Wolbachia: master manipulators of invertebrate biology

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Abstract | *Wolbachia* are common intracellular bacteria that are found in arthropods and nematodes. These alphaproteobacteria endosymbionts are transmitted vertically through host eggs and alter host biology in diverse ways, including the induction of reproductive manipulations, such as feminization, parthenogenesis, male killing and sperm–egg incompatibility. They can also move horizontally across species boundaries, resulting in a widespread and global distribution in diverse invertebrate hosts. Here, we review the basic biology of *Wolbachia*, with emphasis on recent advances in our understanding of these fascinating endosymbionts.

Mutualism
A symbiotic relationship in which both partners benefit.

Commensalism
A symbiotic relationship in which neither partner benefits or is harmed.

Parasitism
A symbiotic relationship in which one partner benefits at the expense of the other.

Clade
A group of genetically related organisms that includes an ancestor and all of its descendants.

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Anton de Bary[1] originally defined symbiosis as the living together of dissimilar organisms. This definition encompasses a broad range of interactions, from mutualism (beneficial) to commensalism (neutral) and parasitism (harmful). These broad categories are actually a continuum, and shifts in symbiotic interactions along the continuum can occur during evolution and even between individual organisms under changing circumstances. Furthermore, symbiotic relationships can have a mixture of mutualistic, commensal and parasitic features[2,3]. To better understand the nature of microbial symbioses with eukaryotic hosts, it is useful to study microorganisms that participate in diverse symbiont–host interactions. *Wolbachia* are members of the order Rickettsiales, a diverse group of intracellular bacteria that comprises species with parasitic, mutualistic and commensal relationships with their hosts. The related genera *Anaplasma, Ehrlichia* and *Rickettsia*, typically have life cycles that include an invertebrate 'vector' and mammalian 'host', although strictly invertebrate-associated species are also found[4]. However, unlike members of these other genera, *Wolbachia* do not routinely infect vertebrates. *Wolbachia* have attracted considerable interest in the past decade primarily because of their vast abundance, fascinating effects on hosts, which range from reproductive manipulation to mutualism, and potential applications in pest and disease vector control[5]

The type species for the *Wolbachia* genus is *Wolbachia pipientis*, which was first described in the mosquito *Culex pipiens*[6]. Based on 16S ribosomal sequences and other sequence information, *Wolbachia* spp. have so far been divided into eight different supergroups (A–H)[7], although further study is required to confirm the status of some of these[8]. All *Wolbachia* supergroups are monophyletic compared with other Rickettsiales (FIG. 1). Two supergroups (C and D) are commonly found in filarial nematodes, whereas the other six supergroups are found primarily in arthropods, in which A and B are the most common.

Nematode-associated *Wolbachia* show a general concordance between the phylogeny of the bacteria and the phylogeny of their hosts, and all these *Wolbachia* have evolved mutualisms with their hosts. This pattern is also found with many other vertically inherited endosymbionts, such as *Buchnera aphidicola*, the obligate intracellular symbionts of aphids[9]. By contrast, *Wolbachia* that participate in symbiotic relationships with arthropods have a range of phenotypic effects on their hosts, and generally behave as reproductive parasites. There is no concordance between the phylogeny of arthropod *Wolbachia* and the phylogeny of their hosts, which is indicative of extensive lateral movement of *Wolbachia* between host species. Furthermore, resolving the relationships between strains is further complicated by extensive recombination, even among some supergroups[8,10–13].

There has been debate in the *Wolbachia* field as to whether all bacteria within the *Wolbachia* clade should be given the *W. pipientis* designation or whether a different species nomenclature should be applied. Until this issue is resolved, the convention has been to refer to the bacteria as *Wolbachia,* with strain designations that are based on host and supergroup identification.

Until the early 1990s, *Wolbachia* were considered to be members of a rare and inconsequential bacteria genus. However, with the advent of molecular typing methods, *Wolbachia* were found to be widespread and common in insects, and subsequently also in other arthropods (for

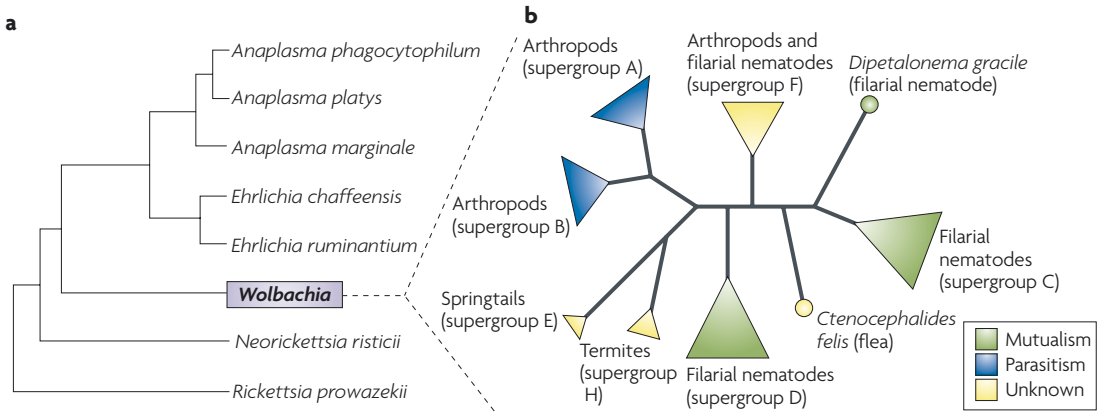

Figure 1 | **Phylogeny of *Wolbachia*. a** | The phylogenetic relationships of *Wolbachia* relative to the closely related Rickettsiales order in the Anaplasmataceae family. **b** | An unrooted phylogenetic tree of the main supergroups of *Wolbachia*. Also shown are the dominant patterns of mutualism and reproductive parasitism across the supergroups. For some supergroups, functional effects of *Wolbachia* have not yet been determined. The G supergroup has been removed because its status is currently unclear[8]. The pattern suggests that the main supergroups of *Wolbachia* participate in either mutualism or reproductive parasitism. Rooting of the *Wolbachia* phylogeny, which could help resolve whether mutualism or reproductive parasitism is ancestral, is problematic owing to long-branch attraction to out-groups. Resolution requires genome-sequence information for additional taxa. Triangle size represents described diversity within each lineage. Circles represent a lineage based on a single *Wolbachia* strain. Part **a** reproduced from REF. 98. Part **b** reproduced, with permission, from REF. 99 © (2007) Society for General Microbiology.

example, mites, spiders, scorpions and isopods), as well as filarial nematodes. A recent meta-analysis estimated that >65% of insect species harbour *Wolbachia*[13], making it among the most abundant intracellular bacteria genus so far discovered, infecting at least 10^6 insect species alone.

