Prophage-encoded *Hm-oscar* gene recapitulates *Wolbachia*-induced male killing in the tea tortrix moth *Homona magnanima*

Hiroshi Arai[1,2*], Susumu Katsuma[3], Noriko Matsuda-Imai[3], Shiou-Ruei Lin[4], Maki N. Inoue[2], Daisuke Kageyama[1*]

[1] National Agriculture and Food Research Organization (NARO), 1-2 Owashi, Tsukuba, Ibaraki 305-0851, Japan
[2] United Graduate School of Agricultural Science, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai, Fuchu, Tokyo 183-8509, Japan
[3] Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-8657, Japan
[4] Crop Environment Section, Tea and Beverage Research Station, Ministry of Agriculture, Taoyuan City 326011, Taiwan

* Co-correspondence:
Hiroshi Arai
National Agriculture and Food Research Organization (NARO), 1-2 Owashi, Tsukuba, Ibaraki, 305-0851, Japan
Email: dazai39papilio@gmail.com / HArai@liverpool.ac.uk

Daisuke Kageyama
National Agriculture and Food Research Organization (NARO), 1-2 Owashi, Tsukuba, Ibaraki, 305-0851, Japan
Email: kagymad@affrc.go.jp

ORCID
Hiroshi Arai: https://orcid.org/0000-0001-9912-3489
Susumu Katsuma: https://orcid.org/0000-0001-7096-3038
Noriko Matsuda-Imai: https://orcid.org/0000-0001-8196-6613
Shiou-Ruei Lin: https://orcid.org/0000-0001-5491-9416
Maki N. Inoue: https://orcid.org/0000-0002-9815-3480
Daisuke Kageyama: https://orcid.org/0000-0002-9026-9825

Abstract

Wolbachia are maternally transmitted bacterial symbionts that are ubiquitous among arthropods. They can hijack host reproduction in various ways, including male killing (MK), where the sons of infected mothers are killed during development. The recent discovery of MK-associated *Wolbachia* genes, i.e., *oscar* in *Ostrinia* moths and *wmk* in *Drosophila* flies, stimulates our interest in the diversity and commonality of MK mechanisms, which remain largely unclear. We recently discovered that a *Wolbachia* symbiont of the moth *Homona magnanima* carries an MK-associated prophage region encoding homologs of *oscar* (*Hm-oscar*) and *wmk* (*wmk*-1–4). Here, we investigated the effects of these genes in the native host. Upon transient overexpression, *Hm-oscar*, but not *wmk*, induced male lethality in *H. magnanima*, in contrast to our observations in *Drosophila*, where the *wmk* homologs, but not *Hm-oscar*, killed the males. *Hm-oscar* disrupted sex determination in male embryos by inducing a female-type *doublesex* splicing and impaired dosage compensation, recapitulating the *Wolbachia* phenotype. Cell-based transfection assays confirmed that *Hm-oscar* suppressed the function of *masculinizer*, the primary male sex determinant involved in lepidopteran dosage compensation. Our study highlights the conserved roles of *oscar* homologs in *Wolbachia*-induced lepidopteran MK and argues that *Wolbachia* have evolved multiple MK mechanisms in insects.

Keywords: symbiosis, *Wolbachia*, Oscar, Wmk, microinjection

Introduction

Arthropods commonly carry microbial symbionts that are passed from mother to offspring (Hurst & Frost, 2015; Werren et al., 2008; Hurst, 2017). The maternally transmitted bacteria, belonging to the genus *Wolbachia* (Alphaproteobacteria), are estimated to be present in at least 40% of all insect species, making them one of the most widespread endosymbiont genera on the planet (Werren et al, 2008; Zug & Hammerstein, 2012). *Wolbachia* have achieved evolutionary success by manipulating host reproduction through various means that enhance endosymbiont transmission (Werren et al., 2008). Such manipulation of the host's reproduction includes cytoplasmic incompatibility (CI), parthenogenesis, feminization, and male killing (MK), each of which affects the biological features, distribution, and evolution of the host. Among these strategies, MK directly skews the sex ratio of the host population toward females by killing male offspring of infected mothers during development. The lack of symbiont transmission through male hosts often leads to the evolution of MK. *Wolbachia* have been shown to induce MK in a wide range of insect taxa (e.g., dipterans, lepidopterans, and coleopterans) and other arthropods (Hurst et al., 1999; Kageyama & Traut, 2004; Hurst et al., 2000). Furthermore, various bacteria, viruses, and microsporidia induce MK phenotypes (Kageyama et al., 2012; Fujita et al., 2021; Kageyama et al., 2023; Nagamine et al., 2023), and recent studies have postulated that these microbes have evolved their MK ability independently (Harumoto & Lemaitre, 2018; Kageyama et al., 2023; Nagamine et al., 2023; Arai et al., 2023a).

The evolution and molecular mechanisms underlying *Wolbachia*-induced MK have attracted considerable attention for decades. It has been hypothesized that the mechanisms underlying MK are associated with sex determination cascades in

insects (Hornett et al., 2022). Indeed, some MK-inducing *Wolbachia* strains have been shown to directly interact with the sex determination system in lepidopteran insects (Sugimoto and Ishikawa, 2012; Sugimoto et al., 2015; Fukui et al., 2015; Arai et al., 2023a). The Lepidopteran sex determination system consists of multiple transcriptional regulators, some of which exhibit sex-linked expression and/or splicing isoforms. Lepidoptera generally have a female heterogametic sex chromosome system (e.g., WZ/ZZ) and employ dosage compensation, which equalizes the sex-linked (Z-chromosome-linked) gene dose between males and females (Kiuchi et al., 2014). Dosage compensation is regulated by the *masculinizer* gene (*masc*), which is critical for male development. *masc* also controls the downstream master regulator of sex determination and differentiation, *doublesex* (*dsx*), which exhibits sex-dependent splicing isoforms (*dsxF* in females and *dsxM* in males) (Kiuchi et al., 2014). In *Ostrinia* and *Homona* male moths, MK-inducing *Wolbachia* strains impair dosage compensation by disrupting the expression of *masc*. Furthermore, they disrupt sex determination in male moths by inducing the "female" isoform of *dsx* (*dsxF*), leading to a "mismatch" between genetic sex (male: ZZ sex chromosome constitution) and phenotypic sex (female: based on *dsxF*) and consequently to male death (Arai et al., 2023a; Fukui et al., 2015; Sugimoto et al., 2015; Sugimoto & Ishikawa, 2012).

More recently, a protein produced in *Wolbachia w*Fur strain named Oscar (Osugoroshi protein containing CifB C-terminus-like domain and many Ankyrin Repeats; Osugoroshi translates to male killing in Japanese) was shown to recapitulate the *Wolbachia*-induced MK phenotype in the native host *Ostrinia furnacalis* (Katsuma et al., 2022). The Oscar protein interacts with and degrades Masc protein, leading to the failure of dosage compensation and the production of female-type *dsx* isoforms in *Ostrinia* male moths (Fukui et al., 2024; Katsuma et al., 2022). Although *oscar* homologs have been identified in several MK-inducing *Wolbachia* strains in Lepidoptera, some MK *Wolbachia* strains in Diptera (*Drosophila*) and Lepidoptera do not carry this gene (Arai et al., 2023b; Arai et al., 2024a). Furthermore, *oscar* homologs are evolutionarily dynamic, with highly variable sequences and structures (classified as type I to type III *oscar* based on sequence homology) (Arai et al., 2024a), making it difficult to assess their functional relevance. In addition to *oscar*, the helix-turn-helix domain-containing putative transcriptional regulator *wmk* is widely conserved among *Wolbachia* strains and induces various toxicities in *Drosophila* flies (i.e., no effect, weak male lethality [30% of males die], strong male lethality [90%–100% of males die], and death of all males and females) (Perlmutter et al., 2019; Perlmutter et al., 2020; Perlmutter et al., 2021; Arai et al., 2023b). Although the mechanisms underlying the *wmk*-induced toxicities and their connection to sex determination systems remain unclear, these findings suggest that *Wolbachia* strains carry multiple factors that cause male lethality. However, the diversity and commonality of these functions in insects remain largely unknown, partly due to the technical challenges in validating gene functions in non-model insects.

We recently discovered a prophage region responsible for a *Wolbachia*-induced MK by comparing closely related non-MK *Wolbachia* (*w*Hm-c) and MK *Wolbachia* (*w*Hm-t) in the tea tortrix moth *Homona magnanima* (Tortricidae) (Arai et al., 2023b; Arai et al., 2024b). The MK-associated prophage element encodes potential MK causative genes–four *wmk* homologs (*wmk*-1 to *wmk*-4) as well as an *oscar* homolog (*Hm-oscar*, encoding 1181 aa protein), which differs significantly in

sequence length and structure from the *w*Fur-encoded *oscar* (encoding 1830 aa protein) (**Fig. 1a**). When these MK candidate genes are transgenically overexpressed in *Drosophila*, *wmk-1* and *wmk*-3 have a lethality of almost 100%, while *wmk-2*, *wmk-4*, and *Hm-oscar* induce no lethal effects when singly overexpressed. Furthermore, co-expression of the adjacent *wmk-3* and *wmk-4* has been shown to induce the death of 90% of male flies and restores female survival, suggesting their combined action (Arai et al., 2023b). However, transgenic expression of genes from a moth-derived male killer in flies does not necessarily replicate the native host–bacteria interaction, and the mechanistic links between prophage-encoded *Wolbachia* genes and MK in the native host *Homona* remain unclear.

