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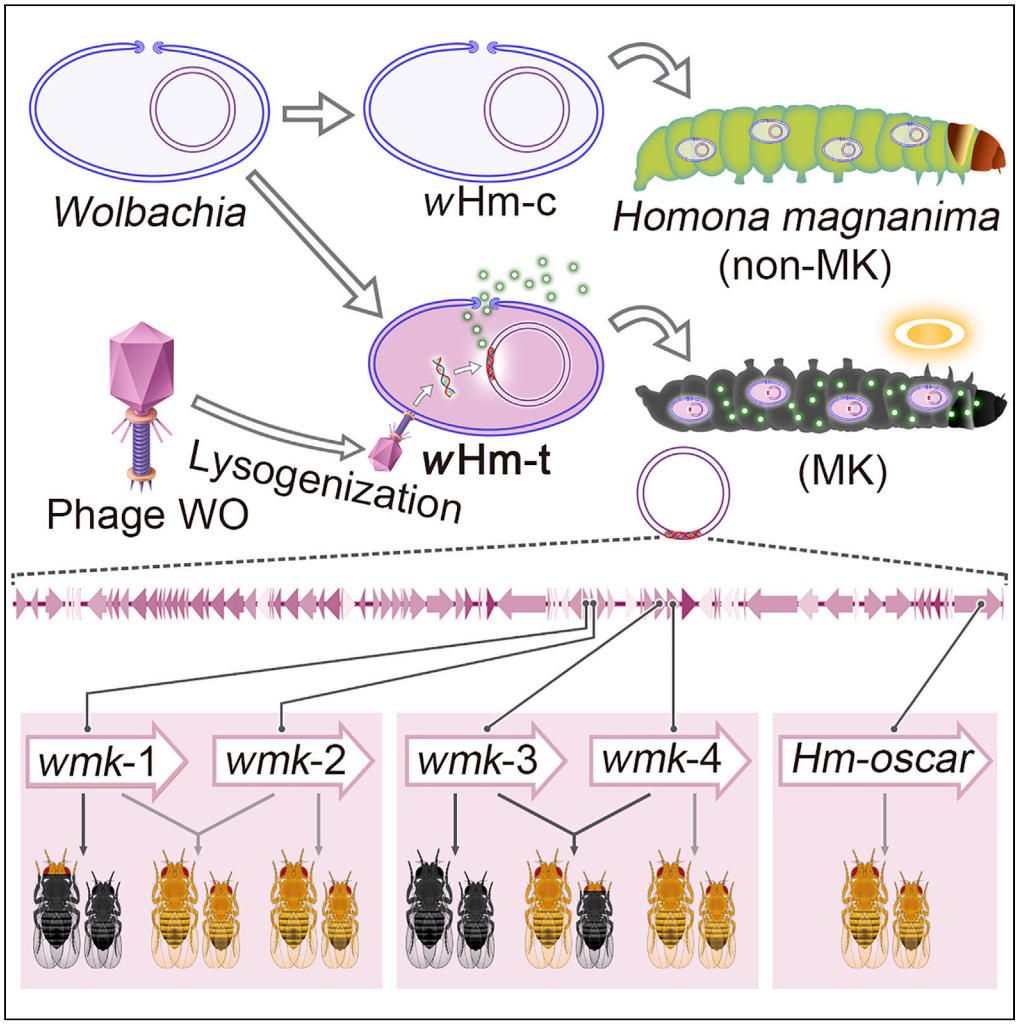

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Highlights

The origin and diversity of the male-killing mechanisms of *Wolbachia* were studied

Bacteriophages are involved in evolution and differences in male-killing mechanisms

Combinations of *wmk* genes induce multiple MK mechanisms and lethality variations

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Combined actions of bacteriophage-encoded genes in *Wolbachia*-induced male lethality

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SUMMARY

Some *Wolbachia* endosymbionts induce male killing, whereby male offspring of infected females are killed during development; however, the origin and diversity of the underlying mechanisms remain unclear. In this study, we identified a 76 kbp prophage region specific to male-killing *Wolbachia* hosted by the moth *Homona magnanima*. The prophage encoded a homolog of the male-killing gene *oscar* in *Ostrinia* moths and the *wmk* gene that induces various toxicities in *Drosophila melanogaster*. Upon overexpressing these genes in *D. melanogaster*, *wmk-1* and *wmk-3* killed all males and most females, whereas *Hm-oscar*, *wmk-2*, and *wmk-4* had no impact on insect survival. Strikingly, co-expression of tandemly arrayed *wmk-3* and *wmk-4* killed 90% of males and restored 70% of females, suggesting their conjugated functions for male-specific lethality. While the male-killing gene in the native host remains unknown, our findings highlight the role of bacteriophages in male-killing evolution and differences in male-killing mechanisms among insects.

INTRODUCTION

Wolbachia (Alphaproteobacteria) is a maternally inherited intracellular bacterium that is present in at least 40% of all insect species, making it one of the most widespread endosymbionts.[1,2] A notable feature of *Wolbachia* is the reproductive manipulation of its host, such as cytoplasmic incompatibility (CI), male killing (MK), parthenogenesis, and feminization, which have contributed to the spread of *Wolbachia* in nature.[1,3–6]

The molecular mechanisms underlying *Wolbachia*-induced phenotypes have attracted extensive attention for decades, and the prophage WO present in the *Wolbachia* genome is suggested as an important factor for the *Wolbachia*-induced reproductive manipulations.[7–12] For example, WO encodes CI-inducing factors (CifA and CifB), which inhibit normal embryonic development by preventing chromosome segregation after fertilization.[10–12] Recently, an MK *Wolbachia* strain *w*Fur was found to induce MK in the moth *Ostrinia furnacalis* through an Oscar protein that contains a CifB C-terminus-like domain and ankyrin repeats.[13] Oscar is thought to achieve MK by disrupting the functions of the protein Masculinizer (Masc), a critical regulator of the sex determination systems of lepidopteran insects. Because Masc is not conserved in insect taxa outside Lepidoptera (e.g., Drosophilidae in Diptera),[13] whether Oscar is conserved among MK *Wolbachia* and broadly functions in various insect taxa remains unclear. In addition, the WO-mediated killing (*wmk*) gene, a putative transcriptional regulator located in the prophage, has been proposed as a candidate MK gene.[7,8] The transgenically overexpressed *wmk* gene, derived from the non-MK *Wolbachia* strain *w*Mel, induces weak male lethality (ca. 30% of males die) in the fruit fly *Drosophila melanogaster*.[7] Moreover, overexpressed *wmk* homologs derived from a non-MK strain, *w*Suz, and an MK strain, *w*Rec,[14,15] killed all males and females of *D. melanogaster*.[8] These results show various toxicities of *wmk* homologs in *D. melanogaster*; however, whether *wmk* is involved in MK has not been clarified. Insects have diverse sex determination systems, and microbes have been thought to accomplish MK by targeting any molecular mechanisms involved in sex determination and differentiation.[16–19] Considering that the expression of *Wolbachia*-induced MK depends on the host's genetic background,[14,20–22] it is possible that *Wolbachia* has acquired multiple mechanisms (i.e., causative genes of different evolutionary origin) to induce MK in a diverse range of host insects.

