A highly divergent Wolbachia with a tiny genome in an insect-parasitic tylenchid nematode

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Wolbachia symbionts are the most successful host-associated microbes on the planet, infecting arthropods and nematodes. Their role in nematodes is particularly enigmatic, with filarial nematode species either 100% infected and dependent on symbionts for reproduction and development, or not at all infected. We have discovered a highly divergent strain of Wolbachia in an insect-parasitic tylenchid nematode, Howardula sp., in a nematode clade that has not previously been known to harbour Wolbachia. While this nematode is 100% infected with Wolbachia, we did not detect it in related species. We sequenced the Howardula symbiont (wHow) genome and found that it is highly reduced, comprising only 550 kilobase pairs of DNA, approximately 35% smaller than the smallest Wolbachia nematode symbiont genomes. The wHow genome is a subset of all other Wolbachia genomes and has not acquired any new genetic information. While it has lost many genes, including genes involved in cell wall synthesis and cell division, it has retained the entire haem biosynthesis pathway, suggesting that haem supplementation is critical. wHow provides key insights into our understanding of what are the lower limits of Wolbachia cells, as well as the role of Wolbachia symbionts in the biology and convergent evolution of diverse parasitic nematodes.

1. Introduction

Bacteria in the genus Wolbachia are the most abundant host-associated microbes on the planet, successfully infecting two old and hyperdiverse groups of invertebrates—arthropods and nematodes [1–3]. Although these symbionts and how they affect their hosts are highly variable, they are united by highly efficient maternal transmission and a strong and broad affinity for germline tissue [4–7]. Wolbachia is especially abundant in arthropods, estimated to infect approximately 40% of terrestrial species [8]. One of the major reasons for this enormous host range is that despite being primarily maternally transmitted over short (i.e. ecological) timescales, new arthropod hosts are repeatedly colonized over longer (i.e. evolutionary) timescales, through mechanisms that are currently not understood [9]. In addition, most arthropod-infecting Wolbachia strains are facultative in their hosts, meaning that their hosts can survive and reproduce without them (although the converse is not true, as Wolbachia cannot live without their hosts). These facultative strains can affect their hosts in diverse ways, such as providing protection against pathogenic viruses [10,11] or manipulating their hosts’ reproduction in order to increase the frequency of infected females (i.e. the transmitting host) [12–14], for example by causing mating incompatibilities between infected males and uninfected females. The combination of protection against and mating incompatibility has generated a great deal of recent interest in using Wolbachia to control pests and disease vectors [15–17]. Yet not all arthropod Wolbachia strains are facultative in their hosts. For example, Wolbachia is an obligate essential symbiont of bedbugs, providing B vitamins that are absent from the

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bedbug’s blood diet [18]; bedbugs that have had their Wolbachia removed via antibiotic treatment have severely impaired development and reproduction.

Patterns of Wolbachia infection in nematodes are markedly different from those in arthropods [3,19,20]. First, the range of nematodes that host Wolbachia is much more restricted. Almost all known nematode hosts are filarial nematodes, which are parasites that require a blood-feeding arthropod and a vertebrate (sometimes human) host to complete their life cycle. Second, all Wolbachia strains that infect filarial nematodes are obligate; hosts that are cleared of infection are unable to successfully develop and reproduce [21,22]. However, why Wolbachia is essential to filarial nematodes is still unknown, despite intensive study. One leading hypothesis is that it provides them with essential nutrients, similar to bedbugs. However, there is still limited evidence for this hypothesis, probably owing to the fact that it is very challenging to manipulate filarial nematodes inside their hosts. In parallel, researchers have used comparative genomics to look for biosynthetic pathways that are conserved across diverse nematode Wolbachia genomes, and that may give clues as to key metabolites that Wolbachia might provide, such as haem [23–27]. Interestingly, a number of filarial nematode species have independently lost Wolbachia without gaining new symbionts, genes or ecologies, making Wolbachia’s essentiality even more mysterious [28–30].

