupae). Single-
549 amplicon production was confirmed with melt curve analyses and relative quantities of
550 *Wolbachia* and *Westeberhardia* calculated with the $2^{-\Delta CT}$ method.
551
552 Experimental crosses
553 We designed seven mating combinations between queens and males from the two
554 populations: 1) JP x JP (n=7), 2) JP x BR (n=26), 3) BR x BR (n=11), 4) BR x JP (n=36), 5) BR
555 x JP_{we-} (n=29), 6) BR x JP^{rif+} (n=13) and 7) BR x JP_{we-}^{rif+} (n=5). Each experimental colony was
556 set up with one dark queen pupa, one freshly hatched male, 10 workers and brood (10
557 eggs, 10 larvae, 10 worker pupae). All individuals were collected from un-manipulated
558 stock colonies, except for JP^{rif+} and JP_{we-}^{rif+} males used in combinations #6 and #7, which
559 were collected from rifampicin-treated colonies. To avoid manipulation effects, all
560 combinations were set up in parallel. In such experimental colonies, mating between
561 queens and males usually occurs within three weeks, after which queens shed their wings

562 and being laying eggs. We monitored colonies for queen wing loss once per week. Once
563 queens had shed their wings, we removed any brood placed in the colony during set up
564 and standardized worker number to 20. Colonies were then monitored once a week for six
565 weeks and all eggs and pupae (queen, worker, wingless male, winged male) counted. A
566 subset of colonies was monitored for a total of 12 weeks (JP x BR: n=19, BR x JP: n=28, BR
567 x JP_{we-}: n=25, BR x JP^{rif+}: n=9, BR x JP_{we-}^{rif+}: n=5). In each colony, the number of adult
568 workers was kept constant at 20 individuals by removing hatched workers or adding
569 workers from stock colonies. From monitoring data, we calculated mean and maximum
570 weekly egg numbers, and total number and sex ratio of produced pupae (male
571 pupae:total pupae).
572
573 To verify that CI is specifically induced by the *w*Cobs-JP strain when it encounters the
574 *w*Cobs-BR strain, we set up three additional mating combinations between queens and
575 males using a third population from Taiwan (TW) which also harbors the *w*Cobs-JP strain:
576 1) JP x TW (n=5), 2) TW x JP (n=6), 3) BR x TW (n=5). Experimental colonies were set up
577 and monitored as described above for six weeks.
578
579 **DNA sequencing and genome assembly**
580 We extracted DNA from a pool of 26 (JP) and 30 (BR) males from two colonies, collected
581 in 2009 in Una, Brazil (BR2009-alpha) from aborted fruits of coconut trees and in 2010 in
582 Naha, Japan (JP2010-OypB) from under bark of coral trees (*Erythrina* sp.). For details see
583 [11]. Briefly, the reads were right-tail clipped (minimum quality of 20) and all reads with
584 undefined nucleotides were removed using a combination of FASTX-Toolkit v0.0.14
585 (http://hannonlab.cshl.edu/fastx_toolkit/, last accessed December 3, 2010) and
586 PRINSEQ++ v1.2 (10.7287/peerj.preprints.27553v1). The resulting paired end reads were
587 assembled with SPAdes v3.13.1 (-k 33,55,77 --only-assembler [66]. The resulting contigs
588 were then binned according to an *ad hoc* BLASTX-based method. First, a proteome
589 database was built from representative genomes (Table S7). Second, the best hit for each
590 scaffold was used for assigning a preliminary bin and a coverage cut-off (10x for BR and
591 30x for JP) was implemented based on the coverage of the longest contigs. Third, each
592 scaffold was manually inspected using the online NCBI web-server *vs.* BLAST+2.9.0. If the
593 best hits to proteins were consistently to *Wolbachia* bacteria across the scaffold, the query
594 sequence was retained. Finally, the assembly graph was loaded into Bandage v0.8.1 [67],
595 the graph(s) matching *Wolbachia* sequences were identified and the sequences were
596 extracted. These extracted sequences were then compared to the *Wolbachia* draft bin. In
597 both cases, the extracted sequences were roughly the same compounded length as the
598 manually-curated bins and added only a couple of kilo base pairs (kbp), suggesting near
599 completeness of the genome. Using these *Wolbachia* bins, we performed a mapping
600 using BOWTIE2 v2.3.4.1 [68] followed by a re-assembly of the mapped reads in SPAdes
601 (with previously-mentioned parameters). This final reassembly was filtered by both
602 coverage and manual screening of scaffolds shorter than 3 kbp. Assembled sequences
603 have been deposited in the European Nucleotide Archive (ENA) under the accessions
604 CACTIU010000000.1 (*w*Cobs-BR) and CACTIV010000000.1 (*w*Cobs-JP).
605
606 **Phylogeny and comparative genomics of *Wolbachia* strains**
607 A draft annotation of the scaffolds was done using Prokka v1.14 [69]. For comparative
608 genomic purposes, we built a subset of the predicted ORFs by filtering out all proteins