In this study, we showed that the prophage-encoded *Hm-oscar* recapitulates *Wolbachia*-induced MK in *H. magnanima*. Furthermore, we clarified the mechanistic links to host sex-determination cascades both *in vivo* and *in vitro* and discussed the underlying mechanisms of MK in Lepidoptera, arguing for the diverse evolutionary origin of *Wolbachia*-induced MK.

Results

Hm-oscar induces female-biased sex ratios

To achieve the transient overexpression of *Hm-oscar* and the four *wmk* genes (*wmk*-1, *wmk*-2, *wmk*-3, and *wmk*-4), constructed mRNA (cRNA) was injected into *Wolbachia*-free *H. magnanima* embryos. Subsequently, the adult moths that emerged from the cRNA-injected embryos were sexed based on their external morphology. When *Hm-oscar* was overexpressed, the sex ratio of adults was strongly female-biased (42 males and 218 females in total, 14.4% ± 11.1% male ratio in 15 replicates), which was in sharp contrast with the ratios observed in the *GFP*-injected (126 females and 123 males in total, 49.1% ± 2.08% male ratio in 10 replicates) and non-injected (NSR) (201 females and 215 males in total, 51.4% ± 3.08% male ratio in 9 replicates) groups (P = 0.001 and 0.002, respectively, Steel–Dwass test, **Fig. 1b**). Compared with the *GFP*-injected group, the sex ratio was not biased by the overexpression of *wmk*-1 (51.1% ± 7.27% male ratio in 6 replicates, P = 0.999), *wmk*-2 (53.2% ± 4.77% male ratio in 6 replicates, P = 0.810), *wmk*-3 (53.3% ± 7.91% male ratio in 5 replicates, P = 0.999), and *wmk*-4 (52.9% ± 3.47% male ratio in 5 replicates, P = 0.773). Although the dual expression of *wmk*-3 and *wm*k-4 induces strong male lethality in *Drosophila* (Arai et al., 2023b), here, it did not skew the sex ratio of *Homona* compared with the ratio detected in the *GFP*-injected group (57.9% ± 7.67% male ratio in 5 replicates, P = 0.704). Similarly, the dual expression of the tandemly arrayed *wmk*-1 and *wmk*-2 did not bias the sex ratio (49.4% ± 2.82% male ratio in 5 replicates) compared with the value in the *GFP*-injected group (P = 1.000). The sex ratio data of all replicates are available on the supplementary data file.

Males are killed mainly at the embryonic stage

The sex of unhatched embryos and hatched larvae (neonates) was determined by karyotyping for W chromatin, with the presence and absence of this substance indicating females and males, respectively. MK *Wolbachia w*Hm-t kills *Homona* males during embryogenesis, resulting in a female-biased sex ratio in hatched larvae (neonates) and a male-biased sex ratio in unhatched embryos (Arai et al., 2023b; Arai et al., 2020). In line with this finding, the sex ratios of hatched larvae (34 females

and 6 males) and unhatched embryos (27 females and 47 males) of the MK *w*Hm-t infected line (W^{T12}) were also female- and male-biased, respectively (P = 0.013 and 0, respectively). In the *Hm-oscar*-injected group, the sex ratio of the hatched larvae (neonates) was strongly female-biased (21 females and 3 males, P = 0.0001 in the binomial test) (**Fig. 2a**). In contrast, the unhatched mature embryos showed a male-biased ratio (18 females and 38 males, P = 0.005), suggesting that male mortality primarily occurred during the embryonic stage, reflecting the *w*Hm-t-induced MK phenotype. In the non-injected group (NSR), the sex ratios of hatched larvae (28 females and 28 males) and unhatched embryos (23 females and 25 males) were not biased (P = 0.553 and 0.443, respectively).

Female-type sex determination in male embryos that are destined to die

Splicing patterns of the downstream sex determinant *dsx* were assessed in each of the *H. magnanima* embryos that were *Hm-oscar*-expressed, *GFP*-expressed, *w*Hm-t-infected, or non-expressed (i.e., non-injected, NSR). In *GFP*-expressed and NSR embryos, females and males (which were identified by the presence and absence of W chromatin, respectively) exhibited a female- and male-type splicing variant of *dsx*, respectively. However, both *w*Hm-t-infected and *Hm-oscar*-expressed embryos induced female-type *dsx* splicing regardless of the presence or absence of W chromatin (**Fig. 2b**). Notably, male embryos expressing *Hm-oscar* also exhibited weak male-type *dsx* splicing in addition to the female-type splicing, resembling the previously observed pattern in male embryos infected with low-titer *w*Hm-t (Arai et al., 2023a).

***Hm-oscar* impairs dosage compensation in male embryos**

To confirm the effects of *Hm-oscar* on dosage compensation, we quantified the expression of Z-linked genes using RNA-seq and qPCR. Unlike other lepidopteran species, the family Tortricidae, including *H. magnanima*, generally possess a large Z chromosome that is homologous to *B. mori* chromosomes 1 (Z) and 15 (autosome). RNA-seq analysis revealed that, in *Hm-oscar*-injected embryos, Z-linked genes (homologs on the *B. mori* chromosomes 1 and 15) were more expressed in males than in females (**Fig. 3a**), which was not observed in the *GFP*-injected group (**Fig. 3b**). Similarly, as previously reported by Arai et al. (2023a), high levels of Z-linked gene expression were also observed in *w*Hm-t-infected males, but not in NSR males (**Fig. 3c,d**). Statistical data are available on the supplementary data file. The high (i.e., doubled) Z-linked gene expression in both *Hm-oscar*-expressed and *w*Hm-t-infected males was further confirmed by quantification of the Z-linked *Hmtpi* gene (**Fig. 3e**).

***Hm-oscar* suppresses the masculinizing functions of lepidopteran *masc* genes**

To confirm whether *Hm-oscar* suppresses the functions of the primary male sex determinant *masc*, their interactions were assessed by transfection using BmN-4 cells, which are derived from *B. mori* ovaries (Grace, 1967) and exhibit a female-type default sex determination (Kiuchi et al., 2014). Previous studies have shown that transfection of BmN-4 cells with *masc* cDNA from various lepidopteran insects, such as *O. furnacalis*, induces the expression of $Bmdsx^M$ and $BmIMP^M$ (Fukui et al., 2015; Katsuma et al., 2019; Katsuma et al., 2022). Since $BmIMP^M$ regulates the splicing of $Bmdsx^M$ and is exclusively expressed in males, we selected $BmIMP^M$ as a

quantitative marker of masculinization, as described in Katsuma et al. (2022). In contrast with the control groups, which were transfected with the non-inserted pIZ/V5-His vector and showed this default sex determination, the cells transfected with the *Hmmasc*-inserted pIZ/V5-His vector exhibited the male-type sex determination, as indicated by the increased expression of the male-specific splicing variant of the sex-determining gene *BmImp*^{M}, although this increase was not statistically significant (**Fig. 4a**). Furthermore, the masculinizing function of *Hmmasc* was suppressed by co-transfecting the *Hmmasc/Hm-oscar*-inserted pIZ/V5-His vectors, as manifested by the low expression levels of *BmImp*^{M}. *w*Fur-encoded Oscar, which differs from Hm-Oscar in structure and amino acid sequence (**Fig. 4b**), also suppressed *Hmmasc*, the function of this gene was less active than that of *Hm-oscar* (**Fig. 4a**). *Hm-oscar* also suppressed the masculinizing function of *masc* genes derived from various lepidopteran insects (i.e., *O. furnacalis, Spodoptera frugiperda, Bombyx mori, Trichoplusia ni,* and *Papilio machaon*), which are highly divergent in their amino acid sequences (**Fig. 4c,d**). These results suggest that *Hm-oscar* has a broad spectrum of activity in Lepidoptera.

Discussion

In this study, we showed that the phage-encoded *Hm-oscar*, but not *wmk*, induced male lethality and a female-biased sex ratio in *H. magnanima*. Furthermore, the overexpressed *Hm-oscar* impaired male sex determination in *Homona,* recapitulating the *w*Hm-t-induced phenotypes. Cell-based assays confirmed that *Hm-oscar* suppressed the masculinizing functions of *masc*. These results strongly suggested that *Hm-oscar* underlies the *w*Hm-t-induced MK function in *H. magnanima*. In light of our results and previous findings in *Homona*, *Ostrinia,* and *Bombyx* (Arai et al., 2023a; Katsuma et al., 2022; Kiuchi et al., 2014), we hypothesize the following molecular mechanisms for *w*Hm-t-induced MK: The Hm-Oscar protein targets and inhibits the function of the HmMasc protein, thereby resulting in female sex determination and disrupting dosage compensation, which ultimately leads to male death. Although the full mechanisms remain unclear, unbalanced Z-linked gene expression mediated by improper dosage compensation—resulting in Z-linked gene expression levels twice as high as those in normal males—appear to be lethal for lepidopteran males (Kiuchi et al., 2014; Fukui et al., 2015; Visser et al., 2021).

The *Wolbachia*-encoded *oscar* homologs identified so far show diversity in sequence length and are classified as type I, II, and III based on their amino acid sequence homologies. Type I and II Oscar proteins, derived from MK-inducing *Wolbachia*, contain many ankyrin repeats and a proteinase domain (Arai et al., 2024a). Although long ankyrin repeat sequences are postulated to be critical for the function of *w*Fur-encoded Oscar protein (1830 aa, type I) (Katsuma et al., 2022), our study revealed that the functions of Hm-Oscar protein (1181 aa, type II), which encodes fewer ankyrin repeats, were comparable with those of Oscar protein carried by *w*Fur. Interestingly, type II *oscar* gene is also present in the feminizing *Wolbachia w*Fem in *Eurema* butterflies (Arai et al., 2024a). *oscar* homologs, which inhibit the masculinizing function and induce female sex determination, may have a conserved function in *Wolbachia*-induced MK and feminization in Lepidoptera.