Here, we identified an MK-associated prophage WO region encoding an Oscar and two tandemly arrayed *wmk* homologs by comparing the genomes of the MK and non-MK *Wolbachia* strains in the tea tortrix moth

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Homona magnanima (Tortricidae, Lepidoptera).[23,24] We previously found three phylogenetically distinct non-MK *Wolbachia* strains (*w*Hm-a, *w*Hm-b, and *w*Hm-c) from the Japanese population of *H. magnanima*. Strain *w*Hm-a does not induce CI or any other apparent phenotypes, *w*Hm-b induces CI, and *w*Hm-c does not induce CI but has positive effects on hosts (i.e., pupal weight gains).[23] We also found an MK *Wolbachia* strain (*w*Hm-t) that induces early MK (i.e., embryonic MK) from the Taiwanese population of *H. magnanima*. Intriguingly, non-MK *w*Hm-c and MK *w*Hm-t are phylogenetically closely related.[24] The expression of a single *wmk* gene in the WO region specific to the *w*Hm-t either caused complete death or had no effect on *D. melanogaster*; however, co-expression of the tandemly arrayed *wmk* genes induced strong male lethality and recovered female survival in *D. melanogaster*. Conversely, the *oscar* homolog did not induce any lethal effects in *Drosophila*. Our studies revealed the presence of multiple MK mechanisms and a complex pattern of varying male and female lethality owing to the combination of *wmk* genes. Based on these results, we propose a hypothesis on the mechanism underlying *Wolbachia*-induced MK and the evolutionary origin of MK.

RESULTS

Comparative genomics identified an MK-associated prophage region, WO*w*Hm-t76

Genome assembly and polishing using PacBio RSII and Illumina data generated a 1,542,158 bp complete genome of the MK-inducing *w*Hm-t isolated from an isofemale line W^{T12} of *H. magnanima* (Table S1). Single-cell analysis using an isofemale line of *H. magnanima* (collected from Yakushima Is, Japan, female ratio = 0.52, n = 1542) harboring non-MK *w*Hm-a, *w*Hm-b, and *w*Hm-c strains generated a complete genome of *w*Hm-c (1,447,668 bp), as well as draft genomes of *w*Hm-a (1,174,342 bp, 20 contigs) and *w*Hm-b (1,302,323 bp, 9 contigs; Table S1). A high level of genome rearrangements was observed among *w*Hm-t, *w*Hm-a, and *w*Hm-b (<50% homologies), whereas phylogenetically related *w*Hm-t and *w*Hm-c showed a high level of syntenic conservation (>92% homology) in most parts of the genomes and were almost perfectly colinear with several inversions (Figure S1). *w*Hm-t and *w*Hm-c had identical genes (84.9%, n = 1210) and orthologs (>90% identity, 94.9%, n = 1353; Table S2). In addition, bacterial density is a crucial factor for the expression of the *Wolbachia* phenotype. Indeed, Arai et al. (24) identified that *w*Hm-t can induce MK only when its density is sufficiently high in *H. magnanima* (Figure S2). In this study, *w*Hm-t in the MK matrilines typically showed higher titers than non-MK *w*Hm-c in *H. magnanima* (p = 0.003 by Steel–Dwass test; Figure S2), but *w*Hm-c did not induce MK even when its density was comparable to or higher than that of *w*Hm-t, suggesting that a high bacterial dose is not sufficient to induce MK in *H. magnanima*.

Notably, a prophage-associated 76 kb insertion (hereafter referred to as WO*w*Hm-t76) was present in the *w*Hm-t genome but not in the closely related *w*Hm-c (Figures 1A and S1). In addition, the WO*w*Hm-t76 region was absent in *w*Hm-a and *w*Hm-b (Figure S1). The WO*w*Hm-t76 region was annotated to harbor 83 genes (wHmt_12340 to wHmt_13160), which include *Wolbachia* density/virulence-associated gene homologs such as octomom genes (n = 7, Chrostek et al.[25]), ankyrin repeat-containing genes (n = 6), and tandemly arrayed *wmk* homologs (n = 4; Figure 1B). Of the four *wmk* homologs, *wmk*-1 (wHmt_12830, 561 bp) and *wmk*-2 (wHmt_12840, 361 bp) had one helix-turn-helix (HTH) domain. Conversely, *wmk*-3 (wHmt_12920, 897 bp) and *wmk*-4 (wHmt_12930, 1006 bp) harbored two HTH domains, as originally found in the *wmk* (WD0626) of the *w*Mel strain (Figure 1C).[7] Although the sequence lengths of these genes differ, the four *wmk* homologs have highly homologous nucleotide and amino acid sequences (Figure S3, Table S3). Notably, the *w*Hm-t specific ankyrin repeat- and papain-like proteinase domain-containing phage gene *w*Hmt_13140 (3558 bp, Figures 1B and 1C) showed high homology to the Wolbachia *oscar* gene (WP250126898.1, Katsuma et al.[13]) that induces MK in *O. furnacalis* (Figures 1B–C, Table S3). Further resequencing analysis confirmed that the WO*w*Hm-t76 region was conserved in *w*Hm-t (n = 15; Taiwan) but not in *w*Hm-c (n = 9; Japan; Figure 1D). Other than WO*w*Hm-t76, the deleted or inserted loci in *w*Hm-t genome were IS5 family transposases. To confirm whether WO*w*Hm-t76 exists consistently in *w*Hm-t, the field-collected *H. magnanima* were subjected to PCR targeting the gene *w*Hmt_13140 (i.e., *Hm-oscar*) located in the WO*w*Hm-t76 region. The 32 MK matrilines established from a single population of Taiwan were all positive, whereas the 13 non-MK matrilines established from the same population were negative for the *Hm-oscar* gene. Conversely, all the Japanese *H. magnanima* derived from 18 populations consistently infected with the non-MK *w*Hm-c were negative (116 females and 129 males) for the *Hm-oscar* gene (Figure 1E).

Comparison of *wmk*, *oscar*, and *cif* homologs between *Wolbachia* strains

MK *w*Hm-t as well as three non-MK *Wolbachia* (*w*Hm-a, *w*Hm-b, and *w*Hm-c) encoded multiple *wmk* homologs regardless of their phenotype (Figure 2A). Other than the four *wmk* homologs present in the

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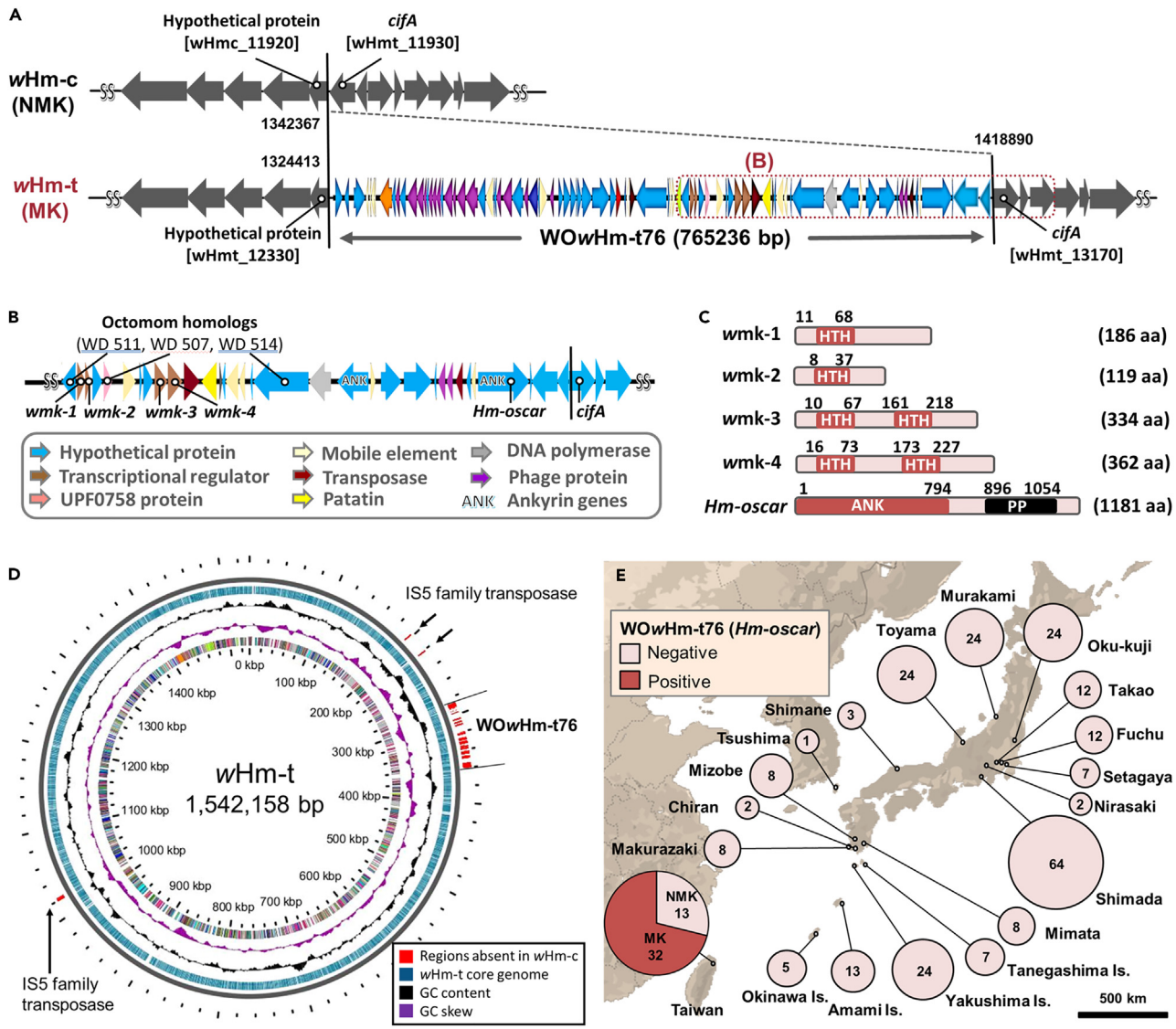