Together with their pandemic distribution, another interesting feature of *Wolbachia* is the various host manipulations they induce (FIG. 2). The effects of *Wolbachia* infection include: feminization of genetic males; parthenogenetic induction, which results in the development of unfertilized eggs; the killing of male progeny from infected females; and sperm–egg incompatibility, which is referred to as cytoplasmic incompatibility (CI). Each of these reproductive alterations is adaptive for the bacterium by enhancing the production of infected females, and collectively, these strategies are referred to as reproductive parasitism. In addition to parasitism, *Wolbachia* have also evolved mutualistic interactions with their filarial hosts, and show a range of other host effects. *Wolbachia* are highly adapted for living within invertebrate cells, which probably partly explains their wide distribution. For example, they are known to use the spindle apparatus during cell division[14] and dynein and kinesin motors to shuttle within host cells[15,16], which allows efficient transmission during cell division and within the germ cells.

Key questions that relate to *Wolbachia* include: what is their genetic architecture and what genetic tools do they possess; how do they alter the reproductive and cellular processes of their hosts; how are their infections maintained globally; do they accelerate host evolution; and can they be applied to pest and disease control? The focus of this Review will be on recent studies that are relevant to these questions.

Meta-analysis
A method for combining results from separate, related studies.

Feminization
A process in which a male acquires female characteristics.

Wolbachia genomics and genetics

Two fully annotated *Wolbachia* genomes are now available: the CI-inducing *w*Mel strain from the arthropod host *Drosophila melanogaster* and the mutualistic *w*Bm strain from the filarial nematode host *Brugia malayi*[17,18]. Several other genomes that are representative of the phenotypic diversity of *Wolbachia* are currently undergoing sequencing or full annotation, whereas others have been partially assembled from invertebrate sequencing projects[19] (TABLE 1). Together with the recent completion of more than ten closely related Rickettsiales genomes, these data are providing valuable comparative support for investigating the evolution of these bacteria.

Wolbachia have small genomes (1.08–1.7 Mb) that are within the range of the Rickettsiales (0.8–2.1 Mb) and are in accordance with a reductive trend following host adaptation. However, *Wolbachia* genomes lack the typical minimal genome content and high stability that is observed in other obligate endosymbionts, such as species of *Buchnera* and *Candidatus* Blochmannia[20]. Unlike most Rickettsiales, *Wolbachia* genomes contain a high number of mobile and repetitive elements. Repeats make up more than 14% of the *w*Mel genome, which in the Rickettsiales is second only to *Orientia tsutsugamushi*. A high number of these repeats are represented by ankyrin (ANK) domains, which are common among eukaryotes but unusual in bacteria, in which they mediate host–pathogen protein interactions[17]. Additional redundancy of the genome comes from extensive duplications of short open reading frames (ORFs) that have a predicted but unknown function (hypothetical proteins) and surface proteins; together these two classes of genes provide *Wolbachia* genomes with most of their genetic distinctiveness[17].