609 annotated as "hypothetical proteins" with genes shorter than 300 base pairs (bp). This
610 helped us avoid wrongly annotated ORFs overlapping pseudogenes. To infer the
611 phylogenetic relationship between *Wolbachia* strains found to infect the analysed BR and
612 JP populations as well as among other *Wolbachia* bacteria, we collected the amino acid
613 sequences for the ribosomal proteins of 23 *Wolbachia* strains from supergroups A (8), B
614 (10), C (2), D (1), E (1), and F (1) (Table S8). Using *Anaplasma phagocytophilum* strain HZ
615 and *Ehrlichia canis* strain Jake as outgroups, we inferred a Bayesian phylogeny using
616 MrBayes v3.2.6 [70]. We ran two independent analyses with four chains each for 300,000
617 generations and checked for convergence. The substitution model JTT+I+G+F was used
618 as suggested by jModelTest v2.1.10.
619
620 To infer the shared protein-coding gene content of *w*Cobs-BR and *w*Cobs-JP strains, we
621 used OrthoMCL v2.0.9 [71,72] with an inflation value of 1.5. The synteny of both strains was
622 assessed using nucmer v3.23 [73] using anchor matches that were unique in both the
623 reference and query (--mum).
624
625 Cif-family gene annotation and phylogenetics
626 *cif* genes were first identified by BLASTP using the protein sequences WP_010962721.1
627 (*cifA*) and WP_010962722.1 (*cifB*) as references. Predicted start codons were manually
628 checked for the presence of a Shine-Dalgarno-like sequence. To identify whether the *cif*
629 genes form each strain belonged to the same type or not, we performed a phylogenetic
630 analysis following Lindsey *et. al.* 2018. Briefly, the previously used protein sequences were
631 aligned with the newly acquired ones from both wCobs-BR and wCobs-JP. The alignments
632 were then back-translated to nucleotides. For *cifB*, the type IV genes were excluded and
633 the 3'-end of the alignment was manually truncated to remove the longer unmatched
634 regions of some *cifB* variants. Bayesian phylogenetic inference was performed with
635 MrBayes v3.2.6 with the GTR+G substitution model (as suggested by jModelTest) running
636 two independent analyses with four chains each for 1,000,000 generations, and checked
637 for convergence. Following [23], Cif protein domains were searched for using HHpred's
638 v3.2.0 webserver (https://toolkit.tuebingen.mpg.de/tools/hhpred) with default parameters
639 and the following databases: SCOPe70 v2.07, COG/KOG v1.0, Pfam-A v32.0, and SMART
640 v6.0. hits with a probability >=80% were considered.
641
642 All genomic data files are available at the zenodo repository
643 https://doi.org/10.5281/zenodo.3561160
644
645 Histological sectioning and fluorescence in situ hybridization
646 *C. obscurior* (JP) male abdomens were fixed in 4% formaldehyde in PBS and washed with
647 water followed by a dehydration series in n-Butanol (30%, 50%, 70%, 80%, 90%, 96%) at
648 room temperature with shaking for 1 h at each step. Absolute n-butanol was used for the
649 last three hours at 30°C, exchanging the solution each hour. Dehydrated samples were
650 embedded in Technovit 8100 cold polymerizing resin (Heraeus Kulzer, Germany)
651 according to the manufacturer's instructions, cut into semithin sections (8 µm) with a
652 Microm HM355S microtome (Thermo Fisher Scientific, Germany) and mounted on glass
653 slides coated with poly-L-lysine (Kindler, Germany). The tissue sections were incubated for
654 90 min at 50°C in hybridization buffer (0.9 M NaCl, 20 mM Tris/HCl (pH 8.0), 0.01 % SDS)
655 containing 0.5 µM of the *Wolbachia*-specific probes Wolb_W2_Cy5 (5'-