Unlike terrestrial arthropods, nematodes (other than filarial nematodes) have been little surveyed for Wolbachia, or for bacterial symbionts in general. Thus far, only two non-filarial nematodes, the plant-parasitic tylenchids Radopholus similis [31] and Pratylenchus penetrans [32] have been found to host Wolbachia, and virtually nothing is known about either of these infections [33,34]. It would thus be highly informative to survey a much broader diversity of nematodes. This would also help solve the mystery of whether the symbiosis originated in arthropods or in nematodes [32]. To this end, in this study, we report the discovery of a highly divergent strain of Wolbachia in a tylenchid nematode parasite of flies that has the hallmarks of an obligate symbiosis. All worms that we screened are infected with this symbiont. We sequenced its genome and found that it is the smallest Wolbachia genome by far, at approximately 550 kb, approximately 35% smaller than the previous smallest published Wolbachia genomes [25], and representing an intriguing new model for understanding Wolbachia-nematode interactions and evolution.

2. Results

(a) A novel Wolbachia in a parasitic nematode infecting Spelobia flies

Sphearoerid flies, primarily from the genus Spelobia, were abundant at our mushroom baits. While less than 5% of sphearoerids were infected by nematodes, there were four morphologically distinct tylenchid nematodes in these samples, infecting Spelobia ordinaria, Spelobia quinata, an unidentified Spelobia species and Minihinosina fangiocola (electronic supplementary material, table S1). 18S rRNA sequencing confirmed that these four nematodes are distinct (figure 1a). These nematodes are all undescribed species, and may also represent new genera. One of these nematode species (species no. 1) is most closely related to Rubezoinema nematode parasites of fleas [35], while the other three (Howardula species nos 2, 3 and 4) are found in a clade of nematode parasites that infects diverse flies. (Note that Howardula is a polyphyletic group of nematodes that infects a wide range of insects, and in major need of taxonomic revision [35,36].)

16S rRNA screening of nematodes for bacterial symbionts revealed the presence of a divergent Wolbachia in Howardula species no. 3, a nematode with distinctively long and thin motherworms (figure 1b,p). We refer to this Wolbachia strain as wHow. Phylogenetic analysis of the Wolbachia 16S rRNA sequence shows a highly divergent taxon with no affiliation to any of the already known Wolbachia supergroups (electronic supplementary material, figure S1). Using primers targeting wHow 16S rRNA, we found that 100% of Howardula sp. no. 3 individuals harboured Wolbachia (n = 32; electronic supplementary material, table S1). We barcoded all wild-caught flies infected with Howardula sp. no. 3; all but one were an unidentified Spelobia sp. (greater than 99.5% similar to Genbank accession MT863700), while the other was S. quinata [37]. To confirm that the Wolbachia symbiont is restricted to the nematode, we screened 26 uninfected Spelobia sp. hosts of Howardula sp. no. 3 (greater than 99.5% similar to Genbank accession MT863700) collected at the same baits; none were infected with Wolbachia. We did not recover Wolbachia from any of the other sphearoerid-parasitic nematode samples (electronic supplementary material, table S1). We also screened nematodes from four additional species in the fly parasite clade; two parasites of Drosophila (Howardula aonynymphum and Parasytlenchus nearcticus), and nematodes infecting Megaselia haltenata (Diptera: Phoridae) and Fannia sp. (Diptera: Fanniidae) (electronic supplementary material, table S1). We were unable to detect Wolbachia in any of these nematodes, except for samples infecting Fannia and Megaselia; these are probably insect Wolbachia, as their 16S rRNA, wsp and coxA sequences are greater than 99.5% similar to symbionts in arthropod-infecting supergroups A and B.

(b) Wolbachia symbiont of Howardula nematode is highly divergent and has a tiny genome

Whole-genome sequencing using Illumina and Oxford’s Nanopore MinION technology led to the assembly of a circular 553 kb wHow genome, containing 487 coding sequences (CDS), seven pseudogenes and with a BUSCO completeness score of 86.86% (figure 2a, table 1; Genbank accession CP092368.1). Its per cent GC content is 29.5, which lies within the range (28–36%) of Wolbachia symbionts of filarial nematodes [25]. Circularization of the wHow chromosome was confirmed by polymerase chain reaction (PCR) and Sanger sequencing. The genome size of wHow is approximately 35% smaller than the previously published smallest Wolbachia genomes with approximately 863 kb of wCtu and wDecu, symbionts of filarial nematodes [25]. The genome does not contain any identifiable mobile elements, ankyrin or phage-related genes (table 1).