In contrast to the results of this study, we have previously demonstrated that the phage-encoded *wmk*, but not *Hm-oscar*, induces male lethality in *D. melanogaster* (Arai et al., 2023b). Although the means of expression are different

(i.e., transient in *Homona* and transgenic in *Drosophila*), this finding highlighted the differences in the mode/range of action of *Wolbachia* genes between insect species. It has been hypothesized that microbes induce MK in insects by targeting molecular mechanisms involved in sex determination and differentiation (Hornett et al., 2022). Sex determination systems in insects are diverse; for example, Lepidoptera (including *H. magnanima*) and Diptera (including *D. melanogaster*) do not share any known sex-determining genes other than *dsx* (Suzuki, 2018). The different outcomes in *Homona* and *Drosophila* are probably due to their different sex determination systems. Because *oscar* interacts with and suppresses *masc*, *Hm-oscar* could induce mortality in *Homona* males that possess *masc*, but not in *Drosophila* males that lack it. Considering that *oscar* homologs are not present in known MK *Wolbachia* strains in dipteran insects (Arai et al., 2024c; Katsuma et al., 2022), the mechanisms (i.e., causative genes) of *Wolbachia*-induced MK probably differ between insect taxa (e.g., between Lepidoptera and Diptera). While the mechanisms underlying *wmk*-induced lethality remain unclear, the distinct effects associated with this gene between *Homona* and *Drosophila* may also reflect their genetic backgrounds (e.g., presence/absence of host factor(s) that interact with *wmk*). In addition, Katsuma et al. (2022) reported that the *wmk* homologs encoded by *w*Fur did not affect the masculinizing function of *masc in vitro*, indicating that *wmk* likely targets factors other than *masc*. Our results strongly suggested that *Wolbachia* strains have acquired multiple MK genes through evolution.

Wolbachia-induced phenotypes are known to be influenced by the genetic backgrounds of hosts (Hornett et al., 2006; Sasaki et al., 2002; Veneti et al., 2012). Our study showed that the *w*Hm-t-encoded *Hm-oscar* suppresses the function of *masc* in *H. magnanima* more efficiently than the *w*Fur-encoded *oscar*, suggesting that this *Wolbachia* factor has undergone evolutionary tuning to adapt to its natural host. However, the mere presence of *oscar* and *wmk* homologs does not ensure MK expression. For example, *w*CauA, bearing type I *oscar*, did not induce MK in *Ephestia* (*Cadra*) *cautella* collected around 2000, although it did induce MK when transferred to the closely related host *Ephestia kuehniella* (Sasaki et al., 2002). An MK phenotype, presumably induced by *w*CauA, was also recorded in *E. cautella* around the 1970s (Takahashi & Kuwahara, 1970). These findings suggest a suppressor evolution against *w*CauA-induced MK in *E. cautella*. In addition, *Wolbachia* typically encode a number of *wmk* homologs in their genomes, but their effects differ among insects and are not apparent in some species such as *Homona,* possibly due to the diversity in sex determination system or the evolution of suppressors. Nonetheless, given the high copy number of *wmk* homologs in *Wolbachia* genomes, they may still contribute to *Wolbachia* biology as transcriptional regulators and potential virulence factors. Virulence genes often undergo duplication and substitution under strong selective pressure (Hill et al., 2022; Jones & Dangl, 2006). An intense evolutionary arms race between *Wolbachia* and their hosts could have increased and diversified the MK-associated *Wolbachia* genes. Conversely, natural selection favors the rescue of males by suppressing the *Wolbachia*-induced reproductive manipulations (Hornett et al., 2006; Hornett et al., 2014), which may involve changes in the sex determination system because *Wolbachia* strains frequently hijack host reproduction systems. In this context, *Wolbachia* and other MK-inducing microbes may be a hidden driver for the diversification of complex insect sex determination systems.

In this study, we clarified the conserved roles of the *Wolbachia*-encoded *oscar* homologs in Lepidoptera and demonstrated that *Wolbachia* have evolved distinct MK mechanisms (through causative genes) in insect taxa. The diversification of phenotype/virulence-associated genes and the rampant horizontal transmission of phages carrying virulence genes between *Wolbachia* strains may have contributed to the outstanding success of this bacterial genus. In addition to *oscar* and *wmk, Wolbachia* may retain other uncharacterized genes that induce male lethality, and further studies on diverse *Wolbachia*–host systems are highly warranted. Our findings provide insights into the molecular mechanisms and evolutionary relationships between endosymbionts and their hosts, which may also contribute to the design of pest management strategies.

Materials and methods

Experimental model and subject details

Homona magnanima

A laboratory-maintained *H. magnanima* line with a normal sex ratio (NSR) that was negative for *Wolbachia* and other endosymbionts, was used in our experiments. This line was initially collected in Tokyo, Japan, in 1999 and has been maintained inbred for over 250 generations in the laboratory. Larvae were reared using the artificial SilkMate 2S diet (Nosan Co., Yokohama, Japan) at 25°C under a long photoperiod (16L:8D) (Arai et al., 2019).

The laboratory-maintained all female *H. magnanima* W^{T12} line, which was initially collected in Taiwan (Tea Research Extension Station, Taoyuan city) in 2015 (Arai et al., 2020), was also used in this study. This line was maintained for over 50 generations by crossing it with the males of the NSR line.

BmN-4 cell line

BmN-4 cells (provided by Chisa Yasunaga-Aoki, Kyushu University, and maintained in our laboratory) were cultured at 26°C in IPL-41 Insect Medium (Applichem, Darmstadt, Germany) supplemented with 10% fetal bovine serum.

Transient expression of MK-associated phage genes

(i) mRNA synthesis

Codon-optimized *wmk* genes (*wmk-1* to *wmk-4*), conjugated *wmk* pairs using a T2A peptide (i.e., *wmk*-1-T2A-*wmk*-2 and *wmk*-3-T2A-*wmk*-4), and *Hm-oscar* genes synthesized by Arai et al. (2023b) were used for mRNA synthesis. These synthetic genes were ligated into the plasmid pIZ/V5-His (Invitrogen, MA, USA) using the NEBuilder® HiFi DNA Assembly kit (New England Biolabs, MA, USA) following the manufacturer's protocol. The inserts of the vector (i.e., *wmk*-1, *wmk*-2, *wmk*-3, *wmk*-4, *Hm-oscar*, *wmk*-1-T2A-*wmk*-2, *wmk*-3-T2A-*wmk*-4, and *GFP* as a control gene) were amplified using KOD-one (TOYOBO, Osaka, Japan) with the primer set containing the T7 promoter described in Fukui et al. (2015) (i.e., pIZ-F-T7: 5′-TAATACGACTCACTATAGGGAGACAGTTGAACAGCATCTGTTC-3′ and pIZ-R: 5′-GACAATACAAACTAAGATTTAGTCAG-3′) under the following PCR conditions: 20 cycles at 98°C for 10 s, 62°C for 5 s, and 68°C for 15 s. The amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), and 100–200 ng DNA was used for mRNA synthesis. The capped mRNA (cRNA) with poly(A) tail was

synthesized using the mMESSAGE mMACHINE® T7 Ultra kit (Invitrogen, MA, USA) following the manufacturer's protocol with some modifications. In brief, the assembled transcription reaction (10 µL of T7 2X NTP/ARCA, 2 µL of 10X T7 reaction buffer, 2 µL of T7 enzyme mix, and 6 µL of linear DNA template diluted with nuclease-free water) was incubated at 37°C for at least 10 h to maximize RNA yields. After poly(A) tailing, cRNA was purified using ISOGEN II (Nippongene, Tokyo, Japan) and dissolved in up to 10 µL of nuclease-free water to achieve an RNA concentration of approximately 1500–2000 ng/µL. The synthesized cRNA was preserved at −80°C until further use.

(ii) Preparation of *H. magnanima* eggs

A total of 15 males and 10 females of *H. magnanima* were mated in a plastic box (30 cm × 20 cm × 5 cm) for 3–4 days. The collection of egg masses began the day after oviposition was confirmed. In brief, newly oviposited egg masses were collected at 30-min intervals during the dark period using red light (which the moths cannot perceive). Females started to oviposit eggs at least 4 h into the dark period and, within less than 30 min post oviposition (mpo), the egg masses were collected. The collection lasted until the start of the light period. The egg masses were then subjected to microinjection assays.

(iii) Inoculum preparation, microinjection, and maintenance of the injected embryos

A glass needle for microinjection was prepared using glass capillary GD-1 (Narishige, Tokyo, Japan) with a PC-10 puller (Narishige). The glass capillaries were pulled at two temperatures (first stage: 75°C, second stage: 65°C) using two heavy weights and one light weight. The movement position during the second heating stage was set to 3 mm (range 1–10 mm).

The cRNA solution was diluted in a buffer (100 mM potassium acetate, 2 mM magnesium acetate, and 30 mM HEPES-KOH; at pH 7.4) containing 0.2% (W/V) Brilliant Blue FCF (Wako, Osaka, Japan) to obtain an RNA concentration of 1000 ng/µL. Approximately 1–4 µL of dye-containing mRNA solution was aspirated into the glass needles (capillaries), and the edge of each needle was ground using Micro Grinder EG-402 (Narishige) at an angle of 20 degrees for 2 s.