656 CTTCTGTGAGTACCGTCATTATC-3′) [74] and Wolb_Wol3_Cy5 (5′-
657 TCCTCTATCCTCTTTCAATC-3′), [75], as well as the *Cand*. Westeberhardia-specific probe
658 Wcard1_Cy3 (5′- ATCAGTTTCGAACGCCATTC-3′) [10]. DAPI (4′,6-diamidino-2-
659 phenylindole) was used for host DNA counterstaining. After hybridization, samples were
660 washed with buffer (0.1 M NaCl, 20 mM Tris/HCl (pH 8.0), 5mM EDTA, 0.01% SDS) for 20
661 min at 50°C. Subsequently, the washing buffer was removed and washed with distilled
662 water for 20 min at 50°C. Samples were air-dried and covered with VectaShield® (Vector
663 Laboratories, Burlingame, CA, USA) and stored overnight at room temperature. Images
664 were acquired on an AxioImager.Z1 epifluorescence microscope (Carl Zeiss, Jena,
665 Germany).
666
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675
676
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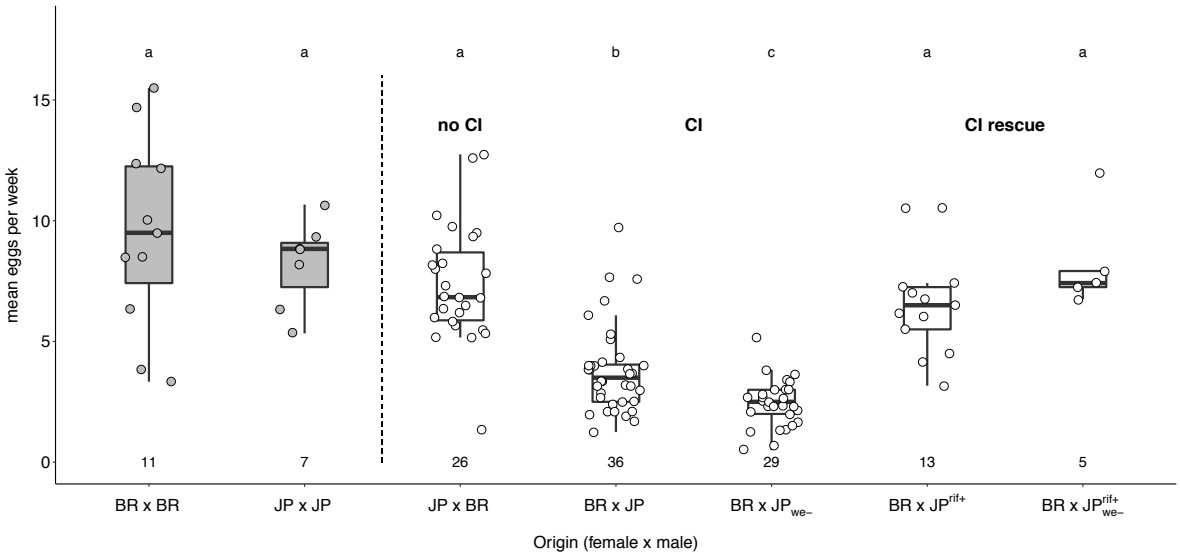

Figure 1: Mean weekly egg numbers produced by intra- and inter-population crosses of the ant *C. obscurior*

Mean weekly egg numbers produced by Brazilian (BR) and Japanese (JP) queens mated to males from their own or from a different population over a period of six weeks. JP ants were collected from colonies that either carried the main endosymbiont *Candidatus* Westeberhardia cardiocondylae (JP) or did not (JP_{we-}). Males used in "CI rescue" crosses were collected from JP colonies with and without *Cand.* Westeberhardia cardiocondylae, which had been treated with the antibiotic rifampicin (JP^{rif+}; $JP_{we-}{}^{rif+}$). Numbers below box plots indicate the number of replicates. Differences between groups were tested with pairwise Mann-Whitney-U-tests followed by Bonferroni-Holms correction of p-values. Letters above boxplots indicate statistically significant differences at $p<0.05$. For Bonferroni-Holms corrected pairwise p-values see Table S1.