For a detailed phylogenetic analysis, we identified a total of 132 single-copy orthologous genes from three close relatives of Wolbachia: Candidatus Mesenlot longicola, Anaplasma marginale and Ehrlichia chaffeensis as well as from across 39 published Wolbachia genomes and wHow (figure 2r; electronic supplementary material, table S2). This confirmed our 16S rRNA phylogenetic analyses and places wHow in a novel, divergent clade, and which probably represents a new Wolbachia supergroup. We obtained similar results
Figure 1. (a) Phylogenetic analysis of nematode 18S rRNA sequences, using maximum likelihood, and implemented with IQ-TREE, and with 1000 bootstrap replicates; bootstrap values ≥65 are indicated. The best-fit model calculated by MODELLER was TIM3 + F + I+G4. The scale bar indicates the distance in substitutions per nucleotide. Coloured circles next to the nematode species indicate the type of host that this nematode infects: with flies (order Diptera) in green, beetles (Coleoptera) in red, wasps and allies (Hymenoptera) in orange, fleas (Siphonaptera) in yellow, plants in grey, and unknown hosts in white. Pink or blue coloured bars show nematodes that harbour *Wolbachia* or *Symbiopectobacterium* symbionts, respectively. (b) Dark-field micrograph of a dissected *Spelobia* sp. infected with nematodes. The dissection reveals an approximately 3 mm long motherworm (right side) as well as a high number of juveniles with a length of roughly 0.25 mm. (b') Magnification of the motherworm. (Online version in colour.)
upon performing phylogenetic analyses using only *Wolbachia* genomes (i.e. without *Ca. M. longicola*, *A. marginale* and *E. chaffeensis*), and a total of 145 single-copy orthologous genes across all 40 *Wolbachia* genomes (figure 2c). We also calculated average nucleotide identity; this confirms that *wHow* is highly divergent, with 71–74% similarity to other *Wolbachia* genomes (electronic supplementary material, table S3).

### (c) Comparative genomics of *wHow*

We compared the content of the *wHow* genome with 45 representative *Wolbachia* genomes, using anvio (electronic supplementary material, figure S2, table S2). There was strong concordance between *Wolbachia* supergroup phylogenetic relationships and gene content, with the exception of symbionts in supergroup D, which were split into two groups, reflecting their different genome sizes. From our pan-genome analysis, we identify a core *Wolbachia* genome of 347 gene clusters that is present in all *Wolbachia* genomes (electronic supplementary material, data file S1). Removing *wHow* from this analysis increased the number of conserved *Wolbachia* gene clusters by approximately 20%. We were therefore intrigued by what genes and gene pathways are retained or lost in *wHow*, as these may reveal features that are critical for *Wolbachia* function, focusing in particular on *Wolbachia* that infect nematodes [25].

To our surprise, *wHow* has completely lost most genes involved in the synthesis of a bacterial cell wall, belonging to the KEGG pathways for peptidoglycan biosynthesis (ko00550) and lysine biosynthesis (ko00340) (electronic supplementary material, figure S3). While these genes, necessary for the production and organization of peptidoglycan, are highly conserved in all other *Wolbachia* [38], *wHow* seemingly has lost the ability to synthesize a bacterial cell wall or sacculus (electronic supplementary material, figure S4), which is commonly observed in symbiotic bacteria with minimal genomes.

