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Nagamine, Keisuke
Department of Environmental and Horticultural Sciences, Minami Kyushu University

Kanno, Yoshiaki
Department of Environmental and Horticultural Sciences, Minami Kyushu University

Sahara, Ken
Faculty of Agriculture, Iwate University

Fujimoto, Toshiaki
Faculty of Agriculture, Iwate University

他

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Male-killing virus in a noctuid moth *Spodoptera litura*

Keisuke Nagamine[a,b,1], Yoshiaki Kanno[a], Ken Sahara[c], Toshiaki Fujimoto[c], Atsuo Yoshido[c,d], Yukio Ishikawa[e], Misato Terao[a], Daisuke Kageyama[b,1], and Yoshinori Shintani[a,1]

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A female-biased sex ratio is considered advantageous for the cytoplasmic elements that inhabit sexually reproducing organisms. There are numerous examples of bacterial symbionts in the arthropod cytoplasm that bias the host sex ratio toward females through various means, including feminization and male killing. Recently, maternally inherited RNA viruses belonging to the family Partitiviridae were found to cause male killing in moths and flies, but it was unknown whether male-killing viruses were restricted to Partitiviridae or could be found in other taxa. Here, we provide compelling evidence that a maternally inherited RNA virus, Spodoptera litura male-killing virus (SlMKV), selectively kills male embryos of the tobacco caterpillar *Spodoptera litura*, resulting in all-female broods. SlMKV injected into uninfected *S. litura* can also be inherited maternally and causes male killing. SlMKV has five genomic segments encoding seven open reading frames, has no homolog of known male-killing genes, and belongs to an unclassified group of arthropod-specific viruses closely related to Tolivirales. When transinfected into larvae, both male and female recipients allow SlMKV to proliferate, but only males die at the pupal stage. The viral RNA levels in embryonic and pupal male killing suggest that the mechanism of male killing involves the constitutive expression of viral products that are specifically lethal to males, rather than the male-specific expression of viral products. Our results, together with recent findings on male-killing partiti-like viruses, suggest that diverse viruses in arthropods tend to acquire male killing independently and that such viruses may be important components of intragenomic conflict in arthropods.

male killing | virus | host–symbiont interactions | selfish genetic element | reproductive manipulation

Significance

Male killing is an adaptive trait of the maternally inherited endosymbionts of various arthropods. Male-killing endosymbionts, called "male killers", include various bacteria, such as *Spiroplasma* and *Wolbachia*, and some microsporidians. In addition to bacteria, partiti-like viruses have recently been shown to be male killers in moths and flies. Here, we found that a virus, belonging to a hitherto unfocused group that is widespread in arthropods, causes male killing in the moth *Spodoptera litura*. This finding suggests that diverse viruses act as selfish cytoplasmic factors in various arthropods, opening new horizons in understanding the biology of virus–arthropod interactions.

A female-biased sex ratio is considered advantageous for the spread of cytoplasmic elements inhabiting sexually reproducing plants and animals (1, 2). Selfish mitochondria that interfere with the production of functional male gametes, the cause of so-called cytoplasmic male sterility, are ubiquitous in plants (3, 4). In animals, mitochondria-induced cytoplasmic male sterility is not pervasive but is known in mollusks (5) and *Drosophila* flies (6). In various arthropods, maternally inherited bacteria belonging to diverse taxonomic groups (e.g., *Wolbachia*, *Rickettsia*, *Arsenophonus*, *Spiroplasma*, and *Flavobacterium*) are known to bias the sex ratio toward females (7). The underlying mechanism of the sex ratio bias can be male killing, feminization, parthenogenesis induction, or meiotic drive (8). Male killing (male-specific lethality in embryos or hatchlings), the most commonly observed mechanism in a variety of arthropods, is thought to be adaptive for cytoplasmic genes where hosts experience sibling–sibling competition for resources, because the death of neonate males, which do not transmit the bacterium, increases the survival prospects of sibling females, which bear the same bacterium and can transmit it (9). Feminization of the male phenotype, a less common mechanism observed in woodlice (10, 11), leafhoppers (12), and butterflies (13), should be adaptive for cytoplasmic genes because nontransmitting males turn into transmitting females. Parthenogenesis induction [which feminizes the male genotype and enables reproduction without males (14)] observed only in haplodiploid insects is also adaptive for cytoplasmic genes. Finally, meiotic drive (either selection for W chromosome-bearing gametes or selection against Z chromosome-bearing gametes during female meiosis) in female heterogametic organisms is obviously adaptive for cytoplasmic genes because of linkage disequilibrium between the W chromosome and the cytoplasm. Meiotic drive has been suspected in *Abraxas* moths (15–17) and *Eurema* butterflies (13), although conclusive evidence is lacking.

In addition to bacterial male killers, microsporidians (unicellular protists) are known to cause male killing in mosquitoes and feminization in amphipods (18, 19). Recently, double-stranded RNA viruses belonging to the family Partitiviridae were found to induce male killing in the fly *Drosophila biauraria* (20, 21) and the moth *Homona magnanima* (22, 23). In *D. biauraria*, the male-killing partiti-like virus (DbMKPV1) contains four double-stranded RNA segments (RNA1 to RNA4), each containing a predicted open reading frame (ORF). Overexpression of these ORFs in *Drosophila melanogaster* using the

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[1]To whom correspondence may be addressed. Email: ef10140@yahoo.co.jp, kagymad@affrc.go.jp, or shintani@nankyudai.ac.jp.

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Gal4/UAS system revealed that the protein PVMKp1, encoded by RNA4, is the cause of the male-killing trait. The other male-killing partiti-like viruses in *H. magnanima*, referred to as Osugoroshi viruses (OGVs), have 27 RNA segments, three of which encode RNA-dependent RNA polymerase (RdRp). Whether multiple viral variants coexist or whether the 27 segments constitute an integral viral component in the male-killing lines (MK line) of *H. magnanima* remains unknown. According to the NCBI database, numerous partiti-like viruses closely related to these male killers are present in diverse species of arthropods. Therefore, male-killing partiti-like viruses will be widely found in other arthropod taxa.

Although bacteria belonging to diverse taxa cause male killing in various arthropods, the generality of virus-mediated male killing, i.e., the taxonomic diversity of male-killing viruses, remains unknown. Here, we aim to provide compelling evidence that a newly identified virus belonging to a hitherto unfocused group that is widespread in arthropods causes male killing in the tobacco caterpillar *Spodoptera litura*, a destructive insect pest of numerous agricultural crops in temperate to tropical regions of South and East Asia and Oceania (24, 25).

Results and Discussion

Maternally Inherited All-Female Trait in *S. litura*. The pupae and adults of *S. litura* are sexually dimorphic (Fig. 1 *A* and *B*). All the *S. litura* larvae collected from a greenhouse in Miyazaki, Japan, developed into female moths (n = 45). After mating with males collected from a field, seven produced only female offspring (207 females in total; *SI Appendix*, Fig. S1*A*). By continuously rearing one matriline (Matriline-1) at a typical laboratory temperature (25 °C), the maternal inheritance of the all-female trait was confirmed for 13 generations, despite the frequent occurrence of broods with an almost 1:1 sex ratio (Fig. 1*C* and *SI Appendix*, Fig. S1*A*). Similarly, the maternal inheritance of the all-female trait was confirmed for one to three generations in the other six matrilines (Matriline-2 to -7; *SI Appendix*, Fig. S1*A*). Of the 15 females collected in the open field, one female produced all-female offspring (n = 33) and 14 females produced offspring with a nearly 1:1 sex ratio (176 females and 195 males in total; Matriline-8 to -22; *SI Appendix*, Fig. S1*B*). Three matrilines derived from the females with a normal sex ratio (Matriline-8 to -10) did not show the all-female trait for 4 to 5 generations (*SI Appendix*, Fig. S1*B*). These results suggest that the all-female trait is not common in *S. litura* from Miyazaki. The frequent appearance of males in all-female matrilines, mentioned above, led us to suspect that the all-female trait is temperature sensitive (26). Thus, we separated a subline of Matriline-1 into two groups that were reared at 22.5 °C (Fig. 1*D* and *SI Appendix*, Fig. S2*A*) and 30 °C (Fig. 1*E* and *SI Appendix*, Fig. S2*B*) for several generations. We found that the all-female trait was stably expressed at 22.5 °C, whereas it was completely lost after two generations reared at 30 °C. Because no sex ratio bias was observed for the normal-sex-ratio matrilines (designated NS lines) at 22.5 °C (*SI Appendix*, Fig. S2*C*), the temperature-dependent sex ratio shift was not considered an inherent characteristic of *S. litura*. In subsequent experiments, *S. litura* were reared at 22.5 °C, unless stated otherwise.