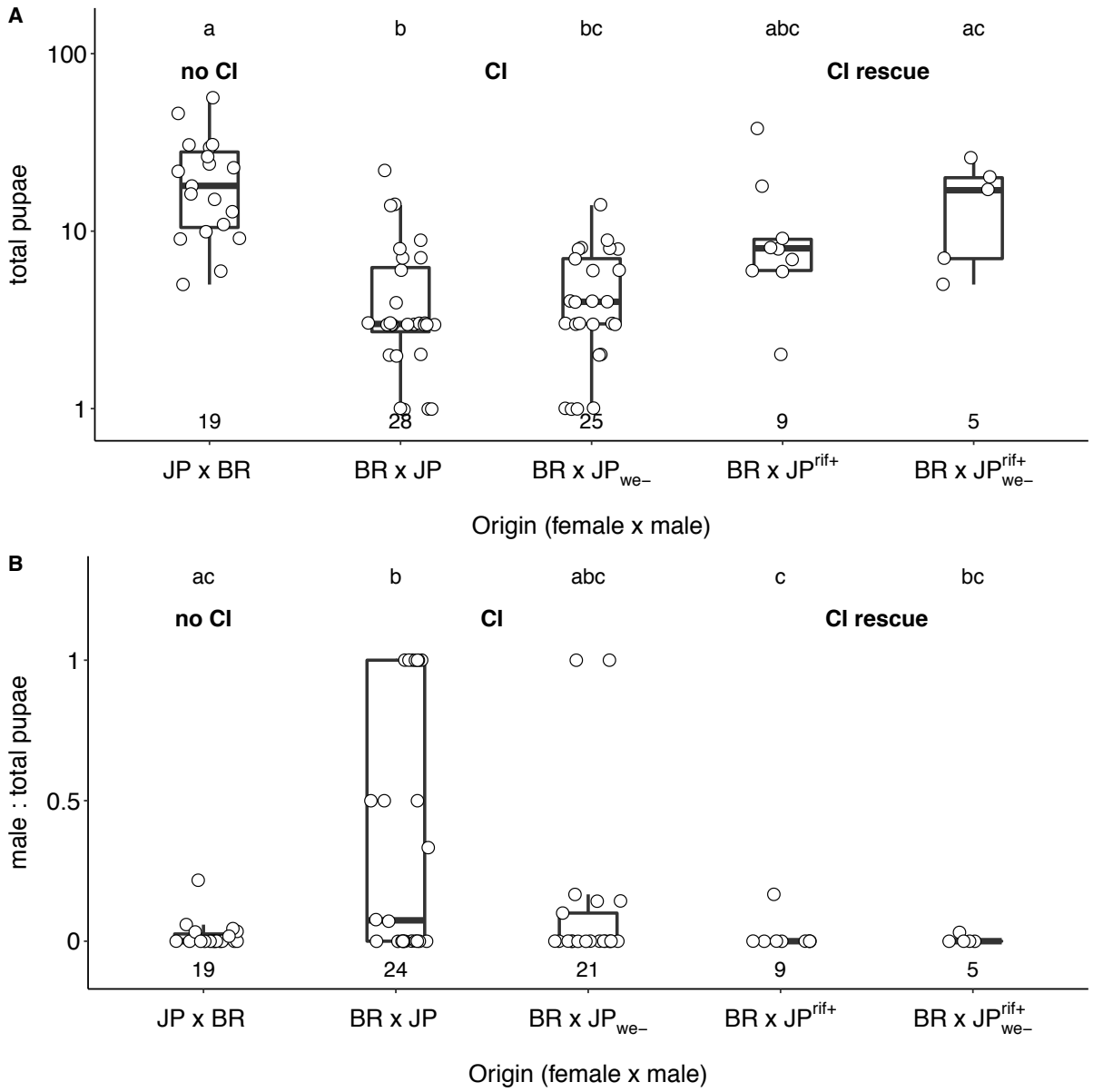

Figure 2: Total pupae numbers and pupae sex ratios produced by intra- and inter-population crosses of the ant *C. obscurior*

Total number of pupae (A) and sex ratio of pupae (B) produced in crosses between Brazilian (BR) and Japanese (JP) queens and males over a period of 12 weeks. JP ants were collected from colonies that either carried the main endosymbiont *Candidatus* Westeberhardia cardiocondylae (JP) or did not (JP_{we-}). Males used in "CI rescue" crosses were collected from JP colonies with and without *Cand.* Westeberhardia cardiocondylae, which had been treated with the antibiotic rifampicin (JP^{rif+}; JP_{we-}^{rif+}). Numbers below box plots indicate the number of replicates. Total pupae numbers are plotted as log +1 for better visualization and differences between groups were tested with pairwise Mann-Whitney-U-tests followed by Bonferroni-Holms correction of p-values. For Bonferroni-Holms corrected pairwise p-values see Table S3. Sex ratios produced by different crosses were compared with a generalized linear model with logit link followed by manual Bonferroni-Holms correction of p-values. For Bonferroni-Holms corrected pairwise p-values see Table S4. Letters above boxplots indicate statistically significant differences at $p<0.05$.