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**Figure 2.** (a) Circular overview of the 553,558 bp *wHow* genome. Red bars indicate the 487 identified CDS (outer ring = positive-sense, inner ring = negative-sense). Blue and pink bars indicate identified transfer RNAs (tRNAs) [33] and ribosomal RNAs (rRNAs) [3], respectively. The GC content is 29.5%. (b + c) Phylogenomic analysis of *Wolbachia*, using maximum likelihood, and implemented with IQ-TREE, and with 1000 bootstrap replicates; bootstrap values ≥65 are indicated. The scale bar indicates the distance in substitutions per nucleotide. Colours correspond to *Wolbachia* supergroups. (b) Rooted phylogenomic tree based on 132 single-copy orthologous genes found in all used *Wolbachia* genomes (*n* = 40) as well as the closest relatives *Candidatus Mesen et longicola*, *Anaplasma marginale* and *Ehrlichia chaffeensis*. The best-fit model calculated by *ModelFinder* was HIVb + F + I + G4. (c) Unrooted tree based on 145 single-copy orthologous genes that are conserved in all used *Wolbachia* genomes (*n* = 40). The best-fit model calculated by *ModelFinder* was HIVb + F + I + G4. Icons depicting *Wolbachia* hosts are from Lefoulon et al. [25], with a new icon for *wHow* host. (Online version in colour.)
Table 1. Genomic characteristics of the wHow Wolbachia strain.

| strain             | wHow |
|--------------------|------|
| host               | Howardula sp. no. 3 |
| genome size (bp)   | 553 558 |
| proteins/hypothetical | 487/9 |
| tRNA genes         | 33 |
| rRNA genes         | 3 |
| % completeness (Rickettsiales) | 86.86 |
| transposases       | 0 |
| ankyrin genes      | 0 |
| phage-related genes| 0 |
| pseudogenes (Prokka/Pseudofinder) | 5/7 |
| signal peptides    | 12 |
| plasmid            | no evidence |
| coding density %   | 86 |

Furthermore, we found a significant loss of genes involved in the cell division pathway in wHow compared to other Wolbachia (electronic supplementary material, figure S3), including the cell division gene ftsZ, which, notably, is used in Wolbachia multilocus sequence typing (MLST) [40]; hcpA, another MLST gene, is also missing from wHow. wHow also has lost a number of genes involved in DNA recombination and repair, including the entire mismatch repair pathway (mutS, mutL, mutH), the recA-associated genes topB, helD and priA, as well as the lexA-associated genes uvrA, uvrB, dinS and ftsK (electronic supplementary material, table S4).

Regarding metabolic pathways that might indicate a nutritional role for Wolbachia, we found that wHow does not have any genes involved in the pyridoxine (vitamin B6), biotin (vitamin B7), and folate (vitamin B9) metabolism pathways, indicating that it does not provide its nematode host with these compounds. Similar to Wolbachia in plant-parasitic nematodes, wHow has almost completely lost riboflavin metabolism genes (vitamin B2), except for ribB. By contrast, we were able to confirm the presence of thiamine metabolism (vitamin B1) in wHow, similar to nematode-associated Wolbachia in supergroups C, J and L. The de novo synthesis pathways for fatty acids, pyrimidines, and purines are also complete, except for two missing genes (purB, dgt) in the latter pathway, indicating their importance for either Wolbachia or its host. wHow has also retained a complete haem metabolism pathway, except for the haem storage protein bacterioferritin (bfr), which is also missing in Wolbachia from supergroups C and J.

The retention of specific transporters can also provide valuable clues into how symbionts interact with their hosts. Wolbachia in supergroups A and B have transporters for haem, phosphate, lipoproteins, zinc, biotin, iron and phospholipids (electronic supplementary material, figure S3). Wolbachia from supergroups C and D have only lost a few individual genes in these pathways, which is unlikely to reduce their ability to transport those molecules. Interestingly, while symbionts from supergroup J show loss in the ability to transport biotin, iron and phospholipids, transport capability is even further reduced in wHow, where only genes for haem, phosphate and zinc transporters are retained. Relative to other Wolbachia, wHow has also lost a large number of genes involved in the production of glycerophospholipids, which play essential roles as membrane constituents or in the formation of specialized membrane domains (electronic supplementary material, figure S3). Finally, most Wolbachia genomes contain a broad range of secretion systems (SS; type I, II and type IV), although symbionts in supergroups C, J and L have lost the type II secretion system (gspD). By contrast, wHow has not only lost the type II SS, but also the type I and type IV SS (electronic supplementary material, figure S3); however, the Sec translocone/signal recognition particle pathway and the twin-arginine targeting systems are still retained, suggesting at least residual abilities to interact with the host. Accordingly, the number of wHow proteins containing a signal peptide is remarkably small, at only 12 (electronic supplementary material, table S5).