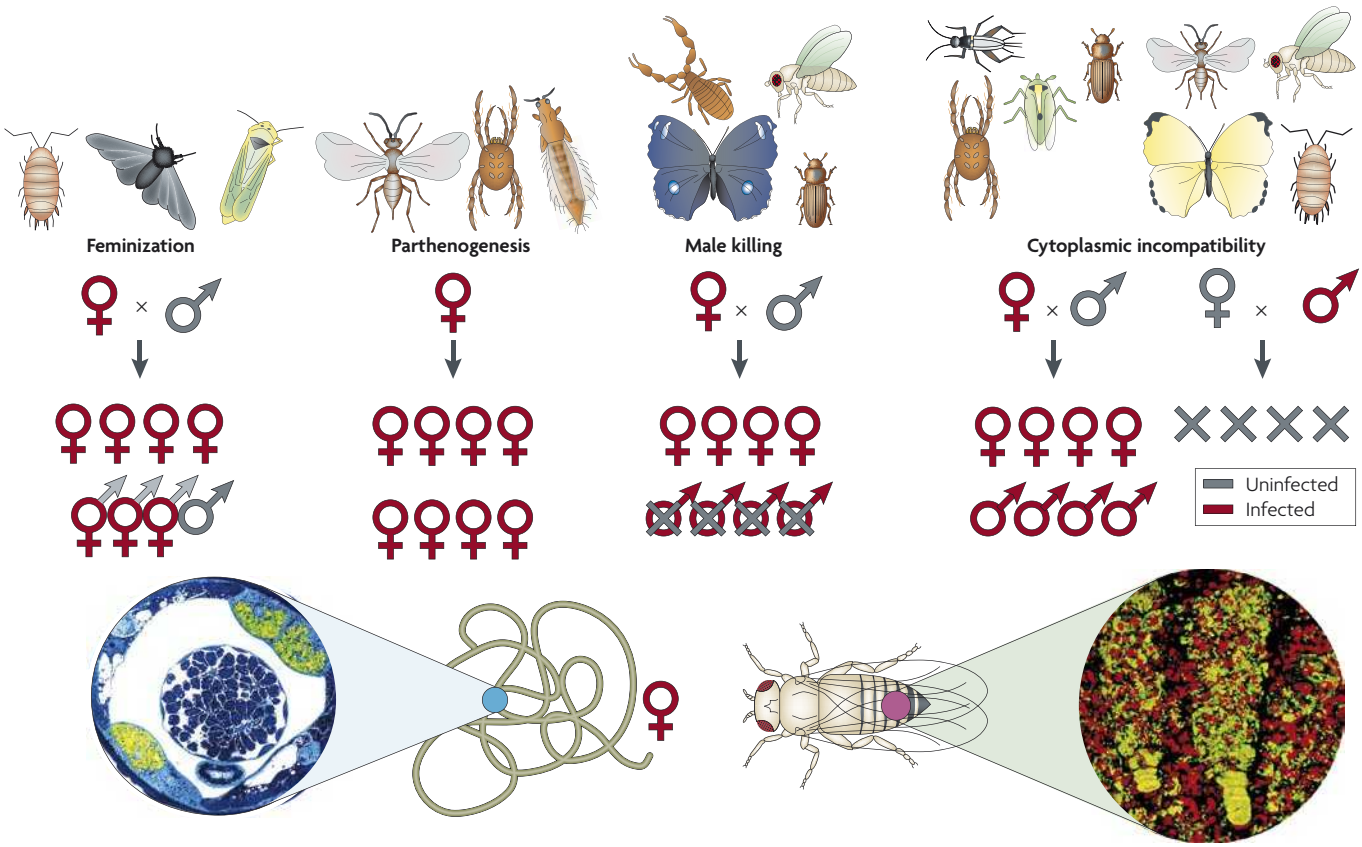

Figure 2 | ***Wolbachia*-induced phenotypes.** *Wolbachia* cause four distinct reproductive phenotypes in a range of arthropod orders (top). Feminization results in genetic males that develop as females (in the Hemiptera, Isopoda and Lepidoptera orders). Parthenogenesis induction eliminates males from reproduction (in the Acari, Hymenoptera and Thysanoptera orders). Male killing eliminates infected males to the advantage of surviving infected female siblings (in the Coleoptera, Diptera, Lepidoptera and Pseudoscorpiones orders). Cytoplasmic incompatibility prevents infected males from successfully mating with females that lack the same *Wolbachia* types (in the Acari, Coleoptera, Diptera, Hemiptera, Hymenoptera, Isopoda, Lepidoptera and Orthoptera orders). A cross section of a male filarial nematode, *Onchocerca ochengi*, that contains *Wolbachia* is shown (bottom left), in which *Wolbachia* are falsely coloured yellow and fill three of the four syncytial lateral cord cells. *Wolbachia* (yellow) are also shown within the ovaries of a female *Drosophila simulans* (bottom right). The image on the bottom left is courtesy of M. Taylor, Liverpool School of Tropical Medicine, UK. The image on the bottom right is courtesy of M. Clark, University of Rochester, New York, USA.

In addition to repetitive elements, a striking peculiarity of the *w*Mel genome compared with other Rickettsiales genomes is the presence of various virus-like elements (51 based on current annotation), including the lambda bacteriophage WO sequences. Homologous WO sequences have only been found in *Rickettsia bellii*, which implies either independent acquisition in both *w*Mel and *R. bellii* or massive loss of these elements in most Rickettsiales. In *Wolbachia*, some of these phage elements are actively transcribed and expressed through a lytic cycle[21]. Several ANK proteins have been found to be integrated in prophage segments, suggesting that bacteriophages have a key role in introducing and then spreading ANK genes within *Wolbachia*.

Overall, the presence or absence of repeats and mobile elements is not consistent with classification into parasitic or mutualistic strains among Rickettsiales. However, it is interesting that *Wolbachia* which participate in mutualistic interactions with nematodes have a lower number of repetitive elements and do not carry phage sequences[18,22]. Some evidence suggests that ANK and phage genes are involved in *Wolbachia* cellular interactions with their hosts. Indeed, although a direct correlation of the ANK and prophage genotype or gene content with the bacteria phenotype remains unclear, their expression profiles seem to be associated with the reproductive phenotypes. This indicates that both ANK and prophage genes are promising candidates for the study of reproductive manipulations[23,24]. Supporting this scenario, a recent study has shown that bacterial ANK repeat proteins in *Legionella pneumophila* are delivered into the eukaryotic cells by the type IV secretion system, where they target host factors that are important for the bacterial infections[25]. Other candidates for the study of *Wolbachia*–host interactions include three major surface proteins that have been identified in *Wolbachia*: Wsp and its two paralogues WspA and WspB. These proteins are homologous to outer-membrane proteins that have been shown to have antigenic function in important pathogenic bacteria (such as species of *Ehrlichia* and *Neisseria*).

Table 1 | ***Wolbachia* genome projects***

Strain	Host	Supergroup	Phenotype‡	Genome size (Mb)	Status	Refs or project leaders
*w*Mel	*Drosophila melanogaster*	A	Cytoplasmic incompatibility	1.27	Complete	94
*w*Bm§	*Brugia malayi*	D	Mutualist	1.08	Complete	18
*w*MelPop	*D. melanogaster*	A	Cytoplasmic incompatibility	1.3	Assembled	S. O'Neill
*w*Pip	*Culex pipiens*	B	Cytoplasmic incompatibility	1.48	Assembled	J. Parkhill and S. Sinkins
*w*Ri	*Drosophila simulans*	A	Cytoplasmic incompatibility	1.44	Assembled	S. Andersson, R. Garrett and K. Bourtzis
*w*Ana	*Drosophila ananassae*	A	Cytoplasmic incompatibility	Unknown	Unfinished	19
*w*Sim	*D. simulans*	A	Cytoplasmic incompatibility presumed	Unknown	Unfinished	19
*w*Au	*D. simulans*	A	Not cytoplasmic incompatibility	Unknown	Unfinished	S. O'Neill
*w*Wil	*Drosophila willistoni*	A	Unknown	Unknown	Unfinished	J. Craig Venter Institute
*w*VitA	*Nasonia vitripennis*	A	Cytoplasmic incompatibility	Unknown	In progress	J. Werren and S. Richards
*w*Uni	*Muscidifurax uniraptor*	A	Parthogenesis	Unknown	In progress	S. Anderson and K. Bourtzis
*w*Bol1	*Hypolimnas bolina*	B	Male killing	~1.6	In progress	A. Duplouy and S. O'Neill
*w*Vul	*Armadillidium vulgare*	B	Feminization	~1.7	In progress	R. Garrett, P. Greve, D. Bouchon and K. Bourtzis
None designated	*Diaphorina citri*	B	Unknown	Unknown	In progress	W. Hunter, Y. Ping Duan, R. Shatters and D. Hall
*w*Dim	*Dirofilaria immitis*	C	Mutualist	~1.0	In progress	C. Bandi and B. Slatko
*w*Ov	*Onchocerca volvulus*	C	Mutualist	~1.1	In progress	M. Taylor, M. Blaxter and B. Slatko

*Several *Wolbachia* genome projects have been completed or are underway. The goal is to compare genomic features among *Wolbachia* to provide insights into mechanisms of genome evolution and mechanisms of host alteration by *Wolbachia*. ‡Genome sequences are becoming available for a range of phenotypic effects on hosts for most of the major supergroups. §The genome of the *Wolbachia* strain that was found in the filarial nematode *B. malayi* is being used to identify possible targets for pharmacological suppression of these *Wolbachia* and their pathogenic host (see Further information for a link to the Anti-*Wolbachia* Project).