The fresh egg masses of *H. magnanima* (collected at less than 30 mpo as described above) were put on a double-sided sticky tape (15 mm × 5 m T4612, Nitoms, Tokyo, Japan) and placed on glass slides. Under a Nikon SMZ1270 microscope (Nicon, Tokyo, Japan), the RNA solution (30–100 fL) was injected into individual eggs using an microinjector IM-400 (Narishige) (balance pressure set at 0.010–0.030; injection pressure set at 0.050-0.100) and a QP-3JOY-2R electric micromanipulator (MicroSupport, Shizuoka, Shizuoka).

The injected egg masses were maintained in a 90-mm plastic Petri dish fitted with a slightly wet filter paper. As high humidity interfered with the development of the embryos, the injected eggs (egg mass) were first maintained at a relative humidity of 30%–50% (0–3 days post injection) and then at a higher humidity until hatching (4–6 dpo, 60% relative humidity). The hatched larvae were reared separately with 1/2 ounces of SilkMate 2S (Nosan Co.) until eclosion. The adult *H. magnanima* moths that emerged from the cRNA-injected embryos were sexed based on their external morphology.

Sexing of embryos/neonates and RNA extraction

To verify the effect of the transient expression of *Hm-oscar* on sex determination in *H. magnanima*, *Hm-oscar*/*GFP*-expressed, non-injected, and *w*Hm-t-infected mature embryos showing black head capsule (1 day before hatching, 5–6 days post oviposition, dpo) were dissected on glass slides using forceps, as described in Arai et al. (2022). Their Malpighian tubules were fixed with methanol/acetic acid (50% v/v) and stained with lactic acetic orcein for W chromatin observations. The remaining tissues not used for sexing were stored in ISOGEN II (Nippon Gene) at −80°C until subsequent extraction. In total, 12 males or females (confirmed based on the presence or absence of W chromatin) were pooled and homogenized in ISOGEN II to extract RNA as described in Arai et al. (2023a). In brief, 0.4 times the volume of UltraPure distilled water (Invitrogen) was added to the ISOGEN II homogenates, which were centrifuged at 12000 × *g* for 15 min at 4°C to pellet proteins and DNAs. The resulting supernatant was mixed with the same volume of isopropanol to precipitate RNAs; then, the resulting solutions were transferred to EconoSpin columns (Epoch Life Science) and centrifuged at 17,900 × *g* and 4°C for 2 min. The RNAs captured in the column were washed twice with 80% ethanol and eluted in 20 µL of UltraPure distilled water (Invitrogen).

Hmdsx detection

Sex-specific *dsx* splicing variants of *H. magnanima* were detected as described in Arai et al. (2023a). In brief, total RNA (100–300 ng) extracted from sex-determined mature embryos was reverse-transcribed using PrimeScript™ II Reverse Transcriptase (TaKaRa, Shiga, Japan) at 30°C for 10 min, 45°C for 60 min, and 70°C for 15 min. Then, cDNA was amplified using KOD-FX Neo (Toyobo Co., Ltd.) with the following two primers: Hmdsx_long3F (5′-TGCCTAAAGTGAAAACGCCGAGGAGCC-3′) and Hmdsx_Mrev (5′-TGGAGGTCTCTTTTCATCCGG-3′). The PCR conditions used were as follows: 94°C for 2 min, followed by 45 cycles of 98°C for 10 s, 66°C for 30 s, and 68°C for 30 s. The amplicons were subjected to electrophoresis on 2.0% agarose Tris-borate-EDTA buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA) gels.

Quantification of Z chromosome-linked genes

The effects on dosage compensation were assessed by measuring differences in gene expression, as described in Fukui et al. (2015) and Arai et al. (2023a). A total of 1.0 µg of the total RNA extracted from *Hm-oscar* or *GFP*-overexpressed mature embryos (108 hpo, approximately 40-50 sex determined embryos) was used to prepare mRNA-seq libraries via the NEBNext Poly (A) mRNA Magnetic Isolation Module (New England Biolabs) and the NEBNext Ultra II RNA Library Prep kit for Illumina (New England Biolabs) following the manufacturer's protocol. The adaptor sequences and low-quality reads (Qscore <20) were removed from the generated sequence data [150 bp paired-end (PE150)] using Trimmomatic (Bolger et al., 2014). The trimmed reads were mapped to the previously assembled transcriptome database for *H. magnanima* (Arai et al., 2023a) using Kallisto (Bray et al., 2016) to generate the normalized read count data (transcripts per million, TPM). While we did not generate new RNA-seq data of the *w*Hm-t-infected and uninfected groups, the expression data included in Arai et al. (2023a) were re-analyzed along with the newly

obtained RNA-seq data above. The binary logarithms of the TPM differences between males and females belonging to each *H. magnanima* group (i.e., *Hm-oscar*/*GFP*-expressed) were calculated to assess the fold-changes in gene expression levels between sexes. As described in Arai et al. (2023a), the transcriptome data of *H. magnanima* were annotated using the *B. mori* gene sets obtained from KAIKOBASE (https://kaikobase.dna.affrc.go.jp). The binary logarithms of the TPM differences between males and females in the *B. mori* chromosomes 1 to 28 were plotted.

To confirm the effects observed in the RNA-seq-based quantification of the Z chromosome genes, expression level of the Z-linked *Hmtpi* gene was quantified by reverse transcription-qPCR as described in Arai et al. (2023a). Briefly, total RNA (100–300 ng) extracted from pooled male or female mature embryos (108 hpo) was reverse transcribed using PrimeScript II reverse transcriptase (TaKaRa) as described above. The cDNA was used to quantify relative gene expression levels, with normalization to the control gene elongation factor 1a (*ef1a*). qPCR was performed using primer sets for *Hmtpi* (HmTpi_F180702_212: 5′-GCTGCGAGTGGGTGATTTTG-3′; HmTpi_R180702_326: 5′-GCGATCACTTTCAGACCCGA-3′) and *Hmef1a* (Hmef1a_F_val1_85: 5′-TTTCCAGGGTGGTTGAGCA-3′; Hmef1a_R_val1_193: 5′-CCGTTAAGGAGCTGCGTCG-3′), and KOD SYBR qPCR Mix (Toyobo, Osaka, Japan) in a LightCycler 96 system (Roche, Basel, Switzerland). The qPCR reaction was made with 5 µl KOD SYBR (TOYOBO), 0.4 µL forward primer [10 pmol/µl], 0.4 µl reverse primer [10 pmol/µl], and 3.2 µl water. The qPCR was performed under the following conditions: 180 s at 95°C, 40 cycles of 8 s at 98°C, 10 s at 60°C, and 10 s at 68°C, followed by heating to 90°C for melting curve analysis. Mean cycle threshold (C_T) values of samples were calculated for at least 13 replicates, and both ΔC_T (C_T Z gene – C_T ef1a) and $\Delta\Delta C_T$ (ΔC_Tmale – ΔC_Tfem) values were calculated.

Transfection assays and quantification of *BmIMP^M*

The coding sequence of *Hmmasc* was amplified from cDNA derived from the RNA extracted from male embryos of the NSR line using KOD-one (TOYOBO) with the following primer set: HmMasc_CDS_HindIIIF: 5′-GCAAAGCTTCAACATGATCTCTCGCCAACCACAATCAACATCA-3′ and HmMasc_CDS_BamHI R: 5′-GCAGGATCCCAACCTACTGATAAGGAGGGAAGTAAGGCTGCTG-3′. The following PCR conditions were applied: 20 cycles of 98°C for 10 s, 62°C for 5 s, and 68°C for 15 s. The amplicon was purified with the QIAquick PCR purification kit (Qiagen) and cloned into the pIZ/V5-His vector using the HindIII and BamHI enzymes (New England Biolabs). The codon-optimized *oscar* gene of *w*Fur (Katsuma et al. 2022) was also cloned into the pIZ/V5-His vector using the KpnI and NotI enzymes (TaKaRa).

To verify the masculinizing function of *Hmmasc*, BmN-4 cells (4 × 10^5 cells per dish, diameter 35 mm) were transfected with 1 µg of each plasmid DNA (pIZ/V5-His having *Hmmasc*) using FuGENE HD (Promega, WI, USA), as described in Katsuma et al. (2022). To clarify whether *Hm-oscar* suppressed the function of *Hmmasc*, 1 µg of plasmid DNA (pIZ/V5-His bearing either *Hmmasc* or *Hm-oscar* and non-inserted vector as negative control) was transfected to the BmN-4 cells using FuGENE HD (Promega). Three days after transfection, the cells were collected

and subjected to RNA extraction via TRI REAGENT® (Molecular Research Center Inc., USA) and cDNA construction with AMV transcriptase (TaKaRa). The degree of masculinization in the BmN-4 cells (default female-type sex determination) was verified by quantifying the expression levels of *BmImp*^{M} (Suzuki et al., 2010), which is involved in male-specific sex determination cascades, using primers BmIMP_F: 5′-ATGCGGGAAGAAGGTTTTATG-3′ and BmIMP_R: 5′-TCATCCCGCCTCAGACGATTG-3′, as described in Fukui et al. (2015). *BmRp49* gene was also amplified as a control gene using primers rp49_F: 5′-CCCAACATTGGTTACGGTTC-3′ and rp49_R: 5′-GCTCTTTCCACGATCAGCTT-3′. Further interactions between *Hm-oscar* and *masc* genes derived from lepidopteran insects [i.e., *Trilocha varians masc* (*Tvmasc*)*, Spodoptera frugiperda masc* (*Sfmasc*), *B. mori masc* (*Bmmasc*), *O. furnacalis masc (Ofmasc),* and *Papilio machaon* masc (*Pmmasc*)] (Katsuma et al., 2022b) were assessed using the same procedures. The expression levels of *BmImp*^{M} were analyzed as described in Katsuma et al. (2022). In brief, the relative expression of *BmImp*^{M} (C_T *BmImp*^{M} / C_T *BmRp49*) was calculated for each experimental group. Then, relative expression of *BmImp*^{M} of each sample was normalized by setting the mean in the *Hmmasc* singly transfected group as 100. The normalized relative expression of *BmImp*^{M} was visualized using R v4.0 with ggplot2 (https://ggplot2.tidyverse.org/).