3. Discussion

Here we report the discovery of a highly divergent and tiny-genomed lineage of Wolbachia infecting a new group of hosts, insect-parasitic nematodes in an undescribed Howardula species (order Rhabditida, suborder Tylenchina, superfamily Phaeurlarioidea). Wolbachia infections have been reported in two other nematode lineages [3], neither of which are closely related to this one. Almost all known nematode hosts are filarial nematodes, which are in a different suborder, Spirurina. While some species of filarial nematode, such as Loa loa, do not harbour Wolbachia, those that do are 100% infected, as in Howardula sp. no. 3. In addition, Wolbachia has been found to infect two plant-parasitic nematode species, Pr. penetrans and R. similis, which are in a different superfamily, Tylenchoidea, in the Tylenchina, so very distantly related to Howardula sp. no. 3. It is difficult to estimate how many millions of years separate these...
lineages; as a comparison, the congeners Caenorhabditis elegans and Caenorhabditis briggsae are estimated to have diverged on the order of 100 Ma [42]. Virtually nothing is known about the plant-parasitic nematode *Wolbachia*, but it is facultative in *Pr. penetrans* [34]. The discovery of *Wolbachia* in an entomoparasitic nematode serves to remind that one should be careful to ascribe *Wolbachia* infections to the correct host. This problem is particularly pertinent with respect to parasitoids of insects and arachnids [43,44], as *Wolbachia* is highly prevalent across terrestrial arthropods. As our newly discovered nematode symbiont is so divergent from arthropod *Wolbachia*, it should not be too difficult to differentiate it; perhaps a greater concern is that identification of *Wolbachia* in entomoparasitic nematodes may be obscured by the presence of other *Wolbachia* that infect the nematode’s insect host.

Figure 3. Localization of wHow inside nematodes via 16S rRNA fluorescence in situ hybridization (FISH). Left side: image of an adult motherworm containing multiple juveniles. FISH staining is confined to a small area within each juvenile. Right side: higher magnification of juveniles. Only a handful of cells are positive for wHow in each juvenile. The stained area is confined to either the apical or caudal side of the juveniles. Blue, DAPI; red, 16S rRNA FISH probe. (Online version in colour.)

Figure 4. Shape and localization of wHow via TEM. (a) low magnification image of a Howardula nematode motherworm containing wHow. (b) higher magnification of the same area within the section highlighting the irregular shapes of wHow. (c) higher magnification showing the lack of typical bacterial cell walls present in these bacteria. (d) is a falsely coloured version of (c) to highlight wHow individuals (coloured red). Mitochondria present in the section have been coloured green. (Online version in colour.)
We found \( w \)How in 100% of wild "Howardula" sp. no. 3 nematodes. In combination with its extremely reduced genome, this suggests that it is essential for its host, although one must be careful not to assume this. For example, \( W. \)Westebaehardia, a recently discovered intracellular bacterial symbiont with a streamlined genome that has retained genes for building a strong insect cuticle, is found in most (approx. 80%), but not all, populations of an invasive ant, as well as its sister species [45], showing that it is not essential, despite a highly reduced genome. Demonstrating that \( w \)How is obligate will of course require experimental confirmation; however, this may be quite challenging, especially as we are not yet able to maintain "Howardula" sp. no. 3 in the laboratory. It is also found at a very low prevalence (approx. 1–5%) at the field site in Victoria where we repeatedly sample, and only in spring and autumn, making it difficult to study. "Howardula" sp. no. 3 was previously collected in eastern North America [46], and it will be useful to screen for \( w \)How across its range.

The distribution of \( w \)How within its worm host also suggests an intimate association, as we observed hybridization patterns that appear to correspond to highly localized regions inside juveniles developing within motherworms. It is tempting to speculate that these localized regions correspond to bacteriomes, but we are currently constrained by limited spatial resolution and challenges in obtaining field samples to confirm this.