Wsp has recently been shown to elicit an immunological response in vertebrate hosts that are infected with filarial nematodes which harbour *Wolbachia*[26]. The function of Wsp in arthropods is unknown.

Role of recombination and diversity

Considerable information on the genetic architecture and evolution of *Wolbachia* is now emerging from studies of strain diversity. The ambiguity concerning the nomenclature of *Wolbachia* has long been a major impediment for organizing and understanding strain diversity, owing to the vast number of insect species that can be infected and the fact that various distinct strains can be found in a single host species. In addition, the discovery of extensive recombination in *Wolbachia* has challenged traditional phylogenetic methods for characterizing strains and their relationships, and has therefore created a need for a standardized and unambiguous typing system. A multilocus sequence typing (MLST) scheme for *Wolbachia* has been recently developed (BOX 1) to provide a standard tool for typing and organizing strain diversity, and promises an unprecedented comparative dataset for studying *Wolbachia* diversity, ecology and evolution[11].

Wolbachia possess remarkable genetic diversity: nucleotide divergence ranges from 6% to 9% at housekeeping genes (according to MLST[11]) and can be up to 30% in prophage ORF7 (REF. 27) and >43% at *wsp*[28]. A key and unexpected finding is that *Wolbachia* undergo extensive recombination between strains, which affects various regions of the genome, including surface proteins (such as Wsp) and housekeeping and prophage genes, as well as intergenic regions[12,28]. The mechanisms and role of recombination in *Wolbachia* evolution are currently under investigation. As breakpoints are not necessarily coupled to gene boundaries, recombination in *Wolbachia* would occur, in part, as a random replacement of homologous sequences. Several theoretical and experimental studies have shown that recombination is a major force that can accelerate genetic and functional diversity and enable bacterial adaptation[29]. In *Wolbachia*, increased recombination is observed in the surface-protein gene *wsp*, and the Wsp protein is likely to mediate interactions with the host cell. Although recombination rates require further elucidation, recombination among *Wolbachia* is compatible with both the high genetic diversity in the genus and the genetic cohesiveness of *Wolbachia* strains with respect to more distant out-groups. Recombination

Box 1 | **Multilocus sequence typing (MLST)**

Wolbachia MLST (see Further information for a link to *Wolbachia* MLST Databases) uses five housekeeping genes (*gatB*, *coxA*, *hcpA*, *ftsZ* and *fbpA*) that are broadly distributed across the genome as a core set of markers for strain genotyping. Each strain is characterized by the allelic profile of the five alleles at the MLST loci, which defines its sequence type (ST). The MLST database contains both sequences and allelic profiles, and host and strain biological information. Each strain is assigned a unique identification that refers to its specific host taxonomical, geographical and biological information. The database is rapidly growing, and to date more than 200 strains have been characterized by MLST, with more than 140 distinct STs, reflecting the extensive diversity of *Wolbachia*. MLST promises to be an invaluable resource for studies of *Wolbachia* genetics, ecology and evolution. The *Wolbachia* surface protein (Wsp), which is divided into four hypervariable regions (HVRs), is used as an additional marker for genetic diversity, and a database has been implemented that allows typing of alleles and individual HVR peptides.

may have a role in moving the genetic machinery for different host manipulations between strains, although this has not been definitively established.

Phenotypic effects and host interactions

A key feature of *Wolbachia* is their ability to live within and manipulate cellular and reproductive processes in invertebrates. This no doubt reflects their long evolutionary history as intracellular bacteria. However, *Wolbachia* seem to be unusual in the suite of manipulations they employ. Only one other bacterial group, the genus *Candidatus* Cardinium (of the Bacteroidetes class), has been found to have a similarly diverse set of reproductive manipulations[30]. Here, we summarize some of the effects of *Wolbachia* infection.

Cytoplasmic incompatibility. CI is the most frequently found *Wolbachia*-induced phenotype and has been described in several arachnids, isopods and insect orders. Sperm from *Wolbachia*-infected males is incompatible with eggs from females that do not harbour the same *Wolbachia* type (or types). CI comprises two distinct components: *Wolbachia*-induced modification of sperm during spermatogenesis and rescue of this modification in embryos infected with the same strain. If the sperm is modified, but the appropriate *Wolbachia* are not present in the developing embryo, embryonic development is disrupted[31].

The molecular mechanisms that underlie CI remain unknown, despite considerable work on the effect and various proposed mechanisms[32]. However, similar cytological manifestations of CI have been described in detail in several host taxa (FIG. 3). Common to each host taxon examined are defects in early embryonic mitosis owing to disruption of the cell cycle, which results in asynchronous development of male and female pronuclei[33–35]. The incompatible cross is due to the asynchrony of the male and female pronuclei at the initial stage of mitosis; the delay of male nuclear envelope breakdown and histone H3 phosphorylation (a histone modification that is required for the initiation of mitosis) indicates that the activity of Cdk1, a key kinase that drives the cell into mitosis, is delayed in the male pronucleus[34]. As a result, chromatids from the female pronuclei are properly condensed and lie at the first metaphase plate, but male pronuclear chromosomes are only in a semi-condensed state. During anaphase, the female chromosomes separate normally, whereas the male pronuclei are either stretched to the centrosome poles or excluded entirely[33,36]. The easiest way to cytologically distinguish an incompatible cross is to look for chromatin bridges between the nuclei at anaphase. A result of the incompatible cross is often haploid development, which has been observed in flies, wasps and mosquitoes. In diploid organisms, this normally results in embryonic lethality, but in haplodiploids, haploidy can result in normal male development[35].

Crossing data indicate that different *Wolbachia* strains can have different modification–rescue mechanisms[31]. Crosses between different strains can therefore sometimes result in bidirectional incompatibility, whereas crosses between infected males and uninfected females yield unidirectional incompatibility. A recent study[37], in which *Wolbachia* from different *Drosophila* species were transferred into a common host genotype (*Drosophila simulans*), indicated that there is a complex relationship between the ability of one *Wolbachia* type to rescue the modification caused by another *Wolbachia* type. One *Wolbachia* type may either fully or partially rescue the modification caused by a different *Wolbachia* variant, or may not do so at all. The authors suggest that rather than only using a simple one-locus modification and rescue model, individual *Wolbachia* strains can have more than one modification–rescue type. There is also evidence to indicate that the genotype of the host can influence the level and form of CI, as at least one *Wolbachia* variant was unable to fully rescue the modification that it induced after transfer into a novel host species, which constituted a so-called 'suicide' infection[37]. However, despite extensive studies, the mechanism of CI remains unknown.