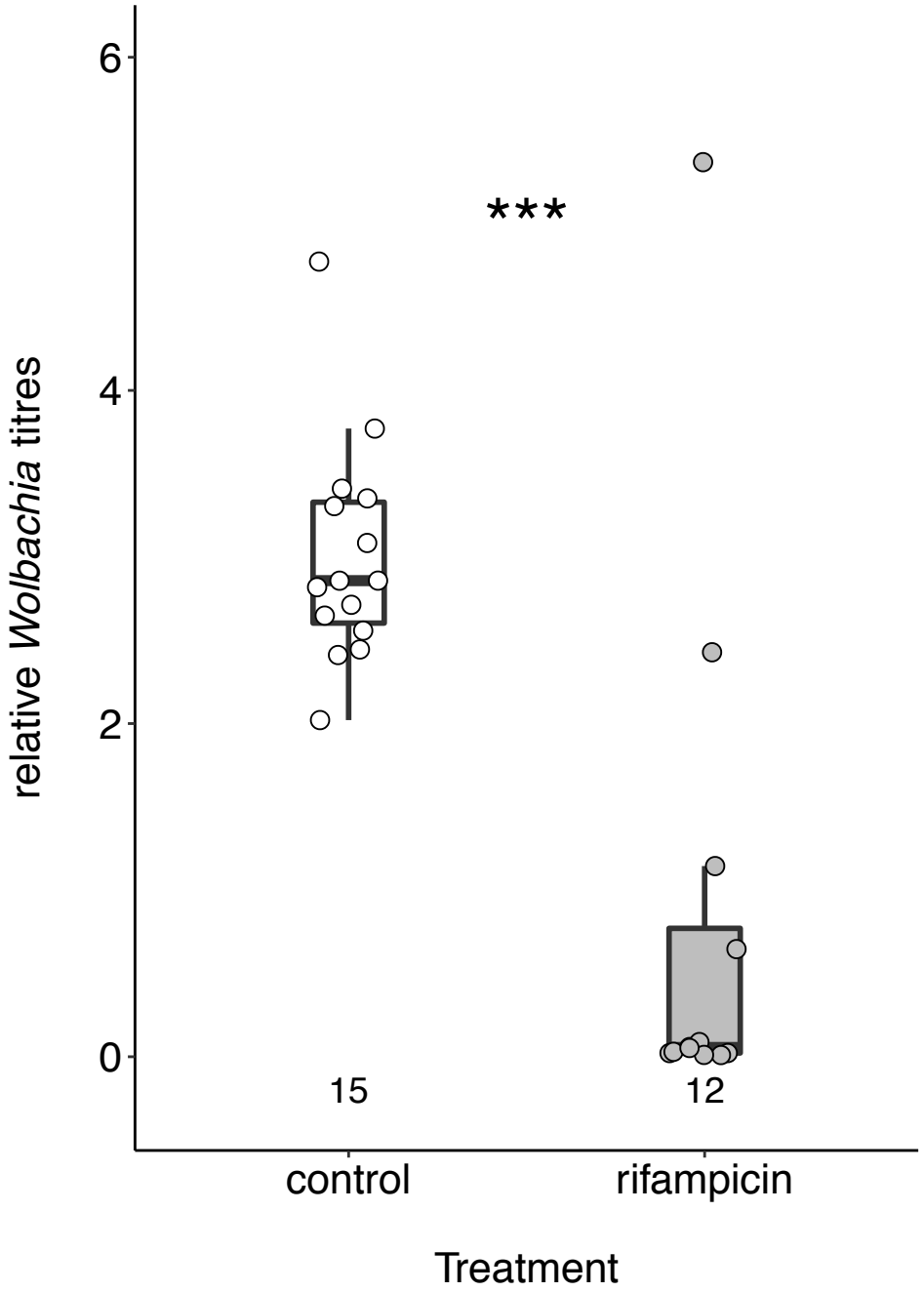

Figure 3: Effects of rifampicin treatment on *Wolbachia* titres in males
All males were collected from colonies that carried the main endosymbiont *Candidatus* Westeberhardia cardiocondylae. Numbers below box plots indicate the number of replicates. Differences in *Wolbachia* titres between control and rifampicin-treated males were tested with a Mann-Whitney-U-Test. Stars indicate statistically significant differences at $p<0.001$ (***).