Figure 1. Genomic comparisons of *Wolbachia* strains in *Homona magnanima*

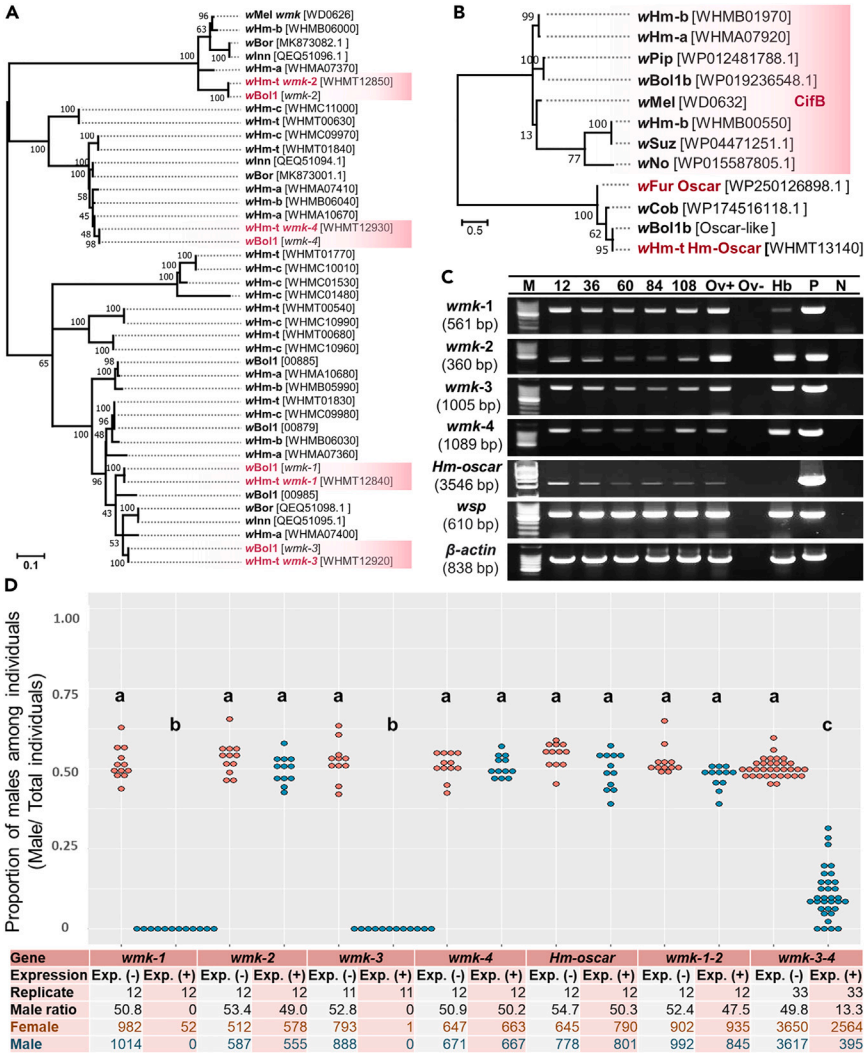
(A) An insertion, the WOwHm-t76 region, in the *w*Hm-t genome. The wHmc_11920 of the *w*Hm-c encodes the IS110 family of transposase and has identical sequences of wHmt_12330 of the *w*Hm-t. wHmc_11930 of *w*Hm-c, encoding cytoplasmic incompatibility factor A (CifA), is identical to wHmt_13170 of *w*Hm-t. The area highlighted by (B) and surrounded by a dashed red line indicates the locus encoding pathogenesis-related genes referred to in the following section.

(B) Virulence-associated genes in the WOwHm-t76 region.

(C) The domain structures of *wmk* and *Hm-oscar* genes annotated by Inter-Pro. HTH: helix-turn-helix. ANK: ankyrin repeat. PP: papain-like proteinase domain.

(D) Genomic loci, absent from *w*Hm-c genomes (n = 9) but conserved in *w*Hm-t genomes (n = 15), are highlighted in red on the *w*Hm-t genome. Homologous genes or loci between *w*Hm-c and *w*Hm-t are not highlighted in red. (E) Detection of the WOwHm-t76 region (i.e., *Hm-oscar* gene) from *Wolbachia* infecting Japanese and Taiwanese *H. magnanima*.

WOwHm-t76 region (i.e., *wmk*-1, *wmk*-2, *wmk*-3, and *wmk*-4), closely related *w*Hm-t and *w*Hm-c strains shared six other *wmk* homologs (100% nucleotide identity for each gene) (Figure 2A). Intriguingly, although absent in the non-MK strains (*w*Hm-a, *w*Hm-b, and *w*Hm-c), the WOwHm-t76-associated *wmk* homologs were found in the genome of the MK *w*Bol1 strain in *Hypolimnas bolina* (GenBank accession: ASM33377v1; Duplouy et al.[26]), with 100% nucleotide identity for each gene (LC701684-LC701687). We confirmed that the *wmk* genes with identical nucleotide sequences were also conserved in the *w*Bol1 strain in a field-collected adult female of Japanese *H. bolina*.



Gene	wmk-1		wmk-2		wmk-3		wmk-4		Hm-oscar		wmk-1-2		wmk-3-4	
Expression	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)	Exp. (-)	Exp. (+)
Replicate	12	12	12	12	11	11	12	12	12	12	12	12	33	33
Male ratio	50.8	0	53.4	49.0	52.8	0	50.9	50.2	54.7	50.3	52.4	47.5	49.8	13.3
Female	982	52	512	578	793	1	647	663	645	790	902	935	3650	2564
Male	1014	0	587	555	888	0	671	667	778	801	992	845	3617	395

Figure 2. Phylogenies and toxicities of *wmk* and *Hm-oscar* genes in the WOwHm-t76 region

(A and B) Phylogenetic trees of *wmk* gene homologs (nucleotide) (A) and Oscar and CifB protein homologs (amino acids) (B). Phylogenetic trees were constructed based on maximum likelihood with bootstrap re-sampling of 1,000 replicates using MEGA7.[27] Homologs of *Wolbachia* strains were quoted from the NCBI database, and those of *w*Hm-a, *w*Hm-b, and *w*Hm-t strains were manually annotated by BLAST and HHpred searches. The *wmk* and Oscar homologs present in *w*Hm-t but absent in the *w*Hm-a, *w*Hm-b, and *w*Hm-c are highlighted in red (*wmk-1*, *wmk-2*, *wmk-3*, *wmk-4*, and Hm-Oscar). *wmk-1*, *wmk-2*, *wmk-3*, and *wmk-4* in the *w*Bol1 genome are also highlighted in red.