Parthenogenesis induction. *Wolbachia*-induced female parthenogenesis (thelytoky) is less common than CI, and so far has only been documented in species with arrhenotokous development (in which males develop from unfertilized eggs), such as mites, hymenopterans (for example, wasps) and thrips[38–40]. Instead of producing sons from unfertilized eggs, infected females produce daughters, which unlike males are able to transmit the bacteria to their offspring. Like CI, *Wolbachia*-induced parthenogenesis is caused by disruption of the cell cycle during early embryonic development, which results in diploid development in unfertilized eggs (thelytoky). In both *Trichogramma* sp. and *Leptopilina clavipes*, anaphase is abortive during the first embryonic division, resulting in one diploid nucleus rather than two haploid

Haplodiploid
A sex-determining mechanism that is found in some insect groups, in which males are haploid and females are diploid.

Parthenogenesis
An asexual form of reproduction that is found in females, in which growth and development of embryos occurs without fertilization by males.

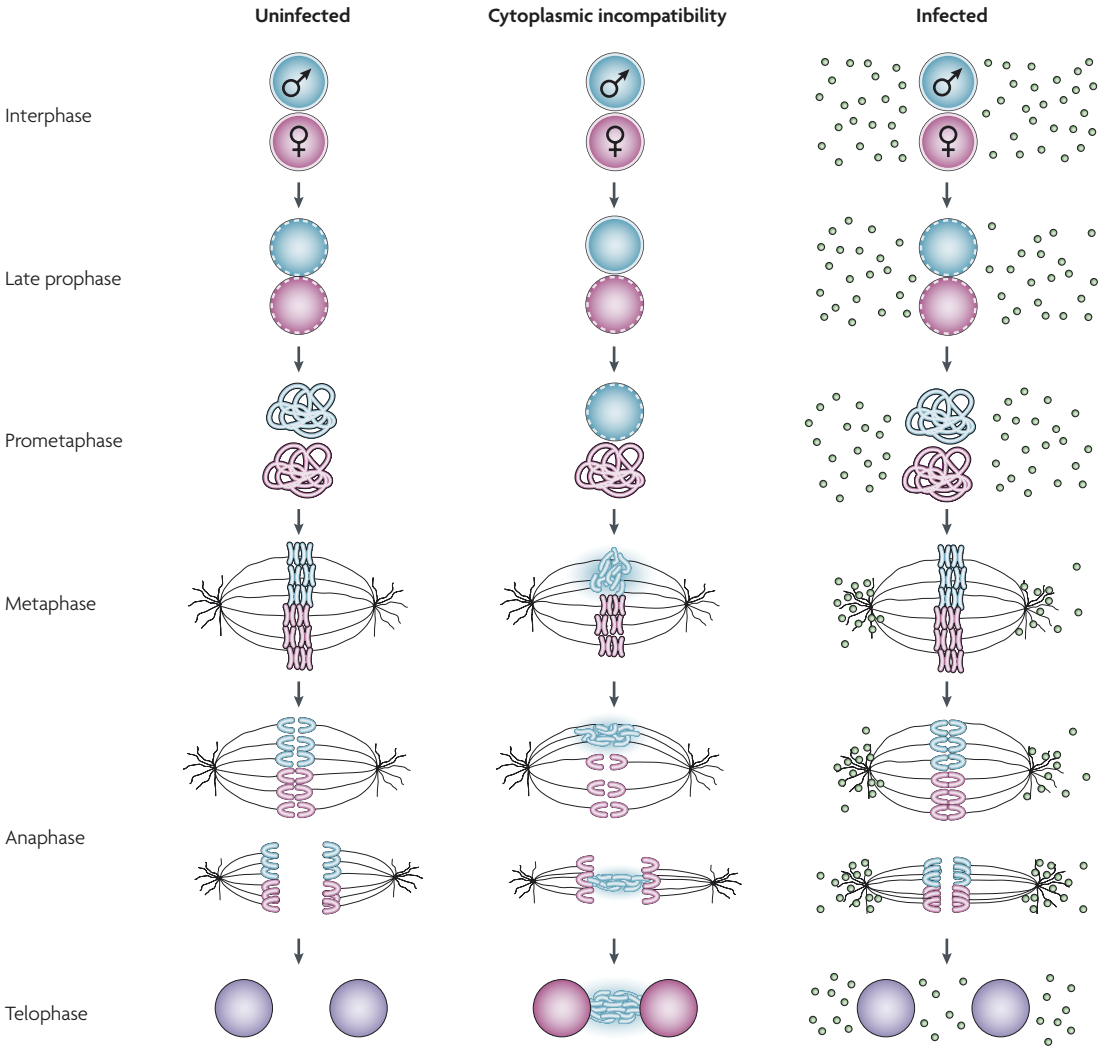

Figure 3 | **The cytological basis of *Wolbachia*-induced cytoplasmic incompatibility.** In a cytoplasmic incompatibility cross (middle column), asynchrony is observed in the development of paternal (blue) and maternal (pink) pronuclei at the first embryonic mitotic division. Breakdown of the nuclear envelope in the male pronuclei, as well as chromatin condensation, lags behind the female pronuclei. At metaphase, paternal chromosomes are not fully condensed, and at anaphase, paternal chromosomes do not properly segregate. Pronuclear synchrony and normal development is restored in an infected embryo (right column). Data from REF. 34.

nuclei[41,42]. In the wasp *Muscidifurax uniraptor*, the first mitotic division is complete and diploid females are produced after fusion of two cell nuclei[43]. Unlike the parthenogenesis that is observed in members of the order Hymenoptera, *Wolbachia*-induced parthenogenesis in the mite *Bryobia praetiosa* seems to be functionally apomictic by altering meiosis, resulting in diploid gametes[39]. In some cases, the infection is polymorphic within a species, and the chromosomal effects are suppressed when sperm fertilize eggs, whereas in others, the infection is fixed within the species, which results in *Wolbachia*-induced parthenogenetic species.

Feminization. *Wolbachia*-induced feminization was first described in isopods, and was more recently described in insects, in which it occurs through different mechanisms. In several isopod species from the order Oniscidea, *Wolbachia* have been shown to proliferate within the androgenic gland, leading to androgenic gland hypertrophy and inhibited function. Consequently, genetic males develop as females[44].