\( W. \)Wolbachia's narrow host range in insect-parasitic tylenchids is puzzling. From \( w \)How's highly reduced genome, we would have expected to find \( W. \)Wolbachia infecting related nematodes, and to co-symbiont with its hosts. Yet we were not able to detect \( W. \)Wolbachia in samples from seven related fly parasitic tylenchid nematode species, except for strains from supergroups A and B that are most likely symbionts of the nematode's insect host; which is similar to a previous study that detected the same strain of \( W. \)Wolbachia in a species of thrips and a nematode of thrips [47]. One possibility is that \( W. \)Wolbachia was lost from other fly parasitic tylenchid nematodes, and perhaps was replaced by another bacterial symbiont [48]. However, as yet, we have not found evidence of any other obligate bacterial symbionts in fly parasitic tylenchids, except for a lineage of \( S. \)Symbiobacterium that has recently colonized \( D. \)Drosophila-parasitic "Howardula" [49], and whose genome, unlike \( w \)How, bears the hallmarks of a recent symbiosis, as it is large (4.5 Mb), with over a thousand pseudogenes. Another possibility is that \( W. \)Wolbachia was perhaps acquired by "Howardula" nematodes relatively recently, from an as yet unknown lineage of hosts where it has been a symbiont for a long time. For example, some species of adelgids, insects that feed on conifer sap, have recently acquired obligate nutritional symbionts that are closely related to obligate bacterial symbionts of fungi [50,51]. It is interesting that the clade of fly parasitic "Howardula" nematodes and allies appears to contain mixtures of species that are free of symbionts, along with ones that have recently acquired putatively obligate symbionts. Many insect-parasitic tylenchids have free-living life stages that feed on fungal or plant material [52–54], and we speculate that the acquisition of symbionts is associated with a loss of this free-living stage, although most nematodes in this group are so poorly studied that it is often not known if they contain a fungal or plant-feeding stage.

The \( w \)How genome is striking in its degree of reduction and at approximately 550 kb is by far the smallest \( W. \)Wolbachia genome described thus far, with features typical of tiny symbiont genomes, including retention of only a small number of genes, few pseudogenes and high coding density. While the genomes of facultative \( W. \)Wolbachia symbionts of arthropods, such as strains that cause cytoplasmic incompatibility, lie in the range of approximately 1–1.5 Mb, genomes of obligate \( W. \)Wolbachia symbionts of filarial nematodes are smaller, ranging from 860 kb to approximately 1.1 Mb. Up to now, the smallest reported \( W. \)Wolbachia genome, described in a recent preprint, is that of a symbiont of \( M. \)Menaenatus chewing lice, within \( W. \)Wolbachia supergroup F, a group that includes symbionts of other blood-feeding insects and some filarial nematodes, at approximately 733 kbp [55]. Another common feature of obligate symbionts is loss of genes involved in DNA replication, repair and recombination, which is thought to drive incredibly rapid rates of substitution [39,56,57]. It is notable that \( w \)How has lost the entire mismatch repair pathway. This may explain why it lies on such a long branch, although it would be useful to obtain sequence information from closer relatives of \( w \)How, in order to accurately infer rates of evolution.

The \( w \)How genome gives us insight into the lower limits of what defines a \( W. \)Wolbachia cell, with many genes that were previously considered core \( W. \)Wolbachia genes missing. Notably, \( w \)How has lost all genes involved in cell wall synthesis and cell division. These genes have also been lost in the smallest known symbiont genomes, such as \( T. \)Tremblaya, \( H. \)Hedgifiniasia and \( C. \)Carsonella, obligate nutritional symbionts of sap-feeding insects, and contributing to their irregular and inconsistent shapes [39], which we also see in our electron micrographs of \( w \)How. All other \( W. \)Wolbachia have retained at least some peptidoglycan genes and the ability to build a cell wall, with a minimum set of genes for cell elongation and division [38,58,59]. How \( w \)How and the other tiny-genomed symbionts complete cell division is not known. One possibility is that this role has been taken over by the host [60], but as yet, we do not have any evidence for this in the Wolbachia-Howardula symbiosis.