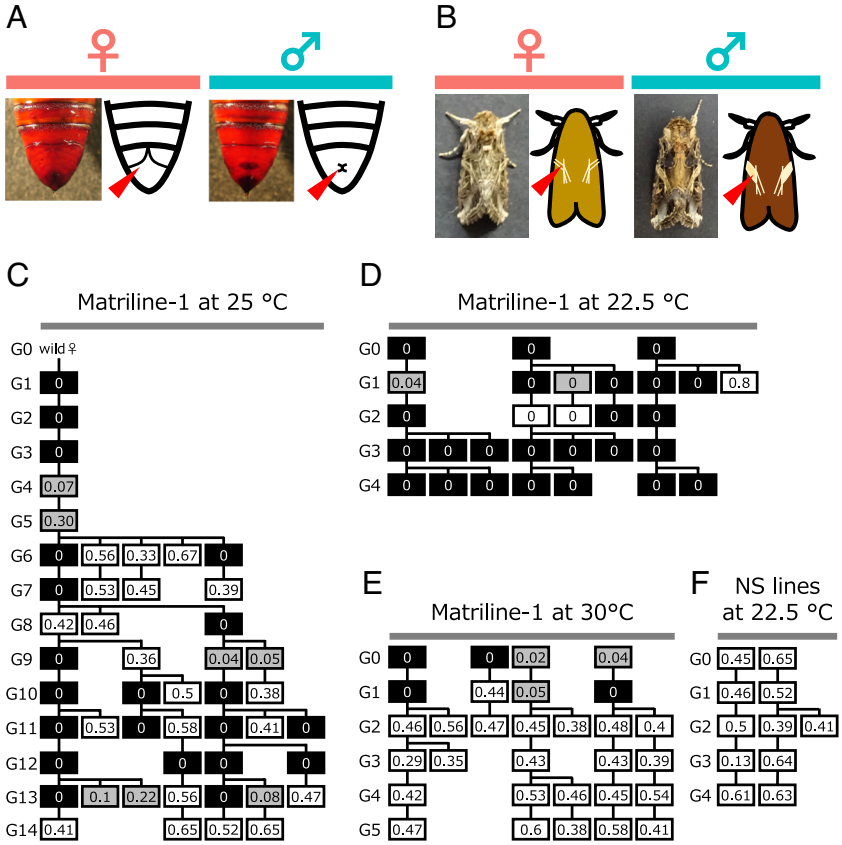


Fig. 1. Sexually dimorphic morphology in pupae and adults of *S. litura* (*A* and *B*) and pedigrees showing brood sex ratios (proportions of males) of Matriline-1 (*C*–*E*) and NS lines (*F*: see *SI Appendix*, Figs. S1 and S2 for details). Key morphological traits (indicated by arrowheads) were used for sexing of pupae (*A*) and adults (*B*). Pedigrees of Matriline-1 (original all-female line) reared at 25 °C (*C*), 22.5 °C (*D*), and 30 °C (*E*) are shown with brood sex ratios. The generation in which the temperature was shifted is designated as G0 (*D* and *E*). Black, gray, and white boxes indicate broods with only females, broods with one or more males but with significantly female-biased sex ratios ($P < 0.05$, binomial), and broods with unbiased sex ratios ($P \geq 0.05$, binomial), respectively (*C*–*E*).

Embryonic Male Killing is the Mechanism of the All-Female Trait. The comparative genomic in situ hybridization of final-instar larvae revealed that the sex chromosome constitution of both the NS females (n = 3) and Matriline-1 females (n = 5) was WZ and that of the NS males (n = 1) was ZZ (*SI Appendix*, Fig. S3). This represents the canonical sex chromosome constitution of lepidopteran species (27) and consistency of chromosomal and phenotypic sex, thus excluding the possibility of the feminization of ZZ males. Moreover, the egg-hatching rate of the Matriline-1 broods (median: 0.39) was nearly half that of the NS broods (median: 0.86; Fig. 2*A*), leading us to assume that males were specifically killed before hatching. To verify this finding, individuals were sexed immediately before and after egg hatching, based on the sex-specific spliced transcripts of the *S. litura* doublesex genes (*SldsxF* and *SldsxM*: Fig. 2 *B* and *C*). At 4 days post oviposition (4 dpo; i.e., the day before egg hatching), the embryos consisted of nearly equal numbers of females and males in a brood of Matriline-1 (16 females and 16 males) and in a brood of an NS line (11 females and 12 males; Fig. 2*D* and *SI Appendix*, Fig. S4). However, after egg hatching, the Matriline-1 brood showed a strong female bias (31 females and no males in the neonate larvae and 30 females and 1 male in the mid-stage larvae). In contrast, the NS line brood consisted of nearly equal numbers of females and males in the neonate larvae (14 females and 10 males) and in the mid-stage larvae (7 females and 9 males; Fig. 2*D* and *SI Appendix*, Fig. S4). These results strongly suggest that male killing occurred prior to eggs hatching (during embryogenesis) in Matriline-1. We also used polymorphism in a Z-linked gene, the *S. litura* homolog of *triosephosphate isomerase* (*SlTpi*), to follow the inheritance of the Z chromosome. Offspring (daughters) of Matriline-1 females inherit their Z chromosome from their fathers (*SI Appendix*, Fig. S5), which excludes the possibility of gynogenesis. Collectively, the mechanism of the all-female trait was determined to be male killing; hence, Matriline-1 is hereafter referred to as the MK line. Our results also indicated that the male-killing mechanism does not affect the splicing of the *dsx* homolog (i.e., the sex-determining gene cascade), which was also unaffected in OGV-induced male killing in *H. magnanima* (28). In contrast, bacterial male killers (*Wolbachia* and *Spiroplasma*) alter the splicing of the *dsx* homolog from male to female in *Ostrinia scapulalis* (29), *Ostrinia furnacalis* (30), and *H. magnanima* (28).