(C) RT-PCR detection for the *wmk* and *Hb-oscar* genes. 12, 36, 60, 84, and 108 indicate hours post oviposition of *Homona magnanima* egg masses. Ov+: female ovary of *H. magnanima* (W^{T12} line); Ov-: non-reverse-transcribed samples (RT minus, control) using RNA extracted from the female ovary of *H. magnanima* (W^{T12} line); Hb: female ovary of *Hypolimnas bolina*; P: DNA extracted from an *H. magnanima* W^{T12} adult female (positive control); N: negative control (water). M: DNA ladders. 1000 bp ladder for *Hm-oscar*, and 100 bp ladder for other genes.

(D) The male ratio of adult progeny obtained from crosses between the *actin–GAL4 Drosophila melanogaster* line and seven *UAS* transgenic fly lines (*wmk*-1, *wmk*-2, *wmk*-3, *wmk*-4, *cifB*-like, *wmk*-1 and *wmk*-2, and *wmk*-3 and *wmk*-4; n = 11–33 independent crosses for each transgene). We counted the number of resulting offspring (females, orange; males, blue) with both *actin–GAL4* and *UAS* (+) and siblings with *UAS* and *Cyo (CyO)* as internal controls (–). The total numbers of adult counts for each genotype and sex are shown at the bottom. Different letters indicate statistically significant differences (Steel–Dwass test, $p < 0.05$). Dot plots show all data points individually. Exp.: expression; (–): not expressed; (+): expressed. Female: adult female progeny; Male: adult male progeny.

MK *w*Hm-t harbored no CifB homologs except for the Hm-Oscar (Figure 2B, Table S3), although it encoded a CifA homolog (wHmt_13170) in a region adjacent to the Hm-Oscar (Figures 1A and 1B). This CifA homolog (wHmt_13170) was present as an identical orphan CifA (whm-c_11930) in the *w*Hm-c genome (Figures 1A and 1B) that has neither Oscar nor CifB. The *cifA* and *cifB* gene pairs are involved in CI induction.[11–13,28–33] The orphan *cifA* genes of *w*Hm-c and *w*Hm-t are likely remnants of the common *cifA-cifB* gene pair that was broken through evolutionary processes. The ancestral *w*Hm-c type *Wolbachia* with the orphan *cifA* gene appears to have acquired the WO*w*Hm-t76 region harboring Oscar and *wmk* through the integration of a certain WO phage, which gave rise to the MK-induced *w*Hm-t. Compared with *w*Hm-t and *w*Hm-c, the CI-inducing *w*Hm-b encoded the *cifA-cifB* genes (Figure 2B, Table S4). Intriguingly, *w*Hm-a, which does not induce CI in *H. magnanima*, also had an intact *cifA-cifB* gene pair. Although its functions remain unclear, the Cifs in *w*Hm-a are insufficient to induce CI in *H. magnanima* but may have a rescue function for CI induced by other *Wolbachia* strains (as observed in Bourtzis et al.[34] and Merçot and Poinsot[35]). PCR assays confirmed that the four *w*Hm-t-specific *wmk* genes as well as the *Hm-oscar* genes were expressed during embryogenesis and in the ovary of *H. magnanima* (Figure 2C). In addition, the *wmk* genes were also expressed in the ovary of *H. bolina*.

Single-gene expression of certain *wmk* genes killed both males and females, and dual expression of tandemly arrayed *wmk* genes resulted in MK *in Drosophila*

To assess whether the four *wmk* genes (*wmk*-1, *wmk*-2, *wmk*-3, and *wmk*-4) and the *Hm-oscar* gene on the WO*w*Hm-*t*-76 region affect insect viability, we synthesized these genes with codon optimization and overexpressed them in *Wolbachia*-free *D. melanogaster* (Figure S4) using the *GAL4/UAS* system.[7,11,36] When *wmk*-1 was overexpressed (12 replicates), no adult males and only a small number of adult females (n = 52 in total, ranging from 0 to 21 individuals per replicate) emerged, which is in sharp contrast with the non-expressing control groups (male ratio: 51.6% $\pm$ 5.11; 982 females and 1014 males in total) ($p = 0.0007$ for sex ratio, Steel–Dwass test, Figure 2D; $p = 0.0003$ for the number of females and $p = 0.0001$ for the number of males; Figure S4). Notably, when *wmk*-3 was overexpressed (11 replicates), almost no adults emerged (no males and females in 10 replicates and only one female in 1 replicate) in contrast with the control group (793 females and 888 males in total). Conversely, sex ratio and viability were not affected by the overexpression of *wmk*-2 (male ratio: 49.2% $\pm$ 4.42, 12 replicates), *wmk*-4 (male ratio: 50.6% $\pm$ 3.38, 12 replicates), and *Hm-oscar* genes (male ratio: 49.8% $\pm$ 5.57, 12 replicates) (sex ratio: $p = 0.8932$ for *wmk*-2; $p = 0.9999$ for *wmk*-4; $p = 0.4618$ for *Hm-oscar* gene; Figure 2D; number of emerged females: $p = 0.9206$ for *wmk*-2, $p = 0.9971$ for *wmk*-4, and $p = 0.5815$ for *Hm-oscar*; number of emerged males: $p = 0.9534$ for *wmk*-2, $p = 0.9998$ for *wmk*-4, and $p = 0.9999$ for *Hm-oscar*) (Figure S4).

To assess whether the dual expression of tandemly arrayed *wmk* pairs has additive or different effects on host viability similar to the *cifA*- and *cifB*-involving CI phenotype of *Wolbachia*, we simultaneously overexpressed each pair of the *wmk* genes (i.e., *wmk*-1 and *wmk*-2 or *wmk*-3 and *wmk*-4) in *Drosophila*, as described by Beckmann et al.[11] In contrast to the single expression of *wmk*-1 or *wmk*-2, dual expression of *wmk*-1 and *wmk*-2 did not bias the sex ratio (male ratio: 47.5% $\pm$ 3.6, 12 replicates, $p = 0.1817$) and generated numbers of adults (935 females and 845 males in total) comparable to the control groups (902 females and 992 males) ($p = 0.9776624$ for females; $p = 0.5997$ for males). Conversely, dual expression of tandemly arrayed *wmk*-3 and *wmk*-4 led to strongly female-biased sex ratios (female ratio: 89.1% $\pm$ 7.84, 33 replicates) compared to the control groups (50.1% $\pm$ 3.00, 33 replicates) ($p = 0.0000$; Figure 2D) and generated larger numbers of adults (2564 females and 395 males) than the flies singly expressing wmk-3 ($p = 0.0004$, Figure S4). Dead pupae (n = 2641 in total, 33 replicates) were conspicuously identified in the cohorts including those that should co-express *wmk*-3 and *wmk*-4. Among the 94 dead pupae harboring *wmk*-3, *wmk*-4, and Gal4, 77 were found to be males through Y chromosome-specific PCR (male ratio: 81.9%) (Figure S4).