In insects, feminization is currently known in two different host species, *Eurema hecabe* (from the Lepidoptera order) and *Zyginidia pullula* (from the Hemiptera order)[45,46]. The exact mechanism of feminization is currently unclear, although in *E. hecabe*, *Wolbachia* seem to interfere with the sex-determination pathway and must continuously act throughout development for complete feminization. Removal of *Wolbachia* during development results in intersexual development[47]. It has been postulated that feminizing *Wolbachia* can lead to the evolution of new sex-determination systems, such as shifts from female heterogamety to male heterogamety[48], although conclusive evidence for this has not been shown.

Heterogamety
The production of dissimilar gametes by an individual of one sex. For example, the production of X- and Y-bearing gametes by the human male.

Male killing. *Wolbachia*-induced male killing has been described in four different arthropod orders: Coleoptera[49], Diptera[50], Lepidoptera[51] and Pseudoscorpiones[52]. In each described infection, *Wolbachia* killing of males occurs mainly during embryogenesis, which can result in more food for the surviving female progeny. Insight into the mechanism of male killing comes from the lepidopteran host *Ostrinia scapulalis*. The all-female broods found in *Wolbachia*-infected *O. scapulalis* were first described as the result of *Wolbachia*-induced feminization. When mothers were treated with tetracycline to remove *Wolbachia*, all-male broods were produced[53]. However, subsequent work has shown the effect to be male killing. In the absence of *Wolbachia*, genetic females die during larval development, whereas in the presence of *Wolbachia*, genetic males become feminized and die during larval development. Thus, *Wolbachia*-induced male killing seems to occur through lethal feminization[54]. It is still unclear at what level *Wolbachia* interfere with sex determination or how this might differ among different host taxa. Recent evidence indicates that some male killers can reach high frequencies, resulting in changes in host mating systems to accommodate the scarcity of males[55]. In addition, host suppressor genotypes to male killing can rapidly spread within infected populations[56].

Multi-potent Wolbachia. Some *Wolbachia* strains can induce more than one phenotype. For example, a *Wolbachia* strain that naturally infects the lepidopteran host *Cadra cautella* normally causes CI, but when transferred into another lepidopteran host, *Anagasta kuehniella*, the same *Wolbachia* strain cause male killing[57]. In *Drosophila bifasciata*, *Wolbachia* also cause male killing, but those males that escape have low levels of CI[58]. *Drosophila recens* is naturally infected by *Wolbachia*, which induce CI, and when the same *Wolbachia* were introgressed into a sibling species, *Drosophila subquinaria*, male killing was immediately triggered[59]. *Wolbachia* have caused male killing in some populations of the butterfly *Hypolimnas bolina*, although a suppressor of male killing has recently become widespread, which has eliminated male killing by allowing *Wolbachia*-infected males with the suppressor allele to survive to adulthood. When mated to uninfected females, these surviving infected males induce CI[56,60]. In both *H. bolina* and *D. subquinaria*, it is clear that emergence of the second *Wolbachia*-induced phenotype is only expressed following release from host suppression. Furthermore, experiments that place different *Wolbachia* in the same host genetic background indicate that some contain multiple modification–rescue mechanisms[37]. Taken together, these studies suggest that some *Wolbachia* carry the machinery for inducing multiple phenotypes, some of which can be expressed only in the permissive host backgrounds in which they are not suppressed. It is currently unclear if these multi-potent *Wolbachia* have similar molecular mechanisms that cause different *Wolbachia*-induced phenotypes.

Other effects. Although not the focus of this Review, *Wolbachia* in nematodes do seem to be mutualistic[61]. The exact benefits of the bacteria to nematodes have not been determined, although antibiotic treatments seem to interfere with moulting of microfilaria and reproduction of mature worms, which is consistent with tissue distribution in the reproductive organs and subcutaneous integument. Inflammatory responses to filarial infections are stimulated by *Wolbachia* antigens, such as the surface protein Wsp, rather than by nematode proteins, and *Wolbachia* may have a role in the redirection of vertebrate immune responses. In an intriguing recent finding, *Wolbachia* infections were found in the tissues of dogs that were infected with heartworms, which were probably released after the death of larva or pre-adult worms[62]. It is unknown how long *Wolbachia* can persist, if at all, outside of their nematode host or if vertebrates can act as an intermediate host for *Wolbachia*.

An interesting possible case of mutualism has also been found in the parasitic wasp *Asobara tabida*, in which antibiotic curing of *Wolbachia* resulted in failure of the ovaries to properly develop[63]. However, related wasps can develop functional ovaries without *Wolbachia*, and therefore it seems unlikely that their requirement in *A. tabida* reflects a mutualistic relationship. A recent study indicates that *Wolbachia* downregulate apoptotic processes in the developing ovaries, and as a result, removal of the bacteria leads to the apoptotic death of ovarian cells[64]. The requirement of *Wolbachia* in *A. tabida* probably reflects a genetic addiction to the symbiont: the reproductive system has evolved in response to the presence of this reproductive parasite (for example, by enhancing apoptosis in ovaries to compensate for bacterial dampening) and consequently removal disrupts normal ovarian development[64]. Support for this interpretation was provided by Starr and Cline[65], who showed that *Wolbachia* can rescue ovarian defects of some mutant alleles in *D. melanogaster*. Because *Wolbachia* are selected to upregulate egg development, which enables their transfer to the next generation, mutations that affect oogenesis may be compensated for by the presence of *Wolbachia*.

It is expected that some *Wolbachia* which infect arthropods have evolved mutualistic relationships with their hosts. This would be consistent with their vertical transmission, which can often favour mutualism. For example, it has been found that *Wolbachia* infection provides protection against RNA viruses in the fruit fly *D. melanogaster* (L. Teixeira, personal communication). *Wolbachia* also have a diverse range of effects that may reflect sophisticated manipulations of the host, including *Wolbachia*-associated alterations in mating preference[66] and responses to olfactory cues[67].