Extragenomic Five RNAs Are Associated with Male Killing. Successive treatment of larvae with tetracycline for generations did not affect the all-female trait of the MK line (*SI Appendix*, Fig. S2 *D* and *E*). Moreover, we found that the male-killing agent was transferrable by injecting a 0.2-μm-filtered homogenate of MK line pupae/adults into NS line pupae/adults. The transinfected sublines (designated tr.NS) began to show female-biased sex ratios from the next generation (Generation 1, G1) and were transformed into all-female matrilines in subsequent generations (*SI Appendix*, Fig. S2*F*). Because most bacteria are suppressed by tetracycline (31) and cannot pass through a 0.2-μm filter, these results led us to hypothesize that a nonbacterial replicating entity, probably a virus, was responsible for the male-killing trait. To search for extragenomic RNAs associated with the male-killing phenotype, we compared the ovarian RNA-seq data of three females with a normal sex ratio and three females with the all-female trait. For the normal-sex-ratio females, a single female was selected from each of the three broods with a 1:1 sex ratio (two from different NS lines and one from the hs.MK line—an MK subline that ceased to express male killing after being maintained at 25 °C for several generations and further confirmed for the absence of male killing at 22.5 °C for several generations). For the females with the all-female trait, a single female was selected from each of the three all-female broods (one from the MK line and two

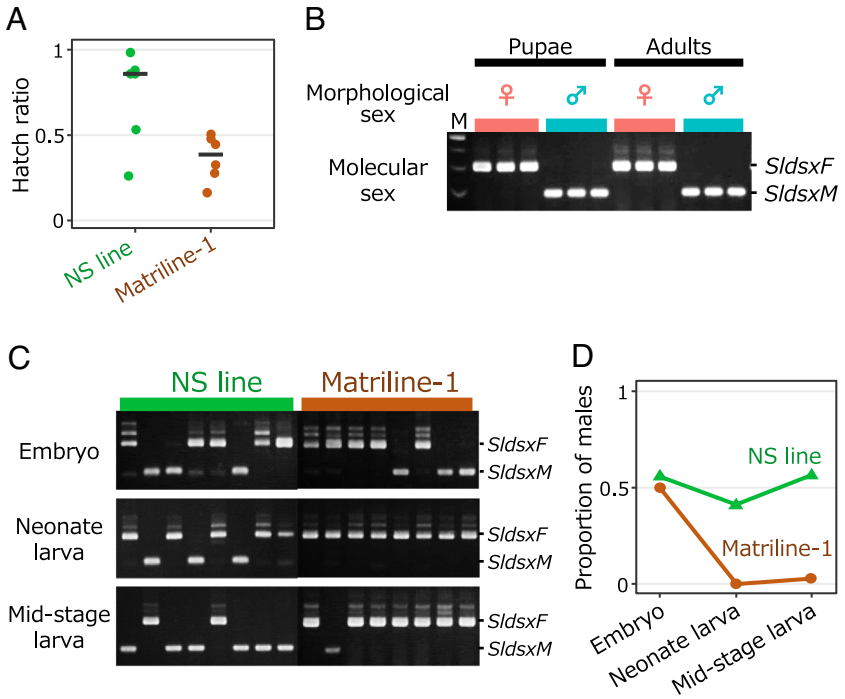
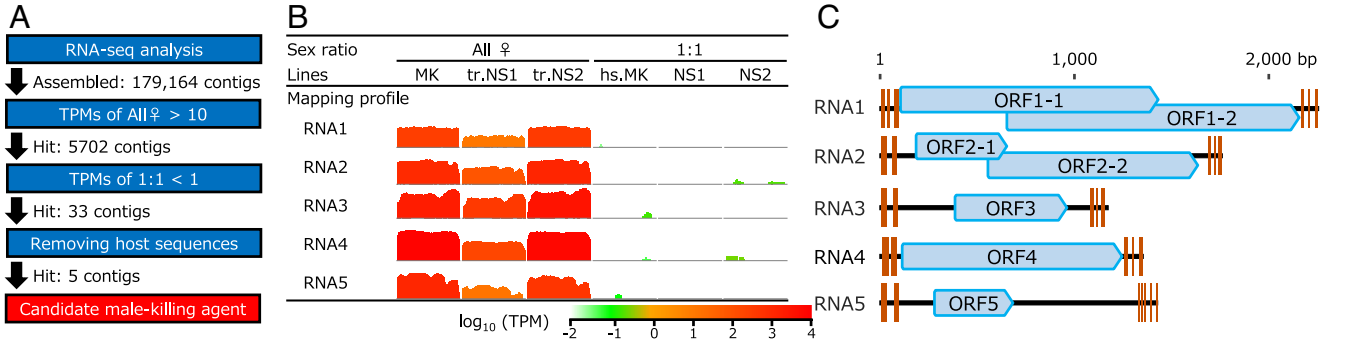


Fig. 2. Embryonic male killing revealed by molecular sexing. (*A*) Egg-hatching rates of NS lines and Matriline-1. Each point represents a single brood (n = 77 to 115). Horizontal black bars represent the medians. (*B*) Splice variants of *Sldsx* examined for females and males based on RT-PCR. The splice variants of *Sldsx* were in accordance with the morphological sex of the pupae and adults. M: the size marker showing 1,000, 750, 500, and 250 bp from *Top* to *Bottom*. (*C*) Examples of the molecular sexing of embryos, neonate larvae (first instar), and mid-stage larvae (second or third instars) based on the splice variants of *Sldsx*. See *SI Appendix*, Fig. S4 for details. (*D*) Proportion of males in a single brood of NS line and Matriline-1 based on molecular sexing in embryos, neonate larvae, and mid-stage larvae (n = 16 to 32, for each point).

from the tr.NS lines; *SI Appendix*, Fig. S2*F*). Assembly of all the raw data obtained from the six samples yielded 179,164 contigs (Fig. 3*A*). The abundances of 33 of these exceeded 10 transcripts per million (TPM) in the three all-female samples and were less than 1 TPM in the three normal-sex-ratio samples (Fig. 3*A* and *SI Appendix*, Table S1). Of the 33 contigs, 28 matched the *S. litura* genome sequence (NCBI RefSeq: GCF_002706865.1); thus, the remaining five contigs (RNA1–RNA5) were considered extragenomic (Fig. 3 *A* and *B*).

RNA Virus with Five Genomic Segments is the Male-Killing Agent. Northern blotting assays using double-stranded DNA probes for RNA1–RNA5 sequences revealed that each RNA sequence was nearly full length (*SI Appendix*, Fig. S6). The presence of conserved sequences at the 5′ and 3′ ends of the five RNAs (Fig. 3*C* and *SI Appendix*, Fig. S7), a common feature of viral segmented genomes, suggests that RNA1–RNA5 are genomic segments that constitute a single virus. RNA1–RNA5 encode seven putative ORFs (Fig. 3*C*). RNA3, RNA4, and RNA5 each encode a single ORF (ORF3, ORF4, and ORF5, respectively), whereas RNA1 and RNA2 each encode two ORFs in different frames (ORF1-1/ORF1-2 and ORF2-1/ORF2-2, respectively). According to a motif search against the Pfam database (32), ORF2-1 contains motifs of a virion membrane protein family and ORF2-2 contains those of a virion glycoprotein family (Fig. 3*D*). A BLASTp search against the NCBI nonredundant protein database revealed that ORF1-2 was homologous to the RdRp (a necessary component of RNA viruses) of Diaphorina citri-associated C virus (Fig. 3*D*). The amino acid sequence of RdRp contains structural motifs A–C, which are required for catalysis and are conserved among viral RdRp sequences (33) (*SI Appendix*, Fig. S8). In the recipient larvae (G0 of the tr.NS lines), the copy number of RNA1 increased from the larval to adult stages (Fig. 3*E*). These features strongly suggested that the five RNAs were derived from an RNA virus. The nucleotide sequences of RNA1–RNA5 were successfully PCR-amplified from complementary DNA prepared from a tr.NS female (using reverse-transcription from total RNA) but were not PCR-amplified from total DNA prepared from the same female (*SI Appendix*, Fig. S9). This indicates that the five sequences exist as extragenomic and transmissible RNA fragments, rather than as host genomic DNA fragments (e.g., in prophage regions or transposons/retrotransposons) that are specific to the MK line. An integral association between the five RNA segments and the male-killing phenotype was suggested based on the transinfection results; the five RNAs behave collectively (without segregation), and the loss of the five RNAs is associated with the loss of the male-killing phenotype (Fig. 4*A* and *SI Appendix*, Fig. S2*G*). We conclude that the causal agent of male killing in the MK line is an RNA virus, hereafter referred to as the Spodoptera litura male-killing virus (SlMKV).