Quantification and statistical analysis

The number of surviving *H. magnanima* injected with either *Hm-oscar, wmk*s, or *GFP* in each replicate were counted at the adult stage. The male ratios (number of adult males/numbers of all adults) under all conditions were compared using the Steel–Dwass test in R v4.0. The sex ratio bias was also assessed based on the total numbers of (i) male and female adults and (ii) male and female embryos under each condition using the binomial test in R v4.0.

Gene expression levels between chromosomes (RNA-seq), a Z-linked *Hmtpi* gene expression in *H. magnanima* (RT-qPCR), and normalized *BmIMP*^{M} expression in BmN-4 cells under all conditions (RT-qPCR) were compared using the Steel–Dwass test in R v4.0.

A phylogenetic tree of Masc proteins used in this study was constructed based on maximum likelihood with bootstrap re-sampling of 1,000 replicates using MEGA7 (Kumar et al., 2016).

Data and code availability

High-throughput sequencing data are available under accession numbers DRA018708 and PRJDB18169 (BioProject). All data generated during this study are included in the manuscript and a supporting excel file. Further information and requests for resources and reagents are accessible from the lead contact, Hiroshi Arai (dazai39papilio@gmail.com/HArai@liverpool.ac.uk).

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Author contributions

H. Arai conducted gene functional validation assays, transcriptome assays, and data analysis; designed experiments; wrote the original manuscript; and revised the manuscript. S. Katsuma conducted transfection assays, quantified the gene expressions in the cells and presented the entire discussion. N. Matsuda-Imai constructed the pIZ/V5 vector having *oscar* gene derived from *Ostrinia furnacalis*. S-R. Lin revised the manuscript and contributed to the discussion. MN Inoue revised the manuscript and contributed to the discussion. D. Kageyama organized the project and revised the manuscript. Lastly, H. Arai and D. Kageyama 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.

Benefit-Sharing

The *w*Hm-t-infected *H. magnanima* was collected from Tea Research and Extension Station (Taoyuan City, Taiwan), and imported with permission from the Ministry of Agriculture, Forestry and Fisheries (No. 27 - Yokohama Shokubou 891 and No. 297 - Yokohama Shokubou 1326). All collaborators are presented as co-authors, and the results have been shared with the provider communities. Moreover, our group is committed to international scientific partnerships as well as institutional capacity building. The authors declare no competing interests.

References

Arai H, Anbutsu H, Nishikawa Y, Kogawa M, Ishii K, Hosokawa M, Lin SR, Ueda M, Nakai M, Kunimi Y, Harumoto T, Kageyama D, Takeyama H, Inoue MN. 2023b. Combined actions of bacteriophage-encoded genes in *Wolbachia*-induced male lethality. *iScience*, *26*, 106842. https://doi.org/10.1016/j.isci.2023.106842

Arai H, Herran B, Sugimoto TN, Miyata M, Sasaki T, Kageyama D. 2024a. Cell-based assays and comparative genomics revealed the conserved and hidden effects of *Wolbachia* on insect sex determination. *PNAS Nexus*, *3*, pgae348. https://doi.org/10.1093/pnasnexus/pgae348

Arai H, Hirano T, Akizuki N, Abe A, Nakai M, Kunimi Y, Inoue MN. 2019. Multiple infection and reproductive manipulations of *Wolbachia* in *Homona magnanima* (Lepidoptera: Tortricidae). *Microbial Ecology*, *77*, 257–266. https://doi.org/10.1007/s00248-018-1210-4

Arai H, Ishitsubo Y, Nakai M, Inoue MN. 2022. Mass-rearing and molecular studies in Tortricidae pest insects. *JoVE (Journal of Visualized Experiments)*, *181*, e63737. https://doi.org/10.3791/63737

Arai H, Lin SR, Nakai M, Kunimi Y, Inoue MN. 2020. Closely related male-killing and nonmale-killing *Wolbachia* strains in the oriental tea tortrix *Homona magnanima*. *Microbial Ecology*, *79*, 1011–1020. https://doi.org/10.1007/s00248-019-01469-6

Arai H, Takamatsu T, Lin SR, Mizutani T, Omatsu T, Katayama Y, Nakai M, Kunimi Y, Inoue MN. 2023a. Diverse molecular mechanisms underlying microbe-inducing male killing in the moth *Homona magnanima*. *Applied and Environmental Microbiology*, *89*, e02095-22. https://doi.org/10.1128/aem.02095-22

Arai H, Ueda M, Hirano T, Akizuki N, Lin SR, Hanh DK, Widada J, Rohman MS, Nakai M, Kunimi Y, Vang LV, Wijonarko A, Inoue MN. 2024b. Conserved infections and reproductive phenotypes of *Wolbachia* symbionts in Asian tortrix moths. *Environmental Microbiology Reports*, *16*, e13219. https://doi.org/10.1111/1758-2229.13219

Arai H, Watada M, Kageyama D. 2024c. Two male-killing *Wolbachia* from *Drosophila birauraia* that are closely related but distinct in genome structure. *Royal Society Open Science*, *11*, 231502. https://doi.org/10.1098/rsos.231502

Bolger AM, Lohse M, Usadel B. 2014. Trimmomatic: A flexible trimmer for illumina sequence data. *Bioinformatics*, *30*, 2114–2120. https://doi.org/10.1093/bioinformatics/btu170

Bray NL, Pimentel H, Melsted P, Pachter L. 2016. Near-optimal probabilistic RNA-seq quantification. *Nature Biotechnology*, *34*, 525–527. https://doi.org/10.1038/nbt.3519

Fujita R, Inoue MN, Takamatsu T, Arai H, Nishino M, Abe N, Itokawa K, Nakai M, Urayama S, Chiba Y, Amoa-Bosompem M, Kunimi Y. 2021. Late male-killing viruses in *Homona magnanima* identified as Osugoroshi viruses, novel members of Partitiviridae. *Frontiers in Microbiology*, *11*. https://doi.org/10.3389/fmicb.2020.620623

Fukui T, Kawamoto M, Shoji K, Kiuchi T, Sugano S, Shimada T, Suzuki Y, Katsuma S. 2015. The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLOS Pathogens*, *11*, e1005048. https://doi.org/10.1371/journal.ppat.1005048

Fukui T, Kiuchi T, Tomihara K, Muro T, Matsuda-Imai N, Katsuma S. 2024. Expression of the *Wolbachia* male-killing factor Oscar impairs dosage compensation in lepidopteran embryos. *FEBS Letters*, *598*, 331–337. https://doi.org/10.1002/1873-3468.14777

Grace TDC. 1967. Establishment of a line of cells from the silkworm *Bombyx mori*. *Nature*, *216*, 613–613. https://doi.org/10.1038/216613a0

Harumoto T, Lemaitre B. 2018. Male-killing toxin in a bacterial symbiont of *Drosophila*. *Nature*, *557*, 252–255. https://doi.org/10.1038/s41586-018-0086-2

Hill T, Unckless RL, Perlmutter JI. 2022. positive selection and horizontal gene transfer in the genome of a male-killing *Wolbachia*. *Molecular Biology and Evolution*, *39*, msab303. https://doi.org/10.1093/molbev/msab303

Hornett EA, Charlat S, Duplouy AMR, Davies N, Roderick GK, Wedell N, Hurst GDD. 2006. Evolution of male-killer suppression in a natural population. *PLOS*

Biology, *4*, e283. https://doi.org/10.1371/journal.pbio.0040283

Hornett EA, Kageyama D, Hurst GDD. 2022. Sex determination systems as the interface between male-killing bacteria and their hosts. *Proceedings of the Royal Society B: Biological Sciences*, *289*, 20212781. https://doi.org/10.1098/rspb.2021.2781

Hornett EA, Moran B, Reynolds LA, Charlat S, Tazzyman S, Wedell N, Jiggins CD, Hurst GDD. 2014. The evolution of sex ratio distorter suppression affects a 25 cM genomic region in the butterfly *Hypolimnas bolina*. *PLOS Genetics*, *10*, e1004822. https://doi.org/10.1371/journal.pgen.1004822

Hurst GDD. 2017. Extended genomes: Symbiosis and evolution. *Interface Focus*, *7*, 20170001. https://doi.org/10.1098/rsfs.2017.0001

Hurst GDD, Frost CL. 2015. Reproductive parasitism: maternally inherited symbionts in a biparental world. *Cold Spring Harbor Perspectives in Biology*, *7*, a017699. https://doi.org/10.1101/cshperspect.a017699