DISCUSSION

By comparing the genomes of an MK *Wolbachia* strain and three non-MK *Wolbachia* strains derived from *H. magnanima*, we identified a 76 kb prophage region WO*w*Hm-t76, which was specifically present in the MK *Wolbachia*. Transgenic overexpression of one of the *wmk* gene pairs (*wmk*-1 or *wmk*-3) encoded by WO*w*Hm-t76 in the non-native host *D. melanogaster* induced complete male lethality and high female lethality. This is similar to the previous finding that overexpression of each *wmk* gene from *w*Suz and *w*Rec killed all *D. melanogaster* individuals.[8] Compared with such strong lethality in *D. melanogaster*, when *wmk* homologs derived from *Drosophila* MK *Wolbachia* (e.g., *w*Bif and *w*Inn) and the butterfly

H. bolina (*w*Bol1) were overexpressed, no effect was observed in *D. melanogaster*.[37] The *wmk* homolog of *w*Bol1 tested in Perlmutter et al.[37] (the sequence therein not available) is not likely to be identical in sequence to those tested in our study (*wmk*-1 to *wmk*-4), which should otherwise induce strong lethality; indeed, *w*Bol1 harbors many *wmk* homologs outside the WO*w*Hm-t76 region. We further demonstrated the recovery of flies by the additional expression of *wmk*-2 or *wmk*-4 in addition to *wmk*-1 or *wmk*-3, which induce strong lethality. Collectively, the virulence of *wmk* in males and females can be attributed to the difference in *wmk* sequences. However, recovery of viability by the co-expression of the tandemly arrayed *wmk* homologs may be explained by the combined action, wherein one *wmk* is toxic to both sexes and the other pair acts as its suppressor. Many microbes, including *Wolbachia*, and some animals employ toxin–antitoxin (TA) systems.[37–47] By definition, TA modules encode a toxin that can globally down-regulate metabolism and an antitoxin that counteracts this action.[44] Some type II TA modules encode HTH domain-containing proteins that form a stable complex and thus inactivate the toxin.[43,45–47] Although it remains to be elucidated, we propose that the adjacent *wmk* pairs form a complex (i.e., a dimer), as reported in the *Wolbachia* CifA-CifB pair,[11,28–33] which can mitigate the virulence of *wmk*.

We can raise two distinct mechanistic possibilities for the *wmk*-induced strong male lethality in *Drosophila*. One possibility is that *Wolbachia* produces *wmk* regardless of its host's sex and the strong male lethality is represented by the different levels of susceptibility to the *wmk* genes between the sexes. The other possibility is that the *wmk* gene functions differently depending on the host sex. Moreover, the level of affinity between the *wmk* pairs may differ depending on the physiological environment, which could account for the differences in virulence between the sexes. These potential mechanisms of *wmk*-induced male lethality appear to be different from that of *Spiroplasma*-induced MK in *D. melanogaster*, where the spaid toxin specifically damages the male X chromosome.[19,36] Further investigation is needed to determine whether *wmk* replicates the effects observed with MK *Wolbachia* in *Drosophila* (e.g., male-specific cell death and malfunctioning of dosage compensation[36,48]).

Intriguingly, we found that the overexpression of *Hm-oscar* did not induce lethality in *D. melanogaster*. MK has been hypothesized to target molecular mechanisms involved in sex determination and differentiation.[16–19] Insect sex-determining systems are diverse; for example, Lepidoptera (including *H. magnanima*) and Diptera (including *D. melanogaster*) do not share any known sex-determining genes other than the widely conserved *doublesex* (*dsx*) gene. The oscar protein encoded by the *w*Fur strain induces embryonic MK in *Ostrinia* moths by targeting the host factor Masc, an essential protein for male sex determination.[13,49] Since Masc is conserved in Lepidoptera but not in Diptera,[13] Hm-Oscar may not be able to induce MK in *Drosophila*. Similar to the effects of *w*Fur in *Ostrinia* moths,[13,49] *w*Hm-t impairs the expression of *masc* and sex determination cascades in *H. magnanima*.[50] This finding suggests that *Hm-oscar* in the WO*w*Hm-t76 region may be involved in the *w*Hm-t-induced MK in *H. magnanima*. Considering that *oscar* homologs have only been identified from the MK *Wolbachia* in lepidopteran insects (i.e., *w*Fur, *w*Bol, and *w*Hm-t),[13] the mechanisms (i.e., causative genes) of *Wolbachia*-induced MK may differ between insect taxa.

We suspect that *Wolbachia* strains have acquired multiple MK genes through their evolution. Generally, virulent genes often undergo duplications and substitutions through strong selective pressure.[51,52] Regardless of their phenotype, MK *Wolbachia* strains (e.g., *w*Rec, *w*Bol1, and *w*Hm-t) and non-MK *Wolbachia* strains (e.g., *w*Mel, *w*Hm-a, *w*Hm-b, and *w*Hm-c) generally possess multiple *wmk* gene cassettes, some of which showed virulence toward the non-native host *D. melanogaster*. The *wmk* homologs encoded by WO*w*Hm-t76 may be able to induce MK in *H. magnanima* by targeting common machinery present in insects. Actually, *wmk* genes of *w*Hm-t were expressed throughout embryogenesis, which is when MK occurred in *H. magnanima* (Figure 2C). We speculate that the *wmk* gene may have originally caused lethality in arthropods without preference for sex. The duplications and nucleotide substitutions in the *wmk* genes may have reduced the virulence and allowed the offspring of *Wolbachia*-infected individuals to survive. As mentioned above, the dual expression of *wmk* gene pairs resulted in high male lethality. An excessively female-biased sex ratio selects for host suppressors against MK in various insects.[16] If MK was quantitatively suppressed by detoxification of *wmk*, the production of a higher amount of *wmk* via the multiplication of *wmk* gene cassettes would counteract the action of host suppressors. The subsequent development of host suppressors would then lead to an increased number of *wmk* genes in the *Wolbachia* genome.[16,22,37,53] An intense evolutionary arms race between *Wolbachia* and its host[37] may have increased the diversity of MK-associated genes (i.e., *wmk* gene pairs and *oscar*) in the *Wolbachia* genome.

In addition, although the two MK strains *w*Bol1 and *w*Hm-t are phylogenetically distantly related,[23,24] they harbor four *wmk* genes in the WO phage region, each of which is identical in nucleotide sequence. As molecular phylogenetic studies have shown previously in WO phages,[54–58] our data suggest that the WO phage may have moved between *w*Hm-t and *w*Bol1. Prophage regions such as WO*w*Hm-t76, which include multiple virulence genes, may be conserved among MK *Wolbachia* strains regardless of their phylogenetic relationships. While the origin (source) of WO*w*Hm-t76 remains unclear, further genome analysis of *w*Bol1 as well as other *Wolbachia* strains will shed light on the evolutionary processes of MK *Wolbachia*. The diversification of the virulence-associated genes, such as the *wmk* and *oscar* genes, as well as rampant horizontal transmission of WO phages between different *Wolbachia* strains may have contributed to the prominent success of *Wolbachia*. Our findings offer insights into the molecular mechanisms and evolutionary associations of MK *Wolbachia*, WO phages, and their host insects.

Limitations of the study

Our study highlights the diversity in MK mechanisms and the bacteriophage as a critical driver of the evolution of MK *Wolbachia*. A limitation of this study is that we did not elucidate the functions and toxicities of the *wmk* and *oscar* homologs in the native host *H. magnanima*. Although our study could highlight the virulence and conjugated functions of the *wmk* genes in the non-native host *D. melanogaster*, the higher expression levels achieved by the Gal4-UAS system do not necessarily recapitulate the natural effects produced by the expression state of *Wolbachia* in insects. Future genome comparisons and functional validation of *wmk* and *oscar* homologs in *H. magnanima* will contribute to an integrated understanding of the diversity of the *Wolbachia*-induced MK mechanisms.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106842.

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AUTHOR CONTRIBUTIONS

H. A. conducted field surveys, genome sequencing, and data analysis; designed fly experiments; wrote the original manuscript; and revised the manuscript. H. A. coordinated the single-cell analysis and presented the entire discussion. Y. N. conducted the single-cell genome sequencing. M. K. conducted the single-cell genome construction. K. I. helped with the genome construction. M. H. supported the entire single-cell analysis. S-R. L. organized the collection of insects in Taiwan and contributed to the discussion. M. U. collected Japanese *H. magnanima*. M. N. provided assistance during all experiments. Y. K. sampled insects and contributed to the entire discussion. T. H. designed and supported the fly experiments. D. K. supported the fly experiments and revised the manuscript. Lastly, H. A., H. T, and M.N. I. took responsibility for the decision to submit the manuscript for publication and managed the experiments and discussion.