Insight into the Male-Killing Mechanism. To investigate the timing of male-specific death, embryonic development of the tr.NS line was examined by gently removing embryos from eggs at 5 dpo (eggs began to hatch a few hours later) and comparing them with hatched larvae and unhatched embryos at 6 dpo. At 5 dpo, the egg mass consisted of eggs with and without black spots (Fig. 5 *A* and *B*)—all the embryos from the black-spotted eggs showed larval morphology with well-melanized head capsules, whereas all embryos from the nonspotted eggs showed larval morphology but had incompletely melanized head capsules (Fig. 5 *C* and *D*). All embryos with well-melanized head capsules exhibited *SldsxF* (n = 8) and were therefore females, whereas of the eight embryos with incompletely melanized head capsules,



D

Gene	Blastp (E < 1e-05)				Motif search (E < 0.01)		
	Annotation	QC	E-value	Species	Annotation	E-value	Pfam
ORF1-1	Hypothetical protein 1	85%	7.00E-34	WIV 35	None	-	-
ORF1-2	RdRp	96%	4.00E-141	DcACV	Viral RdRp	2.00E-36	PF00998
ORF2-1	None	-	-	-	Virion membrane protein	4.00E-07	PF16504
ORF2-2	None	-	-	-	Virion glycoprotein	4.00E-05	PF16506
ORF3	Acetyltransferase	95%	2.00E-29	MaphGV	Unknown function	3.00E-26	PF19234
ORF4	None	-	-	-	None	-	-
ORF5	None	-	-	-	None	-	-

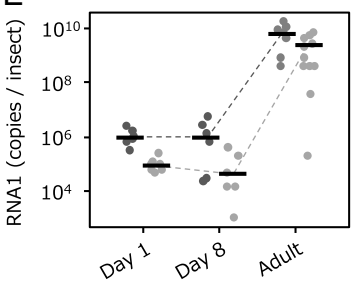


Fig. 3. Spodoptera litura male-killing virus (SlMKV). (*A*) Screening for extragenomic RNAs associated with the all-female trait from the assembled *S. litura*-ovarian RNA-seq contigs. (*B*) Mapped reads of RNA-seq on RNA1–RNA5 (five contigs selected in *A*) for the six *S. litura* samples (see text for details). *X* axis: sequence position in each of the five contigs. *Y* axis: log scale read depth. Color: heatmap based on $\log_{10}$ (TPM). (*C*) Genome structure of SlMKV. Black line: untranslated region (UTR). Blue box: ORF. Brown thin boxes on UTR: highly conserved sequences among the five RNAs. (*D*) Annotation of the seven ORFs based on BLASTp (NCBI nonredundant protein database) and Motif search (Pfam databases). RdRp: RNA-dependent RNA polymerase. MaphGV: Matsumuraeses phaseoli granulovirus (natural host: the moth, *Matsumuraeses phaseoli*). WIV 35: Wuhan insect virus 35 (found from RNA mixture of several insect species). DcACV: Diaphorina citri-associated C virus (natural host: the stink bug, *Diaphorina citri*). (*E*) Viral proliferation after injection of SlMKV. Homogenates prepared from two individuals of a tr.NS line (dark and light gray spots, respectively) were injected into an NS brood. RNA3 copy numbers were estimated at day 1 and day 8 after injection and at the adult stage (33 d or more after injection).

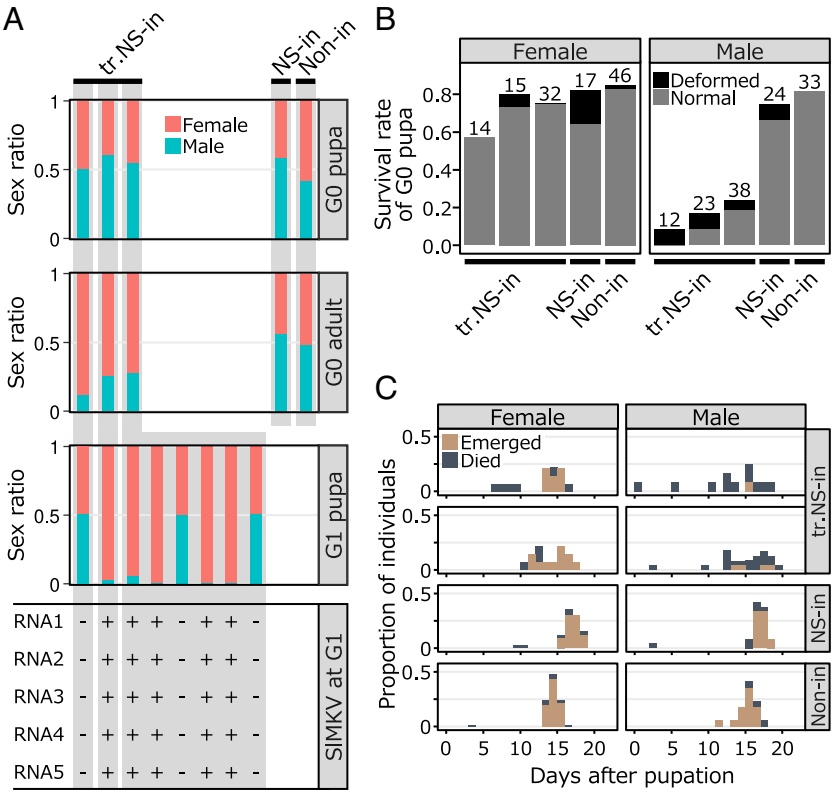
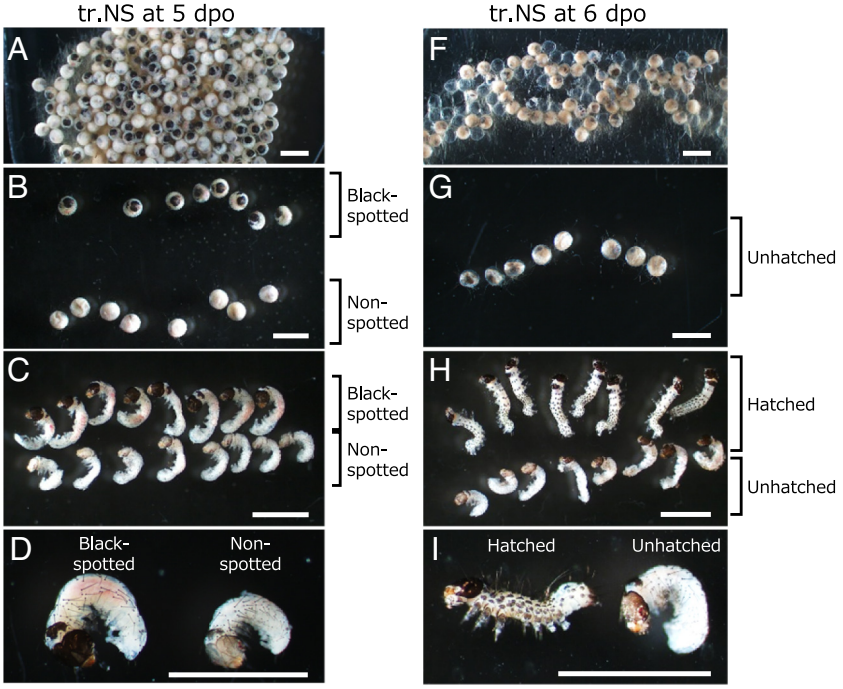