Despite being so highly divergent, the \( w \)How genome is a complete subset of all other \( W. \)Wolbachia, unlike the reduced genome \( W. \)Wolbachia in \( M. \)Menaenatus chewing lice, for example, which has horizontally acquired genes involved in panthoate synthesis [55]. Interestingly, while \( w \)How has lost a huge number of genes, it has retained some, but not all, pathways that are also found in filarial nematode symbionts, which have been independently colonized at least three times by different lineages of \( W. \)Wolbachia [25], all of which have converged on a similar complement of genes. Thus, \( w \)How provides useful clues as to the role of \( W. \)Wolbachia in filarial nematodes, which surprisingly, is still largely unresolved. A number of not mutually exclusive hypotheses have been proposed, including that \( W. \)Wolbachia provides filarial nematodes with essential and limiting nutrients and/or facilitate nematode parasitism by modulating the vertebrate immune response [23,26,61]. Recent beautiful imaging studies in \( B. \)Brugia nematodes have shown that \( W. \)Wolbachia are intricately tied to germline proliferation, prompting the suggestion that they have become essential for development [4,62]. It is especially puzzling that some filarial nematode species have independently lost Wolbcha without acquiring new symbionts, genes, or lifestyles [63].

In this regard, it is striking that \( w \)How has retained all the genes required to synthesize haem, strongly suggesting that this is the key nutrient shaping obligate symbiosis between nematodes and \( W. \)Wolbachia [26,27]. Nematodes are the only animals that have lost the ability to synthesize haem [64], and...
as a result have evolved sophisticated strategies to scavenge haem from their environment. At least two lineages of animal-parasitic nematodes, including filarial nematodes, have independently acquired haem biosynthesis genes via lateral gene transfer[65,66], suggesting that haem is highly limiting at certain times in their life cycle; the presence of haem biosynthesis genes in the genomes of both filarial nematodes and their Wolbachia symbionts makes it challenging to disentangle their respective contributions to nematode haem budgets. Finally, as in How’s nematode host only infects flies and not vertebrates, it also suggests that the interface between nematodes and arthropod hosts is a critical juncture in understanding Wolbachia’s function[67].

4. Material and methods

(a) Fly and nematode collections, DNA extraction, barcoding and next-generation sequencing

Mushroom-feeding woodland flies are infected with diverse tylenchid nematode parasites[46,68]. In order to characterize nematode diversity and to survey nematodes for bacterial symbionts, we used store-bought Agyrus bisporus mushrooms as baits to collect mycophagous flies in the woods near U. Victoria campus, Victoria, British Columbia, in the summers of 2019, 2020 and 2021, focusing in particular on flies in the family Sphaeroceridae, which were especially abundant. This work was also motivated by our recent finding that Drosophila-parasitic Howardula nematodes harbour obligate Symbiobacterium symbionts[49]. We also screened Howardula nematode parasites (probably Howardula husseyi[69]), infecting a laboratory colony of M. hallata, a pest of mushroom houses. Flies were brought back to the laboratory and dissected. DNA was extracted from flies and nematode motherworms separately. DNA extraction methods for PCR, Sanger and next-generation sequencing are described in the electronic supplementary material, methods. Nematodes and host flies were DNA barcoded, using 18S rRNA and mitochondrial cytochrome c oxidase subunit I (COI), respectively (see the electronic supplementary material, table S6).

(b) Screening for Wolbachia

While screening for potential bacterial symbionts by sequencing products amplified with universal 16S rRNA primers, we stumbled on a divergent 16S Wolbachia sequence associated with an undescribed Howardula species infecting mushroom-feeding flies in the genus Spelobia (Diptera: Sphaeroceridae), and which we refer to as wHow. As a number of commonly used Wolbachia-specific primers did not produce any PCR products, including primers designed to amplify 16S rRNA[70], wsp[71], and coxA, fbpA, ftsZ, hcpA MLST primers[40], we designed a new set of primers, amplifying an approximately 340 bp fragment of the wHow 16S rRNA gene (primer names: wol_HA-SPA F1 and wol_HA-SPA R1) (see the electronic supplementary material, table S6); sequences were confirmed by Sanger sequencing PCR products. Universal 16S rRNA screening did not reveal any other potential Howardula sp. No. 3 symbionts (see also[49]).