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY

We support inclusive, diverse, and equitable conduct of research.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Homona magnanima, NSR line, Taiwan	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
H. magnanima, WT12 line, Taiwan	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
H. magnanima, Yakushima line, Japan	This paper	N/A
Drosophila melanogaster, actin5C–GAL4 [actin–GAL4/CyO]	BDSC	Stock number: 4144 (https://flybase.org/reports/FBst0004414.html)
D. melanogaster, UAS-wmk-1	This paper	N/A
D. melanogaster, UAS-wmk-2	This paper	N/A
D. melanogaster, UAS-wmk-3	This paper	N/A
D. melanogaster, UAS-wmk-4	This paper	N/A
D. melanogaster, UAS-wmk-1-t2a-wmk-2	This paper	N/A
D. melanogaster, UAS-wmk-3-t2a-wmk-4	This paper	N/A
D. melanogaster, UAS-Hm-oscar	This paper	N/A
Chemicals, peptides, and recombinant proteins		
SilkMate 2S	Nosan Co., Yokohama, Japan	https://www.nosan.co.jp/english/
H. magnanima sex pheromone	Sumitomo Chemical Co., Ltd., Tokyo, Japan	https://jppaonlinestore.raku-uru.jp/item-detail/972069
SE traps	Sankei Chemical, Kagoshima, Japan	https://jppaonlinestore.raku-uru.jp/item-detail/972059
Universal SYBR Green Master Mix ROX	F. Hoffmann-La Roche, Ltd., Basel, Switzerland	https://www.n-genetics.com/products/1295/1023/13822.pdf
5.0-μm filter	Sartorius AG, Göttingen, Germany	https://www.sartorius.com/en/products-jp/lab-filtration-purification-jp/syringe-filters-jp
1.2-μm filter	Sartorius AG, Göttingen, Germany	https://www.sartorius.com/en/products-jp/lab-filtration-purification-jp/syringe-filters-jp
dsDNA HS quantification kit	Invitrogen, MA, USA	https://www.thermofisher.com/order/catalog/product/jp/ja/Q32851
REPLI-g Mini Kit	Qiagen, Hilden, Germany	https://www.qiagen.com/jp/listpages/ez1-cards/repli-g-mini-kit/
QIAseq FX DNA Library Kit	Qiagen, Hilden, Germany	https://www.qiagen.com/ja-us/products/discovery-and-translational-research/next-generation-sequencing/metagenomics/qiaseq-fx-dna-library-kit
MiSeq Reagent Kit v3	Illumina, CA, USA	https://jp.illumina.com/products/by-type/sequencing-kits/cluster-gen-sequencing-reagents/miseq-reagent-kit-v3.html
Rapid Sequencing Kit (SQK-RAD004)	Oxford Nanopore Technologies, Oxford, UK	https://store.nanoporetech.com/rapid-sequencing-kit.html
NEBNext dsDNA Fragmentase	New England BioLabs, MA, USA	https://international.neb.com/products/m0348-nebnext-dsdna-fragmentase#Protocols,%20Manuals%20&%20Usage
AMPure XP beads	Beckman Coulter, CA, USA	https://www.beckman.jp/reagents/genomic/cleanup-and-size-selection/pcr

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
GenNext NGS Library Prep Kit	TOYOBO Co Ltd., Osaka, Japan	https://www.toyobo-global.com/seihin/xr/lifescience/support/manual/NLQ-101.pdf
NEBNext Multiplex Oligos for Illumina	New England BioLabs, MA, USA	https://international.neb.com/products/e7335-nebnext-multiplex-oligos-for-illumina-index-primers-set-1#Product%20Information
Emerald Amp Max Master mix	TaKaRa Bio Inc., Shiga, Japan	https://www.takarabio.com/products/pcr/pcr-master-mixes/dye-added/emeraldamp-max-products/emeraldamp-max-premix
ISOGEN II	Nippongene, Tokyo, Japan	https://www.nippongene.com/siyaku/product/extraction/isogen2/isogen2.html
PrimeScript™ II Reverse Transcriptase	TaKaRa Bio Inc., Shiga, Japan	https://catalog.takara-bio.co.jp/product/basic_info.php?unitid=U100007713
pUASz1.1	DGRC, Kyoto, Japan	https://dgrc.bio.indiana.edu//stock/1433
NEBuilder® HiFi DNA Assembly	New England BioLabs, MA, USA	https://international.neb.com/applications/cloning-and-synthetic-biology/dna-assembly-and-cloning/nebuilder-hifi-dna-assembly
Deposited data		
Raw sequence reads	This paper	GenBank BioProject: PRJDB13119, SAMD00445397 - SAMD00445400
*w*Hm-a genome data	This paper	GenBank accession number: BQXF01000001 - BQXF01000020
*w*Hm-b genome data	This paper	GenBank accession number: BQXG01000001 - BQXG01000009
*w*Hm-c genome data	This paper	GenBank accession number: AP025639
*w*Hm-t genome data	This paper	GenBank accession number: AP025638
Wolbachia wmk, cif, oscar genes	This paper	GenBank accession number: LC701647 - LC701694
Other datasets (*Wolbachia* density and fly data)	This paper	https://data.mendeley.com/datasets/jpvk5rjksk/1
Experimental models: Organisms/strains		
Homona magnanima, NSR line, Taiwan	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
H. magnanima, WT12 line, Taiwan	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
H. magnanima, Yakushima line, Japan	This paper	N/A
Drosophila melanogaster, actin5C–GAL4 [actin–GAL4/CyO]	BDSC	Stock number: 4144 (https://flybase.org/reports/FBst0004414.html)
D. melanogaster, UAS-wmk-1	This paper	N/A
D. melanogaster, UAS-wmk-2	This paper	N/A
D. melanogaster, UAS-wmk-3	This paper	N/A
D. melanogaster, UAS-wmk-4	This paper	N/A
D. melanogaster, UAS-wmk-1-t2a-wmk-2	This paper	N/A
D. melanogaster, UAS-wmk-3-t2a-wmk-4	This paper	N/A
D. melanogaster, UAS-Hm-oscar	This paper	N/A
Oligonucleotides		
PCR primers for*wsp*	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
PCR primers for*Hm-oscar*	This paper	N/A
PCR primers for*wmk-1*	This paper	N/A
PCR primers for*wmk-2*	This paper	N/A

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
PCR primers for*wmk-3*	This paper	N/A
PCR primers for*wmk-4*	This paper	N/A
PCR primers for*actin*	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
PCR primers for COI	Arai et al.[23]	https://doi.org/10.1007/s00248-019-01469-6
PCR primers for *D. melanogaster* Y chromosome	This study	N/A
PCR primers for*D. melanogaster* Gal4	This study	N/A
Codon-optimized *Hm-oscar*	This paper	N/A
Codon-optimized *wmk-1*	This paper	N/A
Codon-optimized *wmk-2*	This paper	N/A
Codon-optimized wmk-3	This paper	N/A
Codon-optimized *wmk-4*	This paper	N/A
Codon-optimized *wmk-1-t2a-wmk-2*	This paper	N/A
Codon-optimized *wmk-3-t2a-wmk-4*	This paper	N/A
Software and algorithms		
Canu 1.6 and 2.0	Li[59]	https://canu.readthedocs.io/en/latest/
minimap2 v2.17	Walker et al.[60]	https://github.com/lh3/minimap2
Pilon v.1.23	Walker et al.[61]	https://github.com/broadinstitute/pilon
DFAST	Arndt et al.[62]	https://dfast.ddbj.nig.ac.jp/
PHASTER	Li et al.[63]	https://phaster.ca/
SAMtools v.1.9	Petkau et al.[64]	http://www.htslib.org/
Gview	Sullivan et al.[65]	https://server.gview.ca
Easyfig v2	Darling et al.[66]	https://mjsull.github.io/Easyfig/
Mauve 2.4	Ihaka and Gentleman[67]	https://darlinglab.org/mauve/download.html
R software v4.0	Paysan-Lafosse et al.[68]	https://www.r-project.org
InterPro	Gabler et al.[69]	https://www.ebi.ac.uk/interpro
HHpred	Kumar et al.[27]	https://toolkit.tuebingen.mpg.de/tools/hhpred
MEGA7	Kumar et al.[27]	https://www.megasoftware.net/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents are accessible from the lead contact, Hiroshi Arai (dazai39papilio@gmail.com).