Fig. 4. Occurrence of pupal male killing after transinfection of SlMKV into larvae. (*A*) Sex ratio and the presence/absence of RNA1–RNA5 following injection of SlMKV into NS larvae. The third-instar larvae of an NS line were injected with the homogenate of a tr.NS pupa (designated tr.NS-in; with three replicates; *n* = 100, 84, and 90). As a control, the third-instar larvae of an NS line were either injected with the homogenate of an NS pupa (designated NS-in; *n* = 82) or not injected (designated Non-in; *n* = 79). The sex ratios of the pupae and adults of the injected generation (G0) and the pupae of the following generation (G1) are shown. Presence (+) or absence (–) of RNA1–RNA5 in G1 neonate larvae (pooled total RNA from 5 to 10 individuals) is shown at the bottom. (*B*) Survival rates during the pupal stage of G0 for tr.NS-in, NS-in, and Non-in. Numbers above bars indicate sample sizes. Gray: normal-shaped adults upon emergence. Black: deformed adults upon emergence (See text for details). (*C*) Timing of adult emergence and pupal death (days after pupation) in G0 of tr.NS-in, NS-in, and Non-in. Brown: emerged adults. Black: dead pupae.

seven exhibited *SldsxM* (male) and one exhibited *SldsxF* (female). At 6 dpo, nearly half of the eggs had hatched (Fig. 5 *F* and *G*)—hatched larvae exhibited well-melanized head capsules and black spots on the thorax and abdomen, but neither feature was prominent in the embryos from unhatched eggs (Fig. 5 *H* and *I*). All hatched larvae exhibited *SldsxF* (female; n = 8), whereas of the eight embryos from unhatched eggs, six exhibited *SldsxM*, one exhibited *SldsxF*, and one exhibited neither (Fig. 5 *E* and *J*). Collectively, these observations suggest that male embryos can form larval morphology but die before proper melanization and sclerotization, i.e., male killing occurs at a late embryonic stage. Among the developed embryos of the MK line at 4 dpo, male embryos showed significantly higher viral RNA titers than female embryos ($P = 0.00868$ using a generalized linear mixed model with individual differences as random effects; *SI Appendix*, Fig. S10*A*). However, given that the ranges of the viral RNA levels in males and females overlapped at 4 dpo embryos, male lethality is unlikely to be caused by massive SlMKV propagation in males or the high expression of toxins [encoded by an SlMKV gene(s)] in males. Rather, we propose that males (but not females) are susceptible to SlMKV toxicity. Indeed, we found that SlMKV was toxic to male pupae; individuals transinfected at the third to fourth larval instar (G0 of the tr.NS line) successfully pupated regardless of sex, but many males then died during the pupal stage (Fig. 4 *A* and *B*). In addition, many of the surviving males emerged as deformed adults (typically with curled and/or truncated wings; Fig. 4*B*). Most of the injected male pupae died on day 13 to 20 of the pupal stage, whereas male pupae that were not injected or were injected with a homogenate of an NS female emerged on day 14 to 19 (Fig. 4*C*). In females, the timing of emergence was not affected by transinfection (Fig. 4*C*). Thus, it is likely that male killing occurred just prior to adult emergence. All dead male pupae that were injected exhibited *SldsxM* (*SI Appendix*, Fig. S11). SlMKV RNA levels in dead male pupae were not significantly different from those in emerged females ($P = 0.713$ using a generalized linear mixed model with individual differences as random effects; *SI Appendix*, Fig. S10*B*). Thus, the mechanisms of male killing that occurred in the pupae are similar to those that occur naturally in embryos in that they do not involve interference with sex determination, massive proliferation of virus in males, or male-specific expression of a viral gene. This implies that the male-specific toxin in SlMKV targets sex-specific development, including factors downstream (and/or independent) of *SldsxM* during the embryonic and pupal stages. Dosage compensation, an indispensable male-specific process during development (34, 35), may be involved in the male killing, as has been demonstrated for *Wolbachia*-induced male killing in *Ostrinia* (30, 36). We hypothesize that one (or more) of the seven ORFs possessed by SlMKV is responsible for male killing in *S. litura*. Using a BLASTp search (threshold of E-value = 10^{-10}), none of the seven ORFs were found to be homologous to any of the 27 ORFs possessed by OGVs (23) or to the male-killing gene *PVMKp1* of DbMKPV1 (21), the *Spiroplasma* male-killing gene *Spaid* (37), the *Wolbachia* male-killing gene *Oscar* for Lepidoptera (38), or the *Wolbachia* male-killing candidate gene *wmk* for *Drosophila* (39, 40). These results suggest that SlMKV has acquired a unique male-killing mechanism independently of other male killers. The convergence of male-killing ability in different viruses and bacteria suggests the advantage of male killing. For



E

	Black-spotted	Non-spotted
SldsxF	8	1
SldsxM	0	7
N.D.	0	0

J

	Hatched	Unhatched
SldsxF	8	1
SldsxM	0	6
N.D.	0	1

Fig. 5. Embryonic development of an SlMKV-infected tr.NS line. (*A*) Two types of eggs (with or without black spots) were included in the 5 dpo egg mass. (*B*) Separated eggs with (*Upper*) and without (*Lower*) black spots. (*C*) Embryos recovered from the black-spotted eggs had well-melanized head capsules (*Upper*), whereas embryos recovered from the nonspotted eggs had incompletely melanized head capsules (*Lower*). (*D*) Magnified photos of a well-melanized embryo and an incompletely melanized embryo. (*E*) Splicing pattern of *Sldsx* in embryos recovered from the black-spotted eggs and those from nonspotted eggs. (*F*) Nearly half of the eggs had hatched in the 6-dpo egg mass. (*G*) Separated unhatched eggs. (*H*) Hatched larvae (*Upper*) and embryos recovered from the unhatched eggs (*Lower*). (*I*) Magnified photos of a hatched larva and an unhatched embryo. (*J*) Splicing pattern of *Sldsx* in hatched larvae and unhatched embryos. Bars: 1 mm. ND: *Sldsx* not detected.

example, sibling cannibalism, i.e., the feeding of unhatched male eggs by hatched female larvae, increases the fitness of surviving larvae in some insect species harboring male killers (9). Although there are no records of *S. litura* larvae feeding on eggs, it is apparent that cannibalism between larvae occurs (41). This suggests that egg consumption probably occurs and contributes to the fitness of SlMKV-infected *S. litura* females.

SlMKV Is Phylogenetically Close but Structurally Dissimilar to Tolivirales. To infer the phylogenetic position of SlMKV among RNA viruses, we reconstructed a maximum likelihood tree based on the RdRp sequences of SlMKV, OGVs, DbMKPV1, and 724 species representing each genus from 18 orders of (+) ssRNA viruses and four orders of dsRNA viruses. SlMKV formed a clade with the Tolivirales (but did not fall within the Tolivirales, which included Tombusviridae and Carmotetraviridae; Fig. 6*A*). A typical Tolivirales genome is represented by a nonsegmented linear RNA genome that encodes several ORFs, whereas SlMKV comprises five RNA segments, each of which encodes one or two ORFs (Fig. 3*C*). This inconsistency raises the question of whether SlMKV should be classified as a member of Tolivirales. Notably, the clade of SlMKV and Tolivirales was phylogenetically distant from that of Durnavirales, which includes male-killing OGVs and DbMKPV1. This finding suggests that viruses from broader taxa possess male-killing ability, the adaptive trait for cytoplasmic elements. This possibility can be considered, as recent virome analyses of arthropods have revealed a large number of novel viruses from a variety of taxa (42, 43). To infer the phylogenetic position of SlMKV at a finer scale, we constructed a maximum likelihood tree using 1,206 viral RdRp amino acid sequences, including SlMKV, SlMKV-like viruses obtained by a BLASTp search (using a partial sequence of the SlMKV RdRp as the query), and exemplary species from Tolivirales and Nodamuvirales. This analysis revealed that numerous viruses are distributed around SlMKV, filling the gap between SlMKV and the Tolivirales species (Fig. 6*B*). The hosts of the viruses around SlMKV were mainly arthropods, while those of the Tolivirales were mainly plants. We suggest that in the common ancestors of SlMKV and Tolivirales, there was a host shift from arthropods to plants or plants to arthropods, which was accompanied by a drastic reorganization of the genome structure.