Wolbachia possess a number of interesting adaptations to navigate the eukaryotic cell. Recent cytological studies of *Wolbachia* in *Drosophila* revealed interactions between *Wolbachia* and the *Drosophila* cytoskeleton. *Wolbachia* co-opt host molecular motors to move within the host cell. During embryogenesis, *Wolbachia* are in tight association with centrosomes and centrosome-organized microtubules. With each nuclear division,

roughly half of the *Wolbachia* segregate to each spindle pole, ensuring equal segregation of bacteria[14]. During oogenesis, *Wolbachia* often localize to specific regions of the germline, and this localization is dependent on both the molecular motors dynein and kinesin 1 (REFS 15,16). Host motor proteins might have a role in the expression of CI or other *Wolbachia*-induced phenotypes. For example, it has been shown that in the absence of *Wolbachia,* overexpression of myosin II results in paternal-effect defects that are similar to those observed during CI[68].

Progress in revealing the mechanisms by which *Wolbachia* manipulate host cell biology and reproduction has been hindered by the fact that these bacteria cannot be grown outside of host cells (although they can be grown in insect cell culture[69,70]) and that a transformation system does not exist for *Wolbachia*. Investigations are underway to develop transformation in *Wolbachia*, which could greatly accelerate functional studies. The *Wolbachia* origin of replication has recently been identified, which might accelerate the development of transformation methods[71].

Maintenance of the global *Wolbachia* pandemic

Wolbachia represent one of the great pandemics in the history of life, infecting at least 10^6 insect species alone. But how are *Wolbachia* infections maintained globally within invertebrates? Maintenance depends on the rates of acquisition and loss of infections within species relative to the horizontal transfer rates between species. In this way, a *Wolbachia* infection is like any other infection, except that the global arthropod community is the host. Therefore, standard epidemiological conditions apply: to maintain the infection, one successful transfer on average must occur during the course of a *Wolbachia* infection within a host species.

How do *Wolbachia* move between species? We do not fully understand this process, but recent studies have provided some hints. *Wolbachia* can be experimentally transfected between taxa by microinjection into eggs, which indicates that they can become established within cells of diverse arthropods. However, not all hosts are equally permissive and *Wolbachia* strains can differ in their ability to transfect different host species. Such effects need to be investigated systematically. Two recent findings are particularly relevant to the question of intertaxon transfer. *Wolbachia*, like all Rickettsiales, are obligatory intracellular bacteria, and it has been assumed that they cannot survive outside host cells. In tissue culture experiments, however, *Wolbachia* were shown to persist long after the host cells had died[72]. When purified from host cells, *Wolbachia* can remain viable for at least 1 week at room temperature[73]. In addition, Frydman *et al.*[74] have shown that when *Wolbachia* are injected into the body of an adult *D. melanogaster*, they enter the ovary at the somatic stem-cell niche (that is, the microenvironment that supports the stem cells). From this niche, *Wolbachia* reach the somatic stem cells and the germline, and eventually enter developing eggs[74]. Therefore, it seems that some *Wolbachia* strains can briefly exist outside of host cells and traverse cell membranes, which could be important for horizontal transfer within and between species. Studies of *Wolbachia* localization in developing oocytes indicate that some bacterial strains can efficiently localize to the germ pole of the egg, whereas others are widely distributed in the egg cytoplasm and the resulting soma of embryos, as well as at later stages[75]. The data suggest that *Wolbachia* might use two different mechanisms for vertical transmission: efficient localization to the germ pole and somatic distribution with subsequent migration to ovarian stem cells. Somatic distribution might also predispose some strains to more effective horizontal transmission within and between species.

Both the patterns of host distribution and experimental interspecific transfers of *Wolbachia* suggest strain biases in their ability to move between host species[76,77]. Similar phylogenetic constraints to interspecific host shift have been found in the male-killing bacteria *Spiroplasma*, in which the likelihood that bacteria will colonize novel species increases within a single host genus[78].

Wolbachia often establish tight associations with mitochondrial genotypes within a species, which indicates strict vertical transmission. However, in other species, there is a lack of strong linkage disequilibrium between *Wolbachia* and mitochondrial genotypes, which suggests a role of horizontal transfer in intraspecific spreading of *Wolbachia*[77]. Studies of newly established *Wolbachia* infections that involve large taxonomical shifts could give interesting insights into the early evolution of endosymbioses. For example, how do *Wolbachia* genomes evolve following a large intertaxon shift (such as the shift that occurs between insect orders)? When distinct *Wolbachia* strains have similar phenotypes (for example, male killing), does this reflect independent evolution or the acquisition of male-killing machinery by recombination? The availability of MLST data should soon enable these and other crucial questions to be addressed by providing the first large-scale genetic framework for tracing the network of *Wolbachia* movements and analysing the association of *Wolbachia* genotypes with host taxonomy and phenotype.

Finally, an important unresolved question is: why do single *Wolbachia* strains not typically persist over long evolutionary timescales within the same arthropod taxon. This question is based on the observation that closely related host species do not typically show congruent phylogenies with their *Wolbachia* strains, which means that most *Wolbachia* infections do not survive after host speciation events[11]. It is presumed that selection for resistance in hosts eventually leads to loss of parasitic *Wolbachia*. However, resistance to the infection does not evolve easily in females because loss of a common CI-inducing *Wolbachia* infection would effectively sterilize the females owing to incompatibility with infected males. Presumably, loss of infection first requires the evolution of resistance in males, and then the development of resistance in females and eventual elimination of the infection. Losses may also occur when an established *Wolbachia* strain is displaced by another *Wolbachia* strain that is spreading throughout a population.

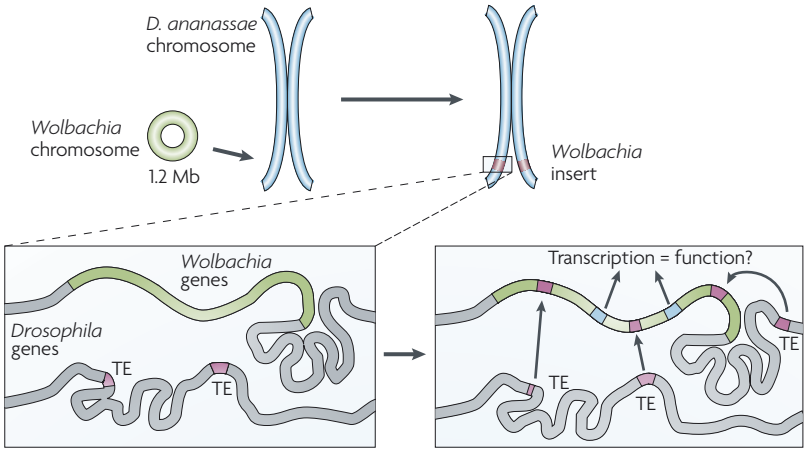

Figure 4 | **Wolbachia-to-host lateral gene transfer in *Drosophila ananassae*.** Almost the entire *Wolbachia* genome (green) has been transferred into the second chromosome of *D. ananassae* (blue). Following this lateral gene transfer, *D. ananassae* transposable elements (TEs) have become inserted within *Wolbachia* genes. At least 28 *Wolbachia* genes are transcribed from within the *D. ananassae* genome, although the functional significance of this is unknown.