Materials availability

The *Drosophila* lines and vectors used in this study are accessible upon request. *H. magnanima* lines are not authorized to share under legislation by the Ministry of Agriculture, Forestry and Fisheries (permission No. 27 - Yokohama Shokubou 891 and No. 297 - Yokohama Shokubou 1326).

Data and code availability

- The sequence read data are publicly available in the DDBJ under accession numbers PRJDB13119 (BioProject) and SAMD00445397 to SAMD00445400 (BioSample). *Wolbachia* genomes (contigs) are available in the DDBJ database under the following accession numbers: *w*Hm-a (BQXF01000001 - BQXF01000020), *w*Hm-b (BQXG01000001 - BQXG01000009), *w*Hm-c (AP025639), and *w*Hm-t (AP025638). Homologs of *wmk*, *cif*, *Hm-oscar*, and Octomom genes and synthetic construct of *wmk* and *Hm-oscar* genes are publicly available in the DDBJ under accession numbers LC701647 to LC701694. Other data are publicly available from the Mendeley data (https://data.mendeley.com/datasets/jpvk5rjksk/1).

- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is accessible from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Homona magnanima

To construct a genome of *w*Hm-t strain, we used the laboratory-maintained all female *H. magnanima* line, W^{T12}, which was initially collected in Taiwan (The Tea Research Extension Station, Taoyuan city) in 2015.[23] The W^{T12} line was maintained over 50 generations by crossing the males of the normal sex ratio line (NSR), which is negative for *Wolbachia*.[23] To construct genomes of *w*Hm-a, *w*Hm-b, and *w*Hm-c strain, we used a 1:1 sex ratio line (referred to as a Yakushima line) triply infected with the *Wolbachia* strains that was newly established from a female collected from Yakushima Island, Kagoshima, Japan in 2018. The Yakushima line was maintained by inbreeding (i.e., sib mating) across more than 20 generations. Larvae were reared using artificial diet SilkMate 2S (Nosan Co., Yokohama, Japan) at 25°C under a long (16L:8D) photoperiodic condition. For the resequencing of *Wolbachia*, we also used *H. magnanima* samples collected from fields using sex pheromone (Sumitomo Chemical Co., Ltd., Tokyo, Japan) with SE traps (Sankei Chemical, Kagoshima, Japan) in Japan (Table S5) and the Taiwanese individuals previously obtained by Arai et al.[23]

Drosophila melanogaster

D. melanogaster line *actin5C–GAL4* (*actin–GAL4/CyO*; BDSC 4414) was obtained from Bloomington *Drosophila* Stock Center at Indiana University (BDSC). Those *D. melanogaster* lines were maintained at 25°C with the standard banana medium. We also maintained transgenic fly lines harboring the codon-optimized *wmk*-1, *wmk*-2, *wmk*-3, *wmk*-4, and *Hm-oscar* genes. One of the two tandemly arrayed *wmk* pairs, either (i) *wmk*-1 and *wmk*-2 or (ii) *wmk*-3 and *wmk*-4, was conjugated using T2A peptide as described by Beckmann et al.[11] Transgenic fly lines were established by the standard microinjection method for phiC31 integrase transformation into the third chromosome at BestGene Inc. (Chino Hills, CA, UAS) using the pUASz 1.1 vector containing the synthesized genes. The newly established transgenic flies shared the genetic background of the fly strain with stock number 24749 (genotype: y1 M{vas-int.Dm}ZH-2A w*; M {3xP3-RFP.attP}ZH-86Fb), and each *wmk* and *Hm-oscar* gene was inserted into the locus (estimated cytological site #86F8) on the right hand side of the third chromosome. Prior to zygotically overexpressing the synthetic gene with a codon optimized for *D. melanogaster*, fly stocks were reared in a tetracycline-containing medium (0.05% [w/v]) for at least one generation and in a standard banana medium for two generations. We confirmed that all the fly lines were negative for *Wolbachia* infection by PCR targeting *Wolbachia wsp* gene (Figure S4).

METHOD DETAILS

Wolbachia quantification

Wolbachia density was quantified as described in Arai et al.[23] Briefly, total DNA was extracted from individual *H. magnanima* abdomen using cell lysis solution (10 mM Tris-HCl, 100 mM EDTA, and 1% SDS, pH 8.0). DNA concentrations were quantified using Nano Photometer NP 80 (Implen, München, Germany) and were adjusted to a concentration of 10 ng/μL with MilliQ water, mixed with *Wolbachia wsp* gene primer sets (Table S6) and Universal SYBR Green Master Mix ROX (F. Hoffmann-La Roche, Ltd., Basel, Switzerland), and subjected to real-time PCR assays using Step One Plus Real-Time PCR System (Life Technologies Japan, Tokyo, Japan) as reported by Arai et al.[23] The cycle conditions were as follows: 10 min at 95°C; followed by 40 cycles of 15 s at 95°C, and 1 min at 60°C.

Genome construction of *Wolbachia*

Wolbachia was purified based on Duplouy et al.[26] and Iturbe-Ormaetxe et al.[70] with several modifications. To pellet *w*Hm-t cells, 20 abdomens of W^{T12} females were individually placed in a plastic tube and homogenized in 1,300 μL of phosphate-buffered saline (PBS; 137 mmol/l NaCl, 8.1 mmol/l Na_2HPO_4, 2.68 mmol/l KCl, 1.47 mmol/l KH_2PO_4, pH 7.4). After centrifugation at 4°C, 3,000 *g* for 5 min, 1,000 μL of the supernatant were filtered using 5.0-μm and 1.2-μm filters (Sartorius AG, Göttingen, Germany). The filtrates were centrifuged at 4°C, 18,000 *g*, for 20 min. Pellets were dissolved in 50 μL of PBS and subjected to DNA extractions using the cell lysis solution. DNA was quantified with NP80 (Implen) and Qubit v4 and dsDNA HS quantification kit (Invitrogen, MA, USA). Extracted DNA was whole-genome-amplified (WGA) with the REPLI-g

Mini Kit (Qiagen, Hilden, Germany) for 16 h at 30°C according to the manufacturer's protocol. The amplicon was sequenced using the PacBio RSII and Illumina (150 bp paired-end) platforms. PacBio RSII data were assembled using Canu 1.6,[71] and subsequent polishing using Illumina data with minimap2 v2.17[59] and Pilon v.1.23[60] generated the complete circular genome of *w*Hm-t.