SlMKV Has Been Detected at Extremely Low Frequencies in *S. litura*. The frequency of SlMKV infection in *S. litura* appears to be extremely low, as only 1 of the 15 females collected in the open field produced all-female offspring in Miyazaki in 2015 (*SI Appendix*, Fig. S1*B*) and was positive for SlMKV (*SI Appendix*, Table S2). Therefore, we assume that the 45 females collected in the greenhouse were siblings produced by a single female that invaded the greenhouse and did not reflect the frequency in the field. In subsequent surveys in the same location in 2019 to 2022, only 2 of 329 females were diagnosed as positive for SlMKV (*SI Appendix*,

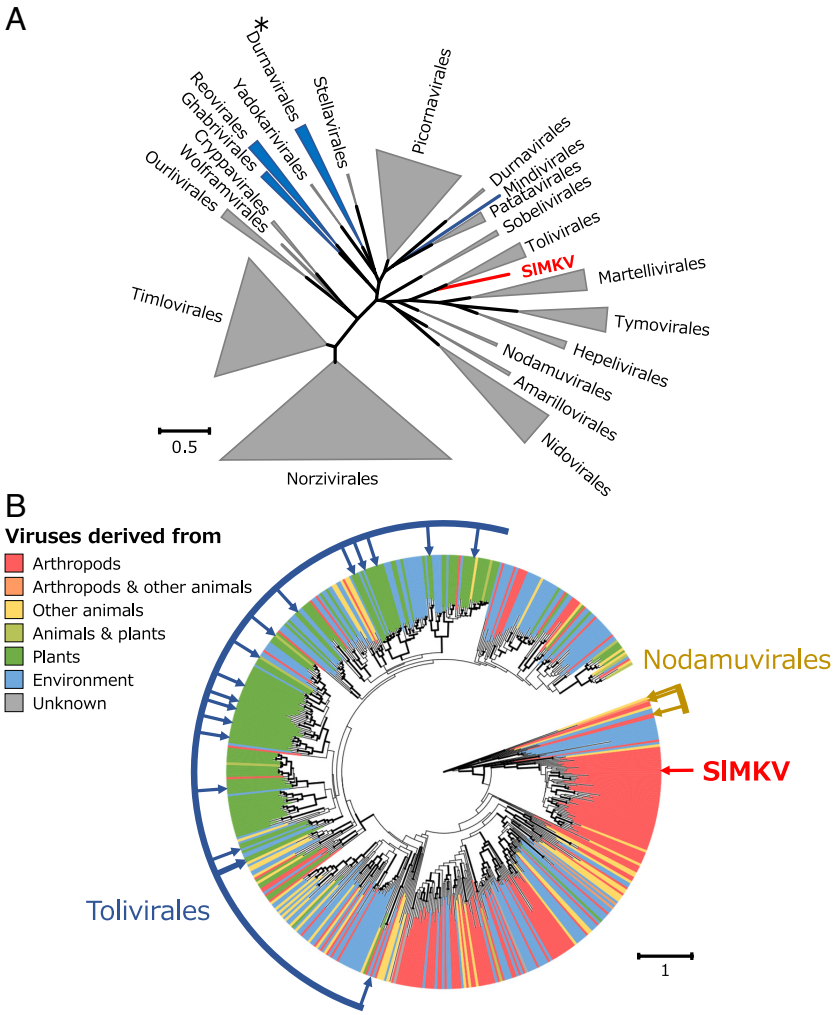


Fig. 6. RdRp-based phylogenetic position of SlMKV. (*A*) An unrooted phylogenetic tree of (+) ssRNA viruses (gray triangles) and dsRNA viruses (blue triangles). SlMKV is shown in red. *The previously published male-killing viruses, OGVs and DbMKPV1, are included in one of the Durnavirales clades. (*B*) A fine-scale phylogenetic tree of SlMKV-related viruses and Tolivirales. Nodamuvirales are used as an outgroup. Each OTU is colored according to the origin, i.e., host taxa (arthropods, plants, animals, or other taxa) or environmental samples (e.g., field soil, field water, and vertebrate feces). Blue arrow: representative species of each genus within the Tolivirales. Yellow arrow: representative species from each genus within the Nodamuvirales. Red arrow: SlMKV. Bold branch: bootstrap value higher than 0.4. The scale bars indicate the amino acid substitutions per site.

Table S2), suggesting that SlMKV is maintained at an extremely low frequency in *S. litura* populations. Notably, *S. litura* cannot overwinter in temperate areas, including the main islands of Japan, owing to cold intolerance and an inability to diapause. They migrate to Japan annually in early summer from tropical, subtropical, or temperate areas in Taiwan or the southern part of China and reproduce for a few generations until late autumn. The fact that SlMKV is susceptible to the high temperatures that are the norm in the summer in the habitats of *S. litura* raises the question of how this virus persists in this species. We speculate that SlMKV is maintained at higher frequencies in areas with constant moderate temperatures (e.g., high-altitude areas in Taiwan and southern China) and that some SlMKV-infected *S. litura* migrate to Japan annually. Searching for SlMKV-positive *S. litura* over a wide geographical area to clarify their field distribution and migration routes may answer this question.

Conclusion

We have shown that in addition to the male-killing Durnavirales (partiti-like viruses), a virus closely related to the Tolivirales can induce male killing in insects. Thus, our study indicates that male-killing viruses are found in a wide range of viral taxa. However, it is unclear how diverse male-killing viruses are and how common they are in different arthropods. When the frequency of a male killer within a population is low, it is often difficult to identify the male-killing trait by examining the sex ratio of the samples en masse. In retrospect, our discovery of the male-killing strain was enabled by limiting the sample size (probably to a single brood) in the greenhouse. Otherwise, the effect would have been undetectable owing to the presence of male larvae from uninfected broods. The establishment of numerous iso-female matrilines and/or individual-based systematic virome analyses with host gender information can indicate the frequency of potential male-killing viruses in different arthropods. Owing to its male-killing nature, SlMKV cannot be inherited paternally. However, before acquiring the ability to kill males, the viruses may have been inherited biparentally. Many heritable viruses show biparental inheritance, but generally with higher fidelity through the egg than through the sperm (44). This asymmetric inheritance may drive the accumulation of sex-specific mutations in SlMKV that are neutral or beneficial for females but deleterious for males, resulting in the evolution of male killing (8). The absence of genes in the SlMKV genome homologous to previously identified male-killing genes also supports the notion that male-killing abilities

were acquired independently by different endosymbionts. Although the molecular mechanisms of virus-induced male killing remain unclear, comparative studies on different systems will shed light on the evolution of male killing. We expect that, in addition to male killing, cytoplasmic incompatibility, feminization, and parthenogenesis may be induced by some viruses, similar to those induced by some endosymbiotic bacteria in arthropod hosts (45–47). Future studies may reveal that certain maternally inherited viruses are involved in intragenomic conflicts over sex ratios and sex determination in arthropods, as has been suggested for some endosymbiotic bacteria (48, 49).

Materials and Methods

Extended materials and methods can be found in *SI Appendix*.