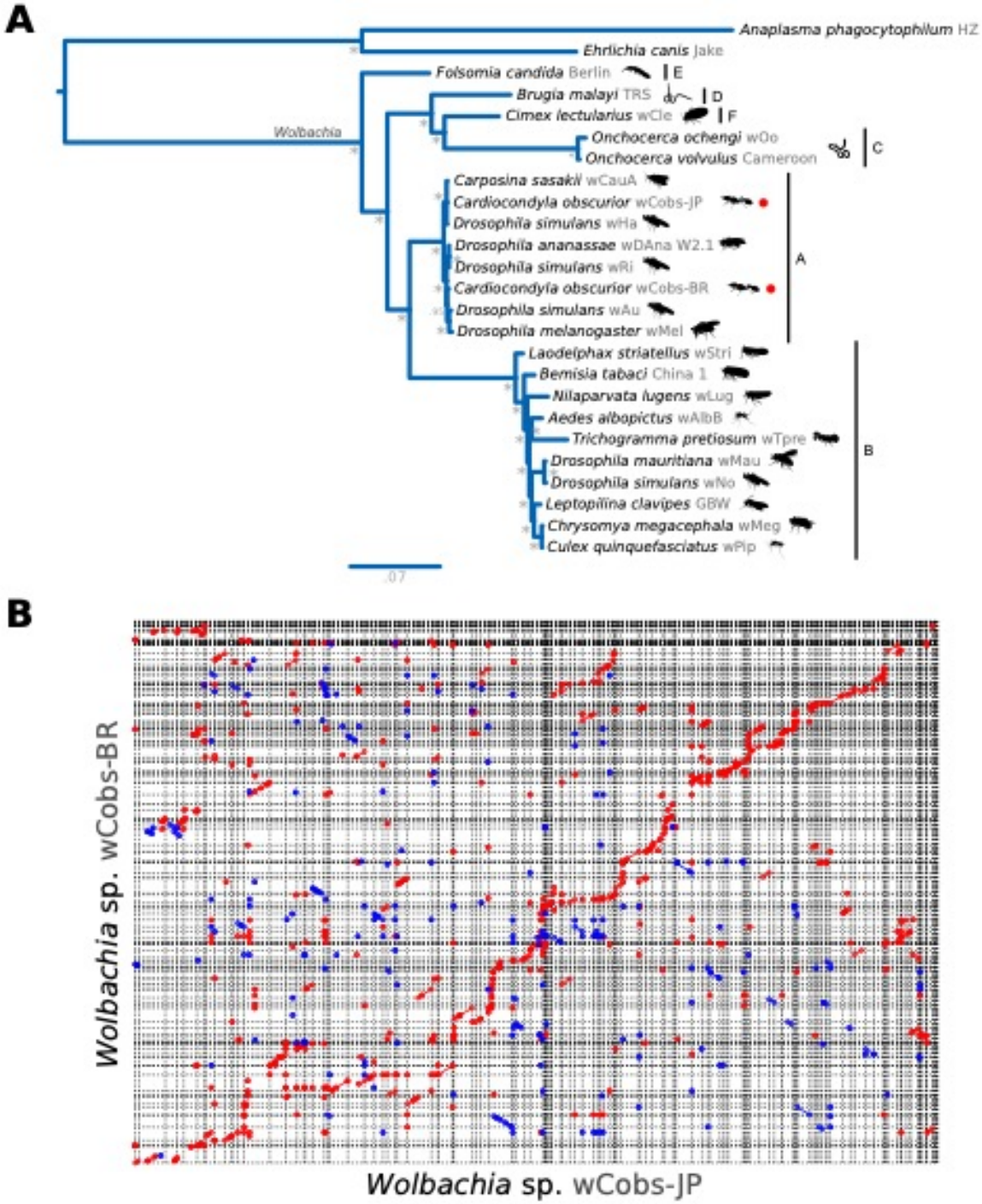

Figure 4: *Wolbachia* spp. phylogeny and genome synteny

(A) Bayesian phylogenetic placement of *Wolbachia* strains sequenced from *C. obscurior* (*w*Cobs-BR and *w*Cobs-JP). *Anaplasma phagocytophilum* and *Ehrlichia canis* were used as outgroups. For *Wolbachia*, names at tips correspond to the host from which they were sequenced/isolated. Red dots are used to highlight the newly sequenced strains. Next to the binomial names, strain names are shown in grey and silhouettes for the host are sketched in black. Horizontal bars delimit *Wolbachia* supergroups (A-F). *=1.0 posterior probability. (B) Genome-wise nucleotide-based synteny plot between *w*Cobs-BR and *w*Cobs-JP. Red is used to indicate a direct match and blue a reverse complement match.