Two other nematodes, infecting Fannia sp., and M. hallata, amplified Wolbachia 16S RNA sequences that upon sequencing were found to be closely related to insect Wolbachia (i.e. supergroups A and B); for these we also amplified and sequenced wsp and coxA, confirming that these Wolbachia were indeed fly and not nematode symbionts. Finally, for nematode species for which we were unable to amplify Wolbachia, we also used the universal 16S RNA primers 63F and 907R. A phylogenetic tree of 16S rRNA sequences was generated as described above containing sequences listed in the electronic supplementary material, data file S2.

(c) Genome assembly, annotation and comparative genomic analysis

See the electronic supplementary material, methods.

(d) Fluorescence in situ hybridization

We designed a 16S rRNA wHow-specific Alexa 594-coupled FISH probe (5’-Alexa-594-GGAGTCTGGACGCTATCCTC-3’, produced by Integrated DNA Technologies Inc.). Flies were sacrificed in 70% ethanol (EtOH) and subsequently dissected in ice-cold phosphate-buffered saline (PBS). Motherworms were fixed in Carnoy’s solution (six parts anhydrous ethanol, three parts chloroform, one part acetic acid) at room temperature overnight, rinsed twice in anhydrous ethanol and then stored at −20°C until further use. Prior to hybridization, samples were re-hydrated twice in 70% and twice in 50% EtOH for two minutes each before transferring them in ddH2O for 3 min. Motherworms were then permeabilized in PBS-Tx (0.3% Triton-X in PBS pH 8.0) for 2 h at room temperature. Samples were then equilibrated in hybridization buffer (5 M NaCl, 1 M Tris-HCl pH 8.0, 20% Formamide, 5% sodium dodecyl sulfate) at 46°C for 1 h and the probe was then hybridized in the same buffer with 200 µM probe. The samples were washed in PBS-Tx at 48°C for 15 min twice and then in 4°C ddH2O for 5 min twice. We finally mounted the samples in ProLong Glass Antifade Mountant with NucBlue (ThermoFisher) and let them set overnight in the dark. Fluorescence confocal microscopy was performed using a confocal microscope (Nikon C2; Nikon Corp., Tokyo, Japan) and images were processed in ImageJ[72]. As negative control, we used both Spelobia sp. guts and a Wolbachia-free nematode species (Howardula aeronymphium, descended from infected Drosophila falkeni collected in West Hartford, CT, USA, in 2006, and maintained in our laboratory since, see also [49]), both of which showed no staining with our probe.

(e) Transmission electron microscopy

We performed TEM of an individual Howardula sp. No. 3 motherworm dissected from a wild-caught fly. Samples were double fixed and EMBed-812 (Epon replacement, Electron Microscopy Sciences) embedded using standard TEM methodology[73]. After initial fixation in Karnovsky’s fixative, the small samples were embedded within low melt agarose (Sigma A9045) and the resulting blocks osmicated, dehydrated, infiltrated with EMBed-812, and then polymerized. TEM sections were stained with uranyl acetate and lead citrate and viewed in a JEOL JEM 1400 TEM at 80 kV. Images were captured using a Gatan SC-1000 digital camera.

Data accessibility. All data can be accessed via Dryad Digital Repository: https://doi.org/10.5061/dryad.98sf7ms [74].

The data are provided in the electronic supplementary material[75].

Authors’ contributions. J.P.D.: conceptualization, data curation, formal analysis, investigation, methodology, project administration, resources, visualization, writing—original draft, writing—review and editing; C.I.C.: conceptualization, data curation, funding acquisition, investigation, methodology, project administration, resources, supervision, writing—original draft, writing—review and editing.

All authors gave final approval for publication and agreed to be held accountable for the work performed therein.

Conflict of interest declaration. We declare we have no competing interests.

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