Insect Culture. As NS lines of *S. litura*, we used offspring of adult females collected at Miyazaki, Japan, or those purchased from Sumika Technoservice Corp., Japan. The founders of all-female lines (G0) were collected as larvae in a greenhouse on the campus of Minami Kyushu University, Miyazaki, Japan, in early September 2015.

Molecular Sexing. Eggs and larvae of *S. litura* were sexed using reverse-transcription PCR on the basis of sex-specific splice variants of *Sldsx*. Genotyping of the Z chromosome was performed using intron sequence polymorphism of *SlTpi*.

Antibiotic Treatment. Larvae were fed with an artificial diet containing 0.06% (w/v) tetracycline hydrochloride during all larval stages for four consecutive generations.

Transinfection. Pupal or adult homogenate were centrifuged at 1,000 g for 5 min and the supernatant was passed through a 0.20-μm syringe filter. A pupal or adult filtrate was injected into the abdomen of NS females in larval, pupal, and adult stages.

Search for the Male-Killing Agent. Total RNAs extracted from the ovaries of normal-sex-ratio broods and all-female broods were subjected to RNA-seq using the TruSeq Stranded Total RNA LT Sample Prep Kit (Illumina) and Illumina Nova-seq 6000.

Northern Blotting. The RT-PCR products corresponding to RNA1–RNA5 and *SlRPS3* were used to produce double-stranded DNA probes incorporating digoxigenin-11-dUTP using DIG-High Prime (Roche). Total RNA from MK and NS females was denatured in a mixture of 50% formamide and 6% formaldehyde at 70 °C for 10 min and separated using 1% agarose-MOPS gel with 6% formaldehyde. Then, the RNA was transferred to the Amersham Hybond N+ nylon membrane (Cytiva). The membranes were baked at 120 °C for 30 min. The hybridization was performed overnight at 42 °C. The chemiluminescent signals were generated using DIG-High Prime DNA Labeling and Detection Starter Kit II (Roche).

Quantitative Real-Time PCR. To quantify the amounts of RNA1–RNA5, the copy numbers of RNA1–RNA5 were estimated by quantitative real-time PCR using a crossing-point analysis on standard curves. The relative levels of RNA1–RNA5 were calculated by the $2^{-\Delta\Delta Ct}$ method using *SlRPS3* as a housekeeping gene. All the primer sequences used in this study are shown in *SI Appendix*, Table S3.

Phylogenetic Analyses. To infer the phylogenetic position of SlMKV among (+) ssRNA and dsRNA viruses, we retrieved the 730 amino acid sequences of the RdRp region from diverse (+) ssRNA and dsRNA viruses in addition to SlMKV, DbMKPV1, and OGVs. The RdRP sequences were aligned using MAFFT, trimmed manually, as well as using TrimAl, and then used for maximum likelihood tree reconstruction using RAxML, by applying the best evolutionary model found by ModelTest-NG. To infer the phylogenetic position of SlMKV among closely related viruses, RdRp amino acid sequences of SlMKV-like viruses were obtained from the NCBI nonredundant protein database using a BLASTp search with a structural motif sequence of the SlMKV RdRp as the query. The viral RdRp sequences of SlMKV, SlMKV-like viruses were clustered into 451 OTUs by using CD-HIT. Then, they were aligned with MAFFT, trimmed manually, as well as using TrimAl, and then used for maximum likelihood tree reconstruction using RAxML, by applying the best evolutionary model found by ModelTest-NG.

Data, Materials, and Software Availability. All the RNA-seq raw data have been deposited with links to BioProject accession number PRJDB13750 (50) in the DNA Data Bank of Japan (DDBJ: https://www.ddbj.nig.ac.jp/) (51) BioProject database. The sequences of *S. litura* genes and SlMKV genomes have also been deposited in the DDBJ (LC740463–LC740470) (52–59). All the data of the figures are available as Datasets S1–S5.

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Author affiliations: [a]Department of Environmental and Horticultural Sciences, Minami Kyushu University, Miyakonojo, Miyazaki 885-0035, Japan; [b]Institute of Agrobiological Sciences, National Agriculture and Food Research Organization, Tsukuba, Ibaraki 305-0851, Japan; [c]Faculty of Agriculture, Iwate University, Morioka, Iwate 020-8550, Japan; [d]Institute of Entomology, Biology Centre of the Czech Academy of Sciences, České Budějovice 370 05, Czech Republic; and [e]Faculty of Agriculture, Setsunan University, Hirakata, Osaka 573-0101, Japan

1. W. G. Eberhard, Evolutionary consequences of intracellular organelle competition. *Q. Rev. Biol.* **55**, 231–249 (1980).
2. L. M. Cosmides, J. Tooby, Cytoplasmic inheritance and intragenomic conflict. *J. Theor. Biol.* **89**, 83–129 (1981).
3. C. D. Chase, Cytoplasmic male sterility: A window to the world of plant mitochondrial–nuclear interactions. *Trends Genet.* **23**, 81–90 (2007).
4. L. Chen, Y.-G. Liu, Male sterility and fertility restoration in crops. *Annu. Rev. Plant Biol.* **65**, 579–606 (2014).
5. P. David *et al.*, Extreme mitochondrial DNA divergence underlies genetic conflict over sex determination. *Curr. Biol.* **32**, 2325–2333.e6 (2022).
6. D. J. Clancy, G. R. Hime, A. D. Shirras, Cytoplasmic male sterility in *Drosophila melanogaster* associated with a mitochondrial CYTB variant. *Heredity* **107**, 374–376 (2011).
7. D. Kageyama, S. Narita, M. Watanabe, Insect sex determination manipulated by their endosymbionts: Incidences, mechanisms and implications. *Insects* **3**, 161–199 (2012).
8. L. D. Hurst, The incidences. Mechanisms and evolution of cytoplasmic sex ratio distorters in animals. *Biol. Rev.* **68**, 121–194 (1993).
9. G. D. D. Hurst, M. E. N. Majerus, Why do maternally inherited microorganisms kill males? *Heredity* **71**, 81–95 (1993).
10. T. Rigaud, P. Juchault, J.-P. Mocquard, The evolution of sex determination in isopod crustaceans. *BioEssays* **19**, 409–416 (1997).
11. B. Herran *et al.*, The shutting down of the insulin pathway: A developmental window for *Wolbachia* load and feminization. *Sci. Rep.* **10**, 10551 (2020).
12. I. Negri, M. Pellecchia, P. J. Mazzoglio, A. Patetta, A. Alma, Feminizing *Wolbachia* in *Zyginidia pullula* (Insecta, Hemiptera), a leafhopper with an XX/X0 sex-determination system. *Proc. Biol. Sci.* **273**, 2409–2416 (2006).
13. D. Kageyama *et al.*, Feminizing *Wolbachia* endosymbiont disrupts maternal sex chromosome inheritance in a butterfly species. *Evol. Lett.* **1**, 232–244 (2017).
14. A. M. Dunn, M. J. Hatcher, R. S. Terry, C. Tofts, Evolutionary ecology of vertically transmitted parasites: Transovarial transmission of a microsporidian sex ratio distorter in *Gammarus duebeni*. *Parasitology* **111**, S91–S109 (1995).
15. L. Doncaster, On an inherited tendency to produce purely female families in *Abraxas grossulariata*, and its relation to an abnormal chromosome number. *J. Genet.* **3**, 1–10 (1913).
16. L. Doncaster, On the relations between chromosomes, sex-limited transmission and sex-determination in *Abraxas grossulariata*. *J. Genet.* **4**, 1–12 (1914).
17. L. Doncaster, Further observations on chromosomes and sex-determination in *Abraxas grossulariata*. *Q. J. Microsc. Sci.* **66**, 397–408 (1922).
18. R. S. Terry *et al.*, Widespread vertical transmission and associated host sex-ratio distortion within the eukaryotic phylum Microspora. *Proc. Biol. Sci.* **271**, 1783–1789 (2004).
19. A. M. Dunn, R. S. Terry, J. E. Smith, Transovarial transmission in the microsporidia. *Adv. Parasitol.* **48**, 57–100 (2001).
20. D. Kageyama, K. Yoshimura, T. N. Sugimoto, T. K. Katoh, M. Watada, Maternally transmitted non-bacterial male killer in *Drosophila biauraria*. *Biol. Lett.* **13**, 20170476 (2017).
21. D. Kageyama *et al.*, A male-killing gene encoded by a symbiotic virus of *Drosophila*. *Nat. Commun.* **14**, 1357 (2023).
22. K. Nakanishi, M. Hoshino, M. Nakai, Y. Kunimi, Novel RNA sequences associated with late male killing in *Homona magnanima*. *Proc. Biol. Sci.* **275**, 1249–1254 (2008).
23. R. Fujita *et al.*, Late male-killing viruses in Homona magnanima identified as Osugoroshi viruses, novel members of *Partitiviridae*. *Front. Microbiol.* **11**, 620623 (2021).
24. J. Kranz, H. Schmutterer, W. Koch, Diseases, pests, and weeds in tropical crops. *Soil Sci.* **125**, 272 (1978).