Hurst GDD, Johnson AP, Schulenburg JHG. v d, Fuyama Y. 2000. Male-killing *Wolbachia* in *Drosophila*: A temperature-sensitive trait with a threshold bacterial density. *Genetics*, *156*, 699–709. https://doi.org/10.1093/genetics/156.2.699

Hurst GDD, von der Schulenburg JHG, Majerus TMO, Bertrand D, Zakharov IA, Baungaard J, Völkl W, Stouthamer R, Majerus MEN. 1999. Invasion of one insect species, *Adalia bipunctata*, by two different male-killing bacteria. *Insect Molecular Biology*, *8*, 133–139. https://doi.org/10.1046/j.1365-2583.1999.810133.x

Jones JDG, Dangl JL. 2006. The plant immune system. *Nature*, *444*, 323–329. https://doi.org/10.1038/nature05286

Kageyama D, Harumoto T, Nagamine K, Fujiwara A, Sugimoto TN, Jouraku A, Tamura M, Katoh TK, Watada M. 2023. A male-killing gene encoded by a symbiotic virus of *Drosophila*. *Nature Communications*, *14*, 1357. https://doi.org/10.1038/s41467-023-37145-0

Kageyama D, Narita S, Watanabe M. 2012. Insect sex determination manipulated by their endosymbionts: incidences, mechanisms and implications. *Insects*, *3*, Article 1. https://doi.org/10.3390/insects3010161

Kageyama D, Traut W. 2004. Opposite sex–specific effects of *Wolbachia* and interference with the sex determination of its host *Ostrinia scapulalis. Proceedings of the Royal Society of London. Series B: Biological Sciences*, *271*, 251–258. https://doi.org/10.1098/rspb.2003.2604

Katsuma S, Hirota K, Matsuda-Imai N, Fukui T, Muro T, Nishino K, Kosako H, Shoji K, Takanashi H, Fujii T, Arimura S, Kiuchi T. 2022. A *Wolbachia* factor for male killing in lepidopteran insects. *Nature Communications*, *13*, 6764. https://doi.org/10.1038/s41467-022-34488-y

Katsuma S, Shoji K, Sugano Y, Suzuki Y, Kiuchi T 2019. Masc-induced dosage compensation in silkworm cultured cells. *FEBS Open Bio*, *9*, 1573–1579. https://doi.org/10.1002/2211-5463.12698

Kiuchi T, Koga H, Kawamoto M, Shoji K, Sakai H, Arai Y, Ishihara G, Kawaoka S, Sugano S, Shimada T, Suzuki Y, Suzuki MG, Katsuma S. 2014. A single female-specific piRNA is the primary determiner of sex in the silkworm. *Nature*, *509*, 633–636. https://doi.org/10.1038/nature13315

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics

analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874. https://doi.org/10.1093/molbev/msw054

Nagamine K, Kanno Y, Sahara K, Fujimoto T, Yoshido A, Ishikawa Y, Terao M, Kageyama D, Shintani Y. 2023. Male-killing virus in a noctuid moth *Spodoptera litura*. *Proceedings of the National Academy of Sciences*, *120*, e2312124120. https://doi.org/10.1073/pnas.2312124120

Perlmutter JI, Bordenstein SR, Unckless RL, LePage DP, Metcalf JA, Hill T, Martinez J, Jiggins FM, Bordenstein SR. 2019. The phage gene wmk is a candidate for male killing by a bacterial endosymbiont. *PLOS Pathogens*, *15*, e1007936. https://doi.org/10.1371/journal.ppat.1007936

Perlmutter JI, Meyers JE, Bordenstein SR. 2020. Transgenic testing does not support a role for additional candidate genes in *Wolbachia* male killing or cytoplasmic incompatibility. *mSystems*, *5*, 10.1128/msystems.00658-19. https://doi.org/10.1128/msystems.00658-19

Perlmutter JI, Meyers JE, Bordenstein SR. 2021. A single synonymous nucleotide change impacts the male-killing phenotype of prophage WO gene wmk. *eLife*, *10*, e67686. https://doi.org/10.7554/eLife.67686

Sasaki T, Kubo T, Ishikawa H. 2002. Interspecific transfer of *Wolbachia* between two Lepidopteran insects expressing cytoplasmic incompatibility: A *Wolbachia* variant naturally infecting *Cadra cautella* causes male killing in *Ephestia kuehniella*. *Genetics*, *162*, 1313–1319. https://doi.org/10.1093/genetics/162.3.1313

Sugimoto TN, Ishikawa Y. 2012. A male-killing *Wolbachia* carries a feminizing factor and is associated with degradation of the sex-determining system of its host. *Biology Letters*, *8*, 412–415. https://doi.org/10.1098/rsbl.2011.1114

Sugimoto TN, Kayukawa T, Shinoda T, Ishikawa Y, Tsuchida T. 2015. Misdirection of dosage compensation underlies bidirectional sex-specific death in *Wolbachia*-infected *Ostrinia scapulalis*. *Insect Biochemistry and Molecular Biology*, *66*, 72–76. https://doi.org/10.1016/j.ibmb.2015.10.001

Suzuki MG. 2018. Sex Determination cascade in insects: a great treasure house of alternative splicing. In K. Kobayashi, T. Kitano, Y. Iwao, & M. Kondo (Eds.), *Reproductive and Developmental Strategies: The Continuity of Life* (pp. 267–288). Springer Japan. https://doi.org/10.1007/978-4-431-56609-0_13

Suzuki MG, Imanishi S, Dohmae N, Asanuma M, Matsumoto S. 2010. Identification of a male-specific RNA binding protein that regulates sex-specific splicing of Bmdsx by increasing RNA binding activity of BmPSI. *Molecular and Cellular Biology*, *30*, 5776–5786. https://doi.org/10.1128/MCB.00444-10

Takahashi F, Kuwahara Y. 1970. Studies on sex pheromones of Pyralididae. III. The inheritance of the abnormal sex ratio condition in a strain of the almond moth, *Cadra cautella* Walker (Phycitinae). *Botyu-Kagaku = Scientific Insect Control*, *35*, 11–21. https://www.cabidigitallibrary.org/doi/full/10.5555/19710503687

Veneti Z, Zabalou S, Papafotiou G, Paraskevopoulos C, Pattas S, Livadaras I, Markakis G, Herren JK, Jaenike J, Bourtzis K. 2012. Loss of reproductive parasitism following transfer of male-killing *Wolbachia* to *Drosophila melanogaster* and *Drosophila simulans*. *Heredity*, *109*, 306–312. https://doi.org/10.1038/hdy.2012.43

Visser S, Voleníková A, Nguyen P, Verhulst EC, Marec F. 2021. A conserved role of

the duplicated Masculinizer gene in sex determination of the Mediterranean flour moth, *Ephestia kuehniella*. *PLOS Genetics*, *17*, e1009420. https://doi.org/10.1371/journal.pgen.1009420

Werren JH, Baldo L, Clark ME. 2008. *Wolbachia*: Master manipulators of invertebrate biology. *Nature Reviews Microbiology*, *6*, 741–751. https://doi.org/10.1038/nrmicro1969

Zug R, Hammerstein P. 2012. Still a host of hosts for *Wolbachia:* analysis of recent data suggests that 40% of terrestrial arthropod species are infected. *PLOS ONE*, *7*, e38544. https://doi.org/10.1371/journal.pone.0038544

Figure legends

Figure 1 Transient expressions of the *Hm-oscar* gene resulted in female-biased sex ratios

(a) Structures of *w*Fur-encoded Oscar and *w*Hm-t-encoded Hm-Oscar and four Wmk proteins. (b) Male ratio of adult progeny obtained from cRNA-injected groups (*wmk*-1, *wmk*-2, T2A-brigged *wmk*-1 and *wmk*-2, *wmk*-3, *wmk*-4, T2A-brigged *wmk*-3 and *wmk*-4, GFP, and *Hm-oscar*; n = 5–15 independent replicates using different egg masses), the non-injected NSR line, and the MK *w*Hm-t-positive W^{T12} line. The total numbers of adult females and males are shown at the bottom. Different letters indicate significant differences (Steel–Dwass test, $P < 0.05$). The dot plots show all data points individually.

Figure 2 Hm-Oscar induced lethality in male embryos with the female-type sex determination

(a) Sex ratio of the hatched larvae and unhatched embryos in the *Hm-oscar*-expressed, *w*Hm-t-infected, and non-infected/expressed groups. Females and males were discriminated based on the presence or absence of W chromatin. The number of individuals is indicated in brackets. (b) Splicing patterns of the downstream sex-determining gene *dsx* of *H. magnanima* embryos (5 days post oviposition). Abbreviations: HmOsc, *Hm-oscar* injected group; GFP, GFP-injected group; W^{T12}, *w*Hm-t-infected line; NSR, non-infected/injected line. M and F indicate W chromatin-negative (ZZ: male genotype) and W chromatin-positive (ZW: female genotype) mature embryos, respectively. *dsx-F* and *dsx-M* represent female and male-specific splicing variants, respectively. L: 100 bp DNA ladder (ExcelBand 100 bp DNA Ladder, SMOBIO Technology, Inc., Hsinchu, Taiwan). 0.1, 0.5, 1.0, and 1.5 kb markers are indicated with arrows. N: negative control (water).