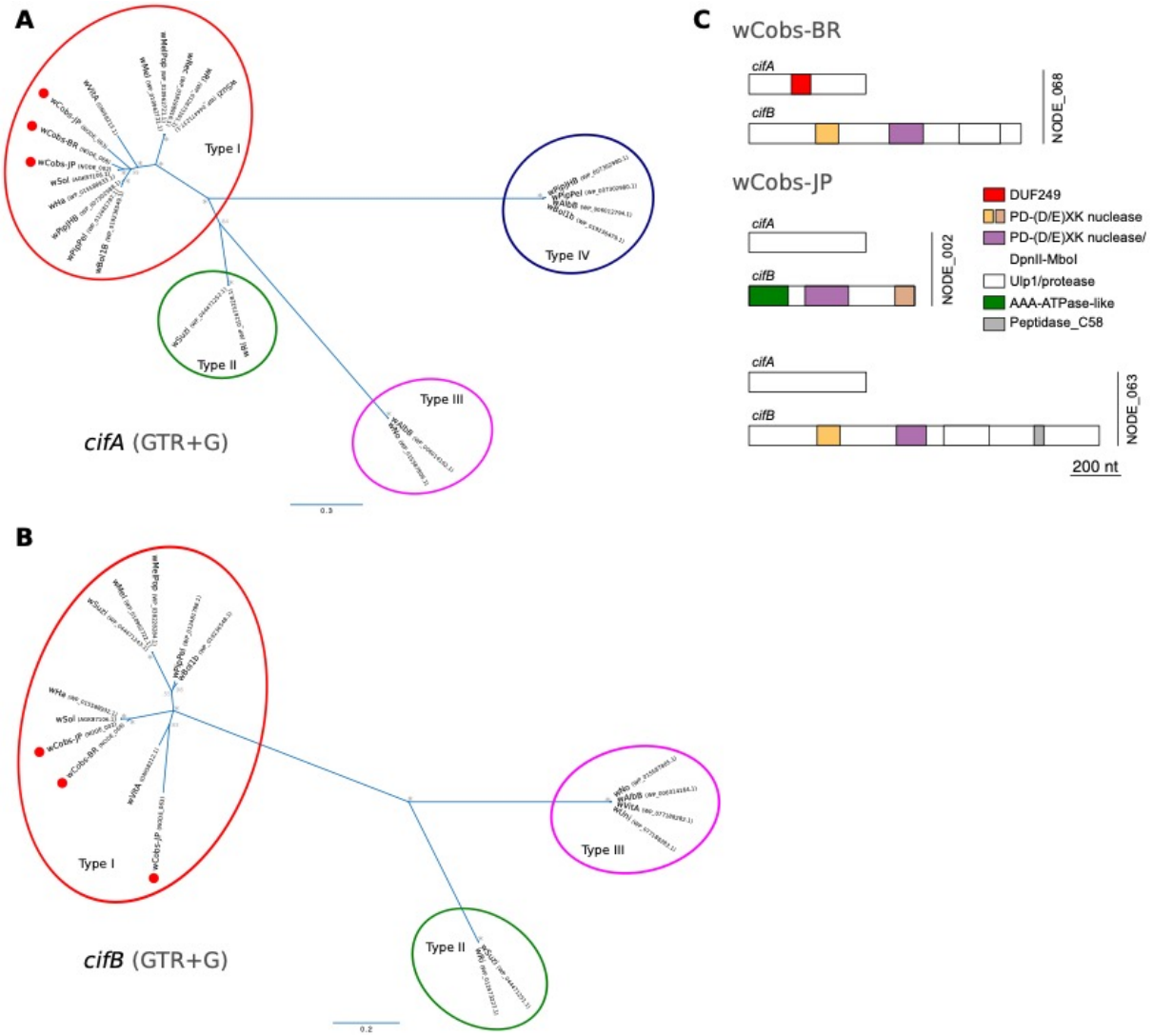

Figure 5. Phylogeny and domain structure of *cif* genes

Bayesian phylogenetic trees for (A) *cifA* and (B) *cifB* genes. Colored ovals are used to highlight the different gene "types". *= 1.0 posterior probability. (C) Domain structure for the *cif* genes of *w*Cobs-BR (top) and *w*Cobs-JP (bottom) strains. For the latter, the two loci are shown and indicated with a vertical bar to the right of the sketches. Color codes and designation of the domains are shown in the legend.