25. EFSA Panel on Plant Health (PLH) *et al.*, Pest categorisation of *Spodoptera litura*. *EFSA J.* **17**, e05765 (2019).
26. G. D. D. Hurst, A. P. Johnson, J. H. G. v. d. Schulenburg, Y. Fuyama, Male-killing Wolbachia in Drosophila: A temperature-sensitive trait with a threshold bacterial density. *Genetics* **156**, 699–709 (2000).
27. W. Traut, F. Marec, Sex chromatin in Lepidoptera. *Q. Rev. Biol.* **71**, 239–256 (1996).
28. H. Arai *et al.*, Diverse molecular mechanisms underlying microbe-inducing male killing in the moth *homona magnanima*. *Appl. Environ. Microbiol.* **89**, e02095-22 (2023).
29. T. N. Sugimoto, Y. Ishikawa, A male-killing *Wolbachia* carries a feminizing factor and is associated with degradation of the sex-determining system of its host. *Biol. Lett.* **8**, 412–415 (2012).
30. T. Fukui *et al.*, The endosymbiotic bacterium *Wolbachia* selectively kills male hosts by targeting the masculinizing gene. *PLoS Pathog.* **11**, e1005048 (2015).
31. S. B. Levy, "Resistance to the tetracyclines" in *Antimicrobial Drug Resistance*, L. Bryan, Ed. (Academic Press, 1984), pp. 191–240.
32. J. Mistry *et al.*, Pfam: The protein families database in 2021. *Nucleic Acids Res.* **49**, D412–D419 (2021).
33. H. Jia, P. Gong, A structure–function diversity survey of the RNA-dependent RNA polymerases from the positive-strand RNA viruses. *Front. Microbiol.* **10**, 1945 (2019).
34. B. S. Baker, M. Gorman, I. Marín, Dosage compensation in *Drosophila*. *Annu. Rev. Genet.* **28**, 491–521 (1994).
35. A. Franke, A. Dernburg, G. J. Bashaw, B. S. Baker, Evidence that MSL-mediated dosage compensation in *Drosophila* begins at blastoderm. *Development* **122**, 2751–2760 (1996).
36. T. N. Sugimoto, T. Kayukawa, T. Shinoda, Y. Ishikawa, T. Tsuchida, Misdirection of dosage compensation underlies bidirectional sex-specific death in *Wolbachia*-infected *Ostrinia scapulalis*. *Insect Biochem. Mol. Biol.* **66**, 72–76 (2015).
37. T. Harumoto, B. Lemaitre, Male-killing toxin in a bacterial symbiont of *Drosophila*. *Nature* **557**, 252–255 (2018).
38. S. Katsuma *et al.*, A *Wolbachia* factor for male killing in lepidopteran insects. *Nat. Commun.* **13**, 6764 (2022).
39. J. I. Perlmutter *et al.*, The phage gene *wmk* is a candidate for male killing by a bacterial endosymbiont. *PLOS Pathog.* **15**, e1007936 (2019).
40. H. Arai *et al.*, Combined actions of bacteriophage-encoded genes in *Wolbachia*-induced male lethality. *iScience* **26**, 106842 (2023).
41. M. Ryuda, H. Nakayama, Y. Hayakawa, A novel gene associated with intraspecific predation in *Spodoptera litura* larvae. *J. Appl. Entomol.* **43**, 563–568 (2008).
42. M. Shi *et al.*, Redefining the invertebrate RNA virosphere. *Nature* **540**, 539–543 (2016).
43. Y.-Z. Zhang, Y.-M. Chen, W. Wang, X.-C. Qin, E. C. Holmes, Expanding the RNA virosphere by unbiased metagenomics. *Annu. Rev. Virol.* **6**, 119–139 (2019).
44. M. F. Camus, B. Alexander-Lawrie, J. Sharbrough, G. D. D. Hurst, Inheritance through the cytoplasm. *Heredity* **129**, 31–43 (2022).
45. J. H. Werren, L. Baldo, M. E. Clark, *Wolbachia*: Master manipulators of invertebrate biology. *Nat. Rev. Microbiol.* **6**, 741–751 (2008).
46. L. C. Rosenwald, M. I. Sitvarin, J. A. White, Endosymbiotic *Rickettsiella* causes cytoplasmic incompatibility in a spider host. *Proc. Biol. Sci.* **287**, 20201107 (2020).
47. M. Pollmann *et al.*, Highly transmissible cytoplasmic incompatibility by the extracellular insect symbiont *Spiroplasma*. *iScience* **25**, 104335 (2022).
48. J. H. Werren, L. W. Beukeboom, Sex determination, sex ratios, and genetic conflict. *Annu. Rev. Ecol. Syst.* **29**, 233–261 (1998).
49. E. A. Hornett, D. Kageyama, G. D. D. Hurst, Sex determination systems as the interface between male-killing bacteria and their hosts. *Proc. Biol. Sci.* **289**, 20212781 (2022).
50. K. Nagamine, D. Kageyama, Y. Shintani, Searching male killing agent from /Spodoptera litura/. DDBJ/EMBL/GenBank. https://ddbj.nig.ac.jp/resource/bioproject/PRJDB13750. Deposited 11 June 2022.
51. A. Fukuda, Y. Kodama, J. Mashima, T. Fujisawa, O. Ogasawara, DDBJ update: streamlining submission and access of human data. *Nucleic Acids Res.* **49**, D71–D75 (2021).
52. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura mRNA for ribosomal protein S3, complete cds. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740463. Deposited 22 November 2022.
53. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura mRNA for protein doublesex, partial cds. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740464. Deposited 22 November 2022.
54. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura mRNA for protein doublesex, partial cds. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740465. Deposited 22 November 2022.
55. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura male-killing virus RNA, segment 3, complete sequence. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740466. Deposited 22 November 2022.
56. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura male-killing virus RNA, segment 2, complete sequence. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740467. Deposited 22 November 2022.
57. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura male-killing virus RNA, segment 1, complete sequence. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740468. Deposited 22 November 2022.
58. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura male-killing virus RNA, segment 4, complete sequence. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740469. Deposited 22 November 2022.
59. K. Nagamine, D. Kageyama, Y. Shintani, Spodoptera litura male-killing virus RNA, segment 5, complete sequence. DDBJ/EMBL/GenBank. https://www.ncbi.nlm.nih.gov/nuccore/LC740470. Deposited 22 November 2022.