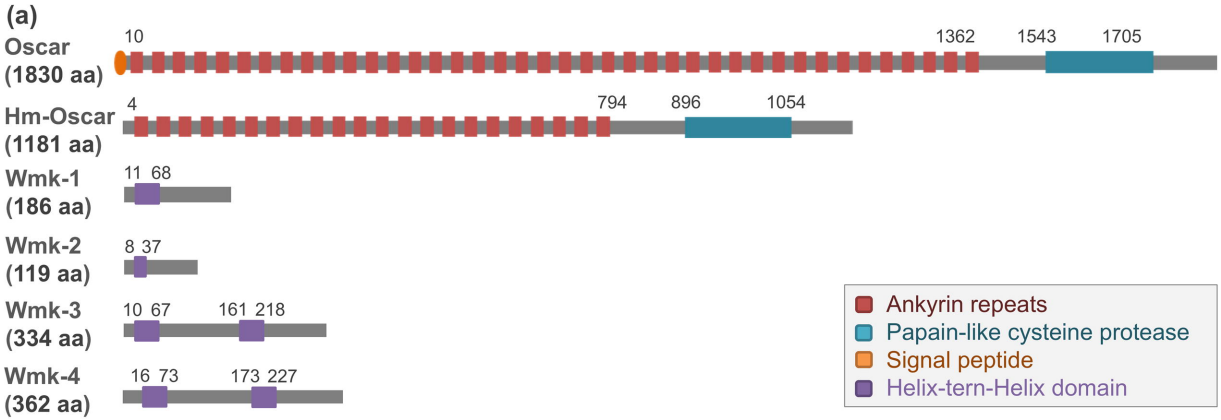
Figure 3 *Hm-oscar*-overexpressed male embryos showed higher levels of Z-linked gene expression

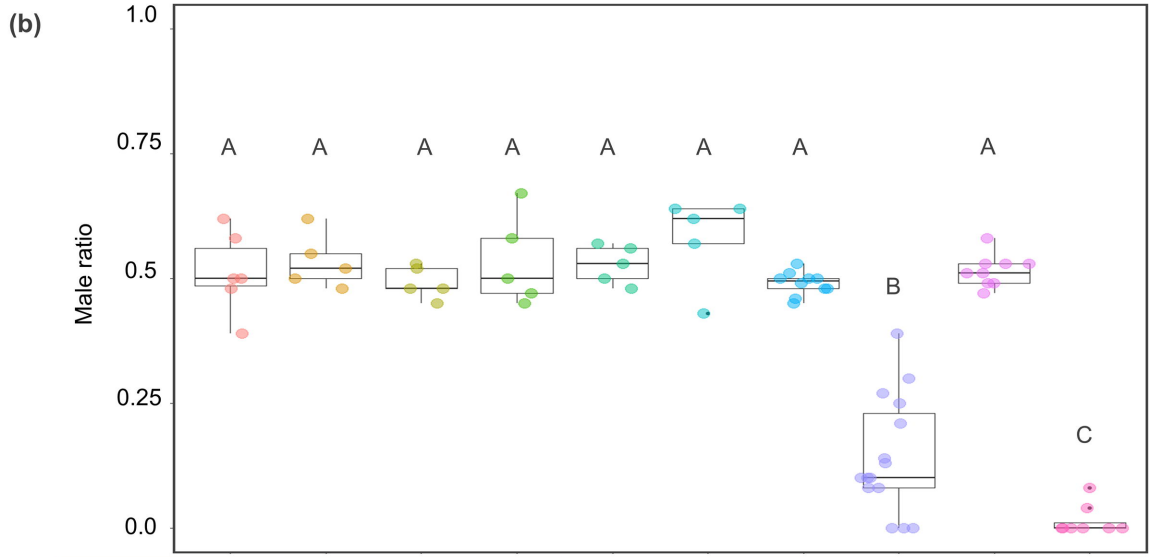
(a-d) Normalized expression levels (TPM) and chromosomal distributions of transcripts in *H. magnanima* embryos. RNA-seq data of embryos (108 hpo) were used to make the following comparisons: *Hm-oscar*-injected males versus *Hm-oscar* injected females (a), GFP-injected males versus GFP-injected females (b), W^{T12} males versus W^{T12} females (c), and NSR males versus NSR females (d). The chromosome number for each *H. magnanima* transcript-derived contig was assigned based on *Bombyx mori* gene models. The X axis represents the chromosome number of *B. mori* (shown as chr01 to chr28), and chr01 and chr15 (highlighted in orange) correspond to the Z chromosome of *H. magnanima* (Arai et al., 2023a). (e) Relative expression of a Z-linked *tpi* gene in *H. magnanima* groups. Statistical significance is highlighted with different alphabets.

Figure 4 Hm-oscar suppressed the masculinizing function of lepidopteran Masc

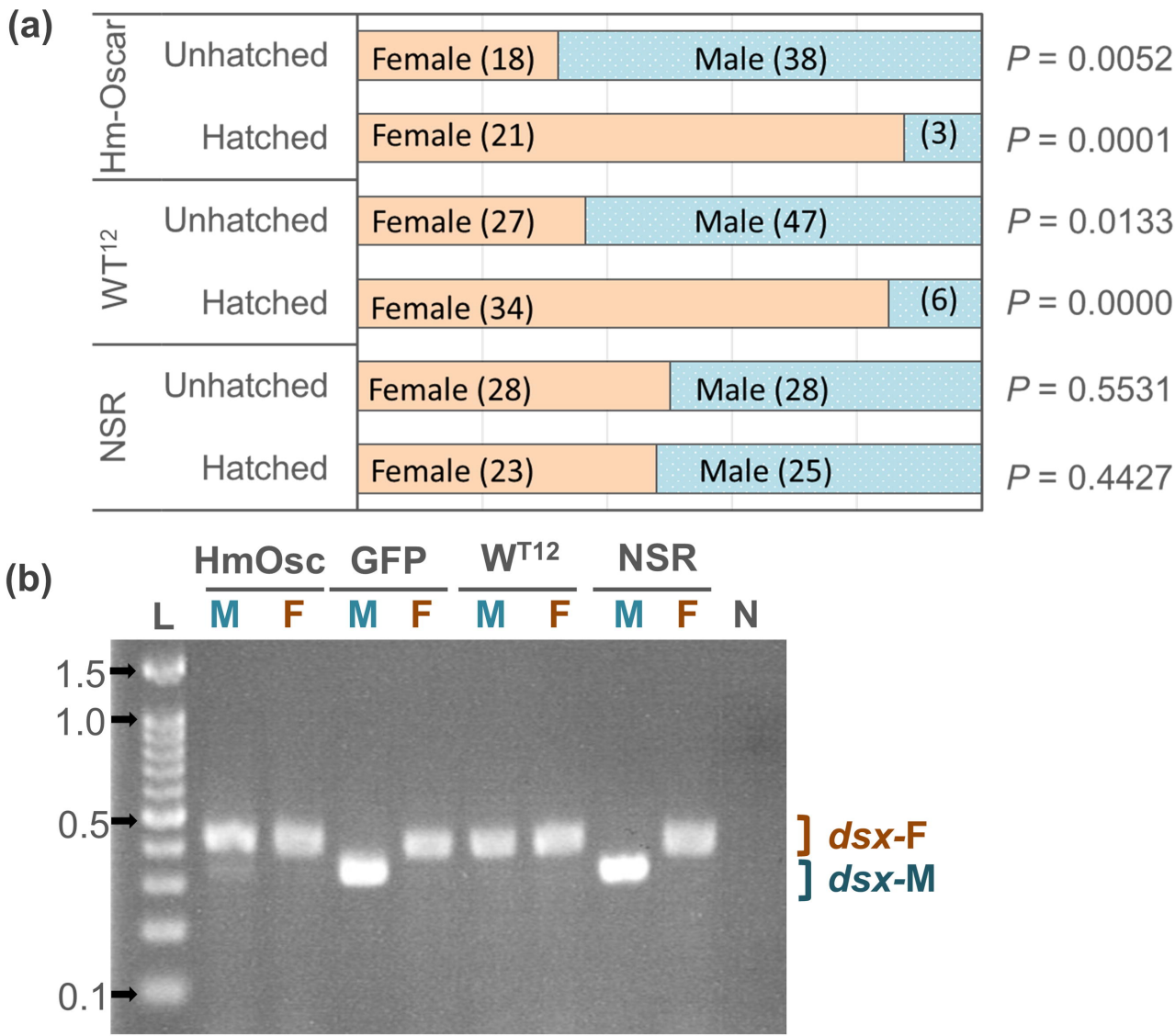
(a) Relative expression levels of the male-specific $BmImp^{M}$ variant in transfected BmN-4 cells. The relative expression of $BmImp^{M}$ in the control (non-inserted pIZ/V5) and *Hm-oscar*/*oscar* co-transfected groups was normalized by setting the mean in the *Hmmasc* single transfected group as 100. (b) Protein structures and homologies