For genome sequencing of *w*Hm-a, *w*Hm-b, and *w*Hm-c, *Wolbachia* cells were purified from the 20 male pupae of Yakushima line as mentioned above. *Wolbachia* cells in 30 μm diameter droplets (0.3 cell/droplet) were lysed and amplified with REPLI-g Mini Kit (Qiagen) as described by Chijiiwa et al.[61] and Nishikawa et al.[72] The WGA amplicons were subjected to library preparation using QIAseq FX DNA Library Kit (Qiagen) and sequenced at MiSeq (75 bp paired-end) with MiSeq Reagent Kit v3 (Illumina, CA, USA). The amplicons were further sequenced at GridION using Rapid Sequencing Kit (SQK-RAD004, Oxford Nanopore Technologies, Oxford, UK). The sequence data from GridION were assembled by Canu 2.0[71] and polished via Pilon v.1.23[60] using MiSeq data. All the *Wolbachia* genomes were annotated via DFAST.[73] Prophage regions were annotated with PHASTER.[62]

Resequencing analysis and detection of the WO*w*Hm-t76 region

A 1 μg sample of DNA extracted from field-collected *H. magnanima* (Table S5) harboring either *w*Hm-t (n = 15, Taiwan) or *w*Hm-c (n = 9, Japan) was incubated with NEBNext dsDNA Fragmentase (New England BioLabs, MA, USA) at 37°C for 24 min. After 5 μL of 0.5M EDTA was mixed and incubated at 65°C for 10 min, fragmented DNA was purified with AMPure XP beads (Beckman Coulter, CA, USA) following the manufacturer's protocol. The purified DNA samples were subjected to library preparation using GenNext NGS Library Prep Kit (TOYOBO Co Ltd., Osaka, Japan) with NEBNext Multiplex Oligos for Illumina (New England BioLabs) for multiplex sequencing. Size selection optimized to 300 bp insert was carried out using AMPure XP beads (Beckman Coulter) following the manufacturer's protocol. Size selected libraries were amplified using 2x Library Amplification Master Mix (TOYOBO) and NEBNext Universal PCR Primers (New England BioLabs) at 94°C for 2 min, 6 cycles at 98°C for 10 sec, 60°C for 10 sec, and 68°C for 15 sec. Libraries, purified with AMPure XP beads (Beckman Coulter) and eluted in 10 mM Tris-HCl (pH 8.0–8.5), were sequenced at Novogene using Illumina platform (150 bp paired end). Resequenced data, mapped to the *w*Hm-t genome (W^{T12}) using minimap2 v2.17,[59] were converted to the consensus genomes using SAMtools v.1.9,[63] followed by synteny analysis using GView.[64] Homologies of the WO*w*Hm-t76 region and other WO phages were visualized using Easyfig v2[65] and Mauve 2.4.[66] The presence or absence of the WO*w*Hm-t76 region in field-collected *H. magnanima* was further assessed using PCR by amplifying a *Hm-oscar* gene (Table S6) in the WO*w*Hm-t76 region with the Emerald Amp Max Master mix (TaKaRa) at 94°C for 3 min, 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 7 min.

Gene expression of *wmk* and *oscar* homologs of *w*Hm-t

RNA was extracted from the W^{T12} egg masses of 12, 36, 60, and 84 h post oviposition (hpo) using ISOGEN II (Nippongene, Tokyo, Japan) following the manufacturer's protocol. For RT-PCR, the RNA (500–1000 ng/reaction) was reverse-transcribed using PrimeScript™ II Reverse Transcriptase (TaKaRa Bio Inc.) at 30°C for 10 min, 45°C for 60 min, and 70°C for 15 min. The cDNA was then amplified using EmeraldAmp MAX PCR Master Mix with specific primers for *wmk* (*wmk*-1, *wmk*-2, *wmk*-3, and *wmk*-4) and *Hm-oscar* genes (Table S6) at 94°C for 3 min, 35 cycles of 94°C for 30 s, 62°C for 30 s, 72°C for 3 min, and a final extension at 72°C for 7 min. The *wmk* gene expression levels were also verified using RNA extracted from an adult female ovary of *Hypolimnas bolina* (collected from Ishigaki Is., Okinawa) harboring *w*Bol1.

Somatic overexpression analysis using *D. melanogaster*

The *wmk* and *Hm-oscar* genes in the WO*w*Hm-t76 region were synthesized with codon optimization for *D. melanogaster* at Integrated DNA Technologies IDT (Coralville, IA, USA). We used IDT Codon Optimization Tool (https://sg.idtdna.com/pages/tools/codon-optimization-tool) to construct genes optimized for expression in *D. melanogaster*. To express two tandemly arrayed *wmk* pairs, either (i) *wmk*-1 and *wmk*-2 or (ii) *wmk*-3 and *wmk*-4 were conjugated using a T2A peptide as described by Beckmann et al.[11] Those synthetic genes were ligated into the plasmid pUASz1.1 (DGRC Stock 1433; https://dgrc.bio.indiana.edu//stock/1433; RRID:DGRC 1433) using NEBuilder® HiFi DNA Assembly (New England Biolabs) following the manufacturer's protocol. Transgenic fly lines were established using the standard microinjection method for phiC31 integrase transformation into the third chromosome at BestGene Inc. (Chino Hills, CA, UAS) using the synthetic constructs.

To transgenically overexpress the *wmk* and *cifB*-like genes, 10 females of *D. melanogaster* line *actin5C–GAL4* (*actin–GAL4/CyO*; BDSC 4414) were crossed with 5 males of homozygous *UAS* transgenic flies harboring *wmk* or *Hm-oscar* genes. These crossing experiments were replicated at least 11 times (numbers shown in Figure 2D). *D. melanogaster* lines were maintained at 25°C with standard banana medium and were randomly used for the crossings. Prior to zygotically overexpressing the synthetic gene with a codon optimized for *D. melanogaster*, fly stocks were reared in a tetracycline-containing medium (0.05% [w/v]) for at least one generation and in a standard banana medium for two generations. We confirmed that all the fly lines were negative for *Wolbachia* infection via PCR targeting the *Wolbachia wsp* gene (Figure S3). The numbers of offspring expressing transgenic (*actin-GAL4/UAS*) and non-expressing genes (*CyO/UAS*) in each replicate were all counted at the adult stage. The numbers of dead pupae in the lines harboring both *wmk*-3 and *wmk*-4 genes were counted and subjected to the detections of *wmk*-3, *wmk*-4, Gal4, and Y chromosome genes using specific primer sets (Table S6) at 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 30 min, and a final extension at 72°C for 7 min.

QUANTIFICATION AND STATISTICAL ANALYSIS

Wolbachia density

We used the average cycle threshold value (Ct) of two technical replicates for each sample and estimated *Wolbachia* density in *H. magnanima* individuals as described by Arai et al.[23] The number of *Wolbachia wsp* copies in 10 ng of sample DNA (i.e., *Wolbachia* density) was calculated based on a standard curve generated using 10^{-4}–10 ng of plasmid DNA harboring a cloned partial *wsp* sequence used in Arai et al.[23] *Wolbachia* densities in the field-collected samples (Table S5) were analyzed using Steel–Dwass test [http://aoki2.si.gunma-u.ac.jp/R/Steel-Dwass.html; source("http://aoki2.si.gunma-u.ac.jp/R/src/Steel-Dwass.R", encoding="euc-jp")] in R software v4.0.[67]

Annotations, homology surveys, and phylogenetical analysis of *Wolbachia* genes

Wolbachia wmk, cifA, cifB and *oscar* genes (accession numbers are shown in Figures 2A and 2B) were used as queries to identify the homologs from *w*Hm-a, *w*Hm-b, *w*Hm-c, and *w*Hm-t genomes using local BLASTn and BLASTp searches (default parameters). Motifs in the *wmk*, *cifA*, *cifB*, and *oscar* gene homologs were surveyed using InterPro[68] (https://www.ebi.ac.uk/interpro/) and HHpred[69] (https://toolkit.tuebingen.mpg.de/tools/hhpred). Phylogenetic trees of *wmk*, *cifB*, and *Hm-oscar* genes were constructed based on maximum likelihood with bootstrap re-sampling of 1,000 replicates using MEGA7.[27]

Fly experiments

The numbers of offspring expressing transgenic (*actin-GAL4/UAS*) and non-expressing genes (*CyO/UAS*) in each replicate were all counted at the adult stage. The number of male and female flies expressing or non-expressing each gene (i.e., *wmk-1, wmk-2, wmk-3, wmk-4, wmk-1* and *wmk-2, wmk-3* and *wmk-4,* and *Hm-oscar*) and the male ratio (number of adult males/numbers of all adults) of all the conditions were compared using the Steel–Dwass test in R software v4.0.[66]