Evolutionary implications of *Wolbachia*

Whether *Wolbachia* have an important role in accelerating the evolution of their hosts[31] is an important and controversial question. For example, induction of sperm–egg incompatibilities (CI) between diverging populations could drive the evolution of new species, and there is increasing empirical and theoretical evidence in support of this proposition. Theoretical studies indicate that bidirectional CI can enhance genetic divergence despite substantial gene flow, which causes the mutual stabilization of divergence in both locally selected genes and *Wolbachia*. Earlier empirical studies showed that bidirectional CI is a major contributor to reproductive incompatibility between sibling species of the insect *Nasonia*[79]. However, bidirectional CI is expected to be less common in nature, and the general thought was that unidirectional CI (which occurs, for example, when only one population is infected) is both unstable to migration between populations and insufficient to maintain genetic divergence. Recent studies dispute this view, however, and indicate that genetic divergence in infection status and locally selected alleles can be maintained when *Wolbachia* impart a fertility cost and/or are incompletely transmitted. In addition, *Wolbachia* that cause CI can readily select for pre-mating isolation, which reinforces genetic identity among populations[80], as observed in natural populations of *D. subquinaria*[81].

There is good evidence that parthenogenesis-inducing bacteria have led to the evolution of parthenogenetic insect species, based on the loss of functional sexuality when these insects are cured of their bacteria[82]. Another way in which *Wolbachia* accelerate host evolution is by causing the rapid evolution of host genes. In particular, elevated rates of evolution are expected in host genes that are expressed in the gonads where *Wolbachia* reside, which would affect their transmission and cellular effects. Support for this idea comes from the interaction between *Wolbachia* in mutant genotypes of *Drosophila*[65,83] and the presence of resistance to some *Wolbachia* infections[56,59]. One particularly exciting recent finding is that lateral gene transfer from *Wolbachia* into invertebrate genomes is common and widespread[84–86]. Approximately one-third of sequenced invertebrate genomes contain recent *Wolbachia* gene insertions, which range in size from short segments (<600 bp; for example, in *Nasonia* species) to nearly the entire *Wolbachia* genome (>1 Mb; in the tropical fruit fly *Drosophila ananassae*)[85]. Low levels of transcriptional activity have been found in some of the *Wolbachia*-inserted genes, whereas others are disrupted by transposons (FIG. 4). A key question is whether such *Wolbachia* insertions can result in the acquisition of novel gene functions. This has yet to be shown, but the high frequency of invertebrate species that are infected with these bacteria and the apparent common occurrence of lateral gene transfers suggest that at least some cases will result in new functional genes. Aside from the acquisition of new genes, the insertional events may contribute to host chromosomal rearrangements, which in turn may play a part in reproductive isolation.

Practical applications of *Wolbachia*

The possible implications and applications of *Wolbachia* to human disease and pest control have been extensively reviewed elsewhere[5,87]. Here, we highlight just a few key developments.

Wolbachia seem to have an important role in filarial pathogenicity, apparently owing to inflammatory responses of infected hosts to *Wolbachia* proteins[88]. This, combined with the dependence of nematodes on *Wolbachia*, has led to considerable interest in targeting these bacteria in new strategies to combat filariasis. Anti-*Wolbachia* therapies are promising for both eliminating filarial nematode infections as well as lessening the effects of infections, and recent studies have supported such therapies. Although short-term antibiotic administration is not sufficient to reduce nematode load[89], combining treatment with traditional vermicides and anti-*Wolbachia* therapy is effective in improving treatment[90–93]. The *Wolbachia* genomes are now being used to identify possible targets for therapeutic agents.

The ability of *Wolbachia* that cause CI to increase in arthropod populations has generated interest in their use as a mechanism to drive desirable traits (for example, resistance to disease) into insect vector populations (reviewed in REF. 5). The use of *Wolbachia*-infected males is also being developed as a mechanism to decrease pest populations by inducing elevated CI[94–96], similar to the use of sterile male programmes to control pest insects. Other creative approaches are under consideration and development, such as using *Wolbachia* to shorten the lives of vectors in which the disease agent requires a long incubation time within the vector, such as Dengue fever in Aedes mosquitoes[97]. Several projects to use *Wolbachia* in disease or vector control are currently funded by the Bill and Melinda

Gates Foundation (see Further information). Whether these methods will be successful remains to be seen, and there are a number of challenges to their development[5]. However, the prospects for using *Wolbachia* to reduce major diseases that affect millions of people show considerable promise.

Conclusions

Wolbachia are a diverse group of intracellular bacteria that show impressive adaptations towards living in invertebrate cells and in manipulating the biology of their hosts. Considerable progress has been made in the past 10 years to elucidate their biology. There has also been growth in the *Wolbachia* research community, particularly in the areas of genomics, cell biology and molecular biology. In addition, a number of new research tools are in place (for example, tissue culturing of *Wolbachia*, a multilocus strain-typing system and genome sequences). However, important questions still remain, including: how do *Wolbachia* manipulate host reproduction; how is the incredible abundance and distribution of *Wolbachia* maintained globally; can *Wolbachia* be effectively used in disease control; do *Wolbachia* have important roles in the evolution of their hosts; and, in particular, do *Wolbachia* accelerate the rates of speciation in invertebrates and contribute to novel gene acquisition. We are now poised for major breakthroughs that could answer these fundamental questions.

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DATABASES

Entrez Genome Project: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=genomeprj
Brugia malayi | *Drosophila ananassae* | *Drosophila melanogaster* | *Drosophila simulans* | *Legionella pneumophila* | *Orientia tsutsugamushi* | *Rickettsia bellii*

FURTHER INFORMATION

John H. Werren's homepage: http://www.rochester.edu/college/bio/labs/werrenlab/
Anti-Wolbachia Project: http://www.a-wol.net/
Bill and Melinda Gates Foundation: http://www.gatesfoundation.org/default.htm
***Wolbachia* MLST databases:** http://pubmlst.org/wolbachia/

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