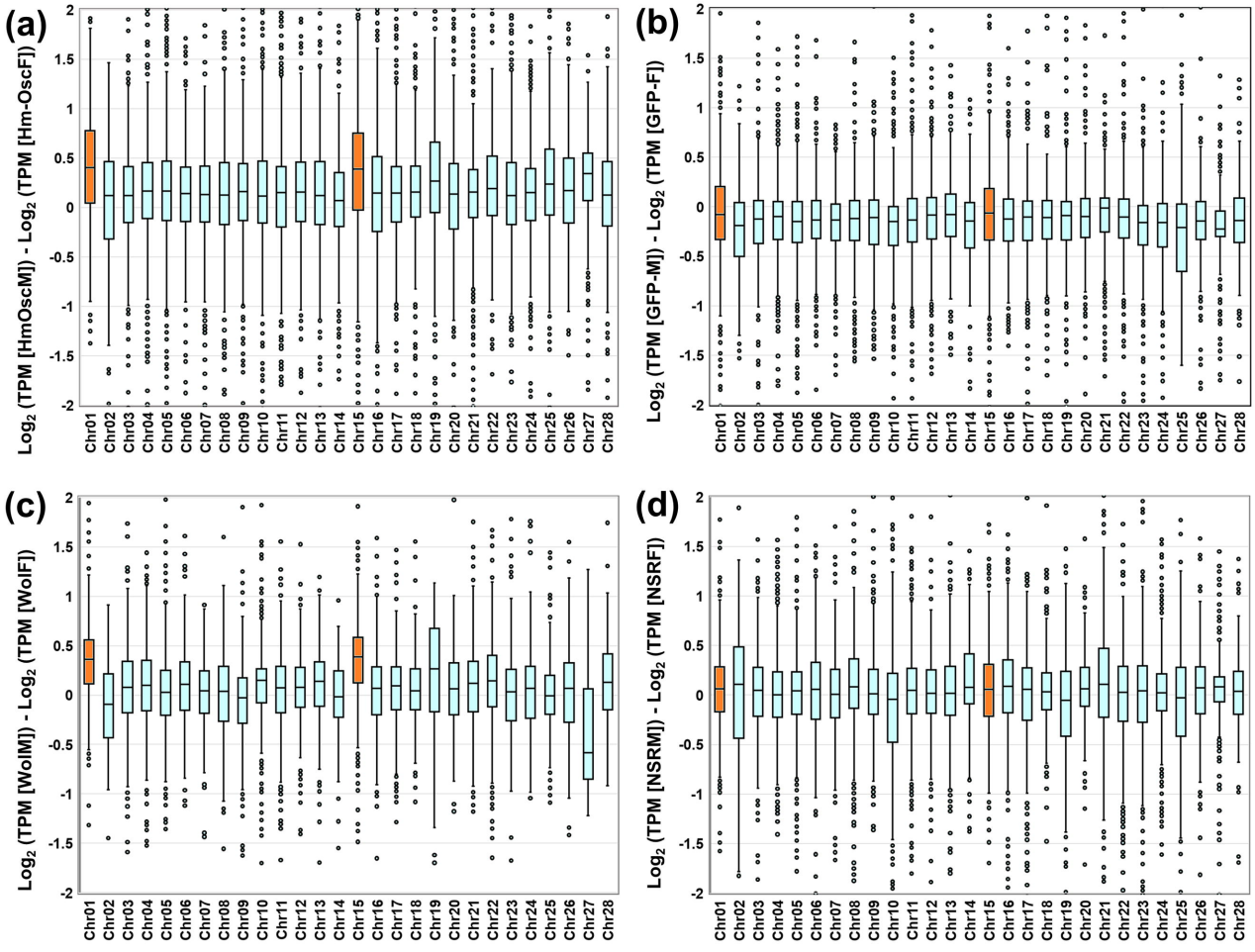
of Hm-Oscar and Oscar proteins. (c) Relative expression levels of the *BmImp*^{M} in *Hm-oscar/masc* transfected BmN-4 cells. The relative expression of *BmImp*^{M} in each condition was normalized by setting the mean in the *Hmmasc* single transfected group as 100. The dot plots show all data points individually. Each experimental condition was replicated three times. Abbreviations: Hm, *Homona magnanima*; Bm, *Bombyx mori*; Of, *Ostrinia furnacalis;* Pm, *Papilio machaon;* Sf, *Spodoptera frugiperda*; and Tv, *Trilocha varians.* (d) Phylogeny and homologies of the Masc protein sequences used in this study.

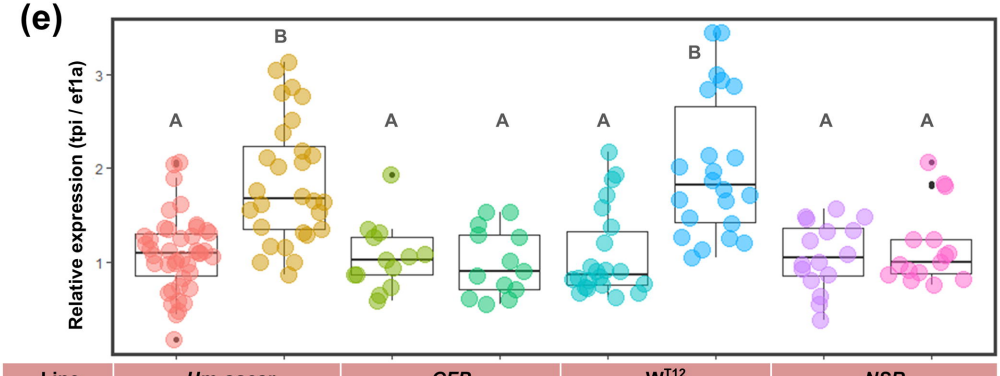




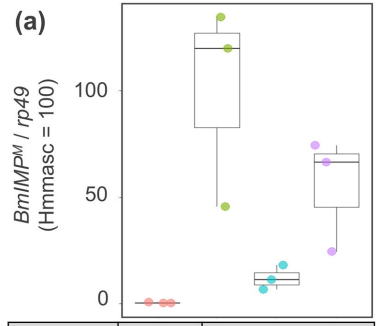
Gene	*wmk-1*	*wmk-2*	*wmk-1-2*	*wmk-3*	*wmk-4*	*wmk-3-4*	*GFP*	*Hm-oscar*	NSR	W^{T12}
Replicates	6	5	5	5	5	5	10	15	9	9
Male ratio	0.51	0.52	0.50	0.51	0.53	0.56	0.49	0.16	0.52	0.01
Female	47	62	72	84	50	72	126	218	201	213
Male	48	67	73	86	56	90	123	42	215	2
Binominal test (P =)	0.5000	0.3624	0.5000	0.4694	0.3137	0.0907	0.4496	0.0000	0.2172	0.0000


(a)
Hm-Oscar
Unhatched
Female (18)
Male (38)
P = 0.0052
Hatched
Female (21)
(3)
P = 0.0001
WT12
Unhatched
Female (27)
Male (47)
P = 0.0133
Hatched
Female (34)
(6)
P = 0.0000
NSR
Unhatched
Female (28)
Male (28)
P = 0.5531
Hatched
Female (23)
Male (25)
P = 0.4427
(b)
HmOsc
GFP
WT12
NSR
L
M
F
M
F
M
F
M
F
N
1.5
1.0
0.5
0.1
dsx-F
dsx-M

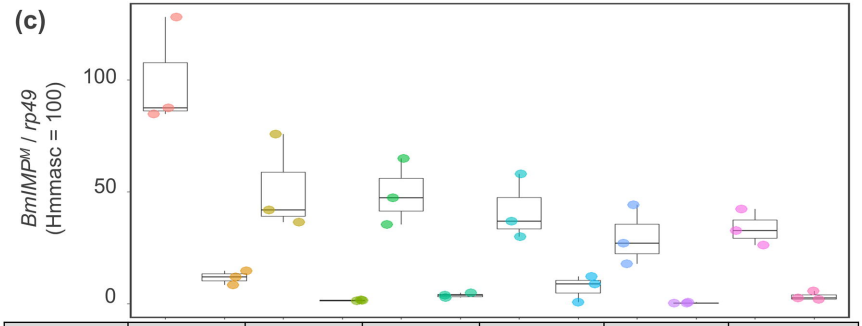




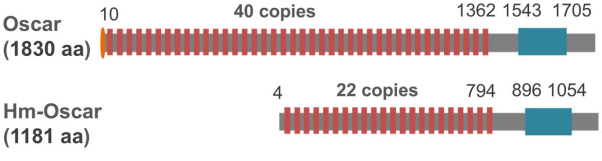
Line	*Hm-oscar*		*GFP*		W^{T12}		*NSR*	
Sex	Female	Male	Female	Male	Female	Male	Female	Male
Replicates	42	28	13	13	22	22	16	15



Masc	None	HmMasc		
Hm-Oscar	-	-	+	-
Oscar	-	-	-	+

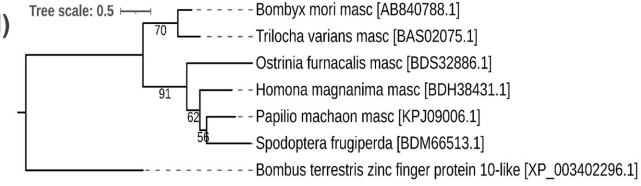


Masc	HmMasc		BmMasc		OfMasc		PmMasc		SfMasc		TvMasc	
Hm-Oscar	-	+	-	+	-	+	-	+	-	+	-	+



	Oscar	Hm-Oscar
Oscar	100	81.44
Hm-Oscar	81.44	100

- Ankyrin repeats
- Papain-like cysteine protease
- Signal peptide



	BmMasc	TvMasc	OfMasc	SfMasc	HmMasc	PmMasc
BmMasc	100	58.36	24.43	29.09	27.6	27.81
TvMasc	58.36	100	23.82	25.66	26.43	26.29
OfMasc	24.43	23.82	100	32.11	32.42	34.36
SfMasc	29.09	25.66	32.11	100	37.37	43.89
HmMasc	27.6	26.43	32.42	37.37	100	41.97
PmMasc	27.81	26.29	34.36	43.89	41.97	100