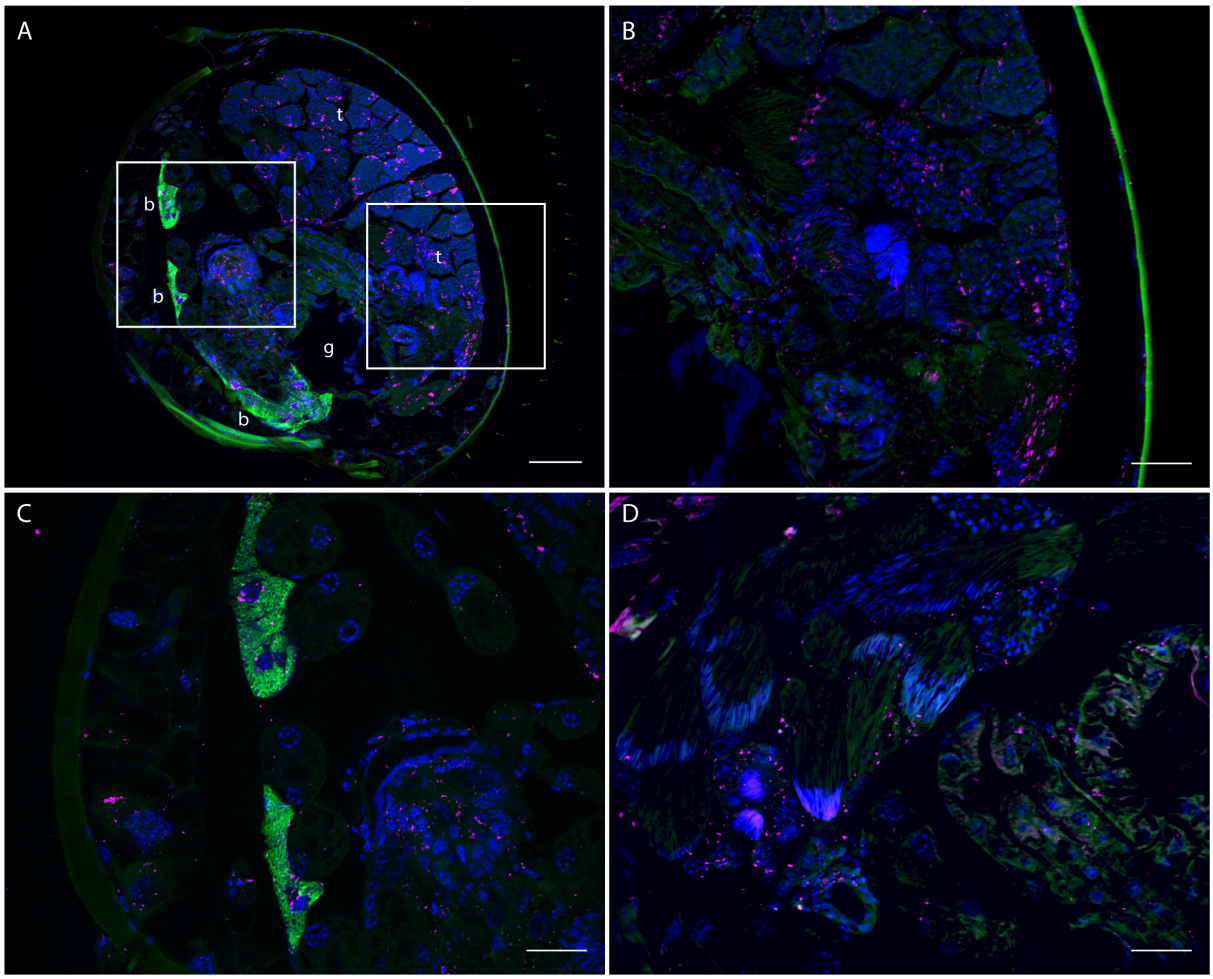

Figure 6: Localization of endosymbiotic bacteria in male adult abdomens of *C. obscurior* via fluorescence in situ hybridization.

Wolbachia were specifically stained with two Cy5-labeled probes (magenta), and *Cand.* Westeberhardia with a Cy3-labeled probe (green). Host cell nuclei are counterstained with DAPI (blue). (A) Transversal section of *C. obscurior* JP male abdomen showing the testes (t), the *Cand.* Westeberhardia-containing bacteriomes (B) and the gut (g). The areas indicated by rectangles are displayed in higher magnification showing *Wolbachia*-infected seminiferous tubules (B) and bacteriomes co-infected with *Cand.* Westeberhardia and *Wolbachia* (C). (D) *C. obscurior* BR male testes and gut epithelium infected with *Wolbachia*. Scale bars: 50 µm (A) and 20 µm (B-D).

Table 1: Wolbachia genome assembly statistics

Genome assembly and draft annotation statistics for the newly sequenced *Wolbachia* strains from *C. obscurior* (*w*Cobs-BR and *w*Cobs-JP).

	Wolbachia	
	wCobs-BR	**wCobs-JP**
Assembled genome size (Mbp)	1.19	1.30
Number of scaffolds	148	182
N50	14,698	16,173
G+C content (%)	35.3	35.1
Predicted CDSs*	1,013	1,058
tRNAs	34	34
rRNAs	3	3
Other ncRNAs genes/motifs	71	54