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Wolbachia causes cytoplasmic incompatibility but not male-killing in a grain pest beetle

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Abstract

The endosymbiotic *Wolbachia* is one of the most common intracellular bacteria known in arthropods and nematodes. Its ability for reproductive manipulation can cause unequal inheritance to male and female offspring, allowing the manipulator to spread, but potentially also impact the evolutionary dynamics of infected hosts. Estimated to be present in up to 66% of insect species, little is known about the phenotypic impact of *Wolbachia* within the order Coleoptera. Here, we describe the reproductive manipulation by the *Wolbachia* strain *w*Sur harboured by the sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera, Silvanidae), through a combination of genomics approaches and bioassays. The *Wolbachia* strain *w*Sur belongs to supergroup B that contains well-described reproductive manipulators of insects and encodes a pair of cytoplasmic incompatibility factor (*cif*) genes, as well as multiple homologues of the WO-mediated killing (*wmk*) gene. A phylogenetic comparison with *wmk* homologues of *w*Mel of *Drosophila melanogaster* identified 18 *wmk* copies in *w*Sur, including one that is closely related to the *w*Mel male-killing homologue. However, further analysis of this particular *wmk* gene revealed an eight-nucleotide deletion leading to a stop-codon and subsequent reading frame shift midsequence, probably rendering it nonfunctional. Concordantly, utilizing a *Wolbachia*-deprived *O. surinamensis* population and controlled mating pairs of *w*Sur-infected and noninfected partners, we found no experimental evidence for male-killing. However, a significant ~50% reduction of hatching rates in hybrid crosses of uninfected females with infected males indicates that *w*Sur is causing cytoplasmic incompatibility. Thus, *Wolbachia* also represents an important determinant of host fitness in Coleoptera.

KEYWORDS
cytoplasmic incompatibility, male-killing, *Oryzaephilus surinamensis*, sawtoothed grain beetle, symbiosis, *Wolbachia*

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1 | INTRODUCTION

Symbiotic bacteria influence the ecology and evolution of animals in various ways (Douglas, 2015; McFall-Ngai et al., 2013). Insects harbour an especially high abundance and diversity of microbial associations that span the entire range from parasitism to mutualism (Drew et al., 2021). While some symbionts exhibit a very strict phenotype, others incur context-dependent impacts along the parasite–mutualist continuum including host fitness benefits or costs (Feldhaar, 2011; Oliver & Martinez, 2014; Zytynska et al., 2021). However, a large proportion of insects are also infected by multiple symbionts that each on their own exhibit different, stable phenotypes, such as nutritional supplementation and reproductive manipulation, but could interfere with each other (Duron et al., 2008). In consequence, host ecology and evolution can be driven by multiple symbionts with possibly different selective interests.

Wolbachia bacteria (α-Proteobacteria) are some of the most common intracellular bacteria known in arthropods and nematodes (Werren et al., 2008). They are predominantly parasitic and transmitted maternally between host generations, but horizontal transmission occurs occasionally. *Wolbachia* employ several distinct strategies to maximize their transmission by influencing the germ line of their host. Thereby, they can rapidly sweep through uninfected populations and then maintain a high prevalence within a population. These mechanisms include cytoplasmic incompatibility (CI), parthenogenesis, male-killing or feminization (Werren et al., 2008). While CI leads directly to a higher proportion of infected individuals, the other mechanisms lead to a higher proportion of female individuals in the population. This in turn increases the fitness of *Wolbachia*, which is predominantly transmitted maternally (Heath et al., 1999). However, *Wolbachia* infection does not necessarily result in reproductive manipulation with negative fitness consequences for the host (Zug & Hammerstein, 2015). Furthermore, *Wolbachia* can even evolve into a mutualist and enhance its host's fitness by supplementing dietary-limited nutrients, such as B-vitamins like riboflavin (Hosokawa et al., 2010; Ju et al., 2019; Moriyama et al., 2015).

CI and male-killing are the predominant strategies of reproductive manipulation in insects (Fialho & Stevens, 2000; Perlmutter et al., 2020). CI generally refers to factors localized in the cytoplasm of sperm and eggs that render them incompatible with each other, resulting in inviable embryos (Beckmann et al., 2019; Shropshire et al., 2019; Shropshire & Bordenstein, 2019). *Wolbachia* causes CI by expressing a "killing" factor in the male sperm. In eggs of uninfected females, this modification leads to nonviable embryos, whereas in infected females a "rescue" factor reverses this modification so that the zygote can develop normally (Shropshire, 2020). While unidirectional CI occurs when infected males mate with uninfected females resulting in fertilized but unviable eggs, bidirectional CI occurs when two individuals are infected by different, yet incompatible *Wolbachia* strains (Werren et al., 2008). Recently, two cytoplasmic incompatibility factor genes (*cifA* and *cifB*) have been identified as key factors in CI-inducing *Wolbachia* strains (LePage et al., 2017). The pair of CI-inducing genes were not found in the chromosomal *Wolbachia* genes, but in the integrated eukaryotic association module of phageWO (LePage et al., 2017). A two-by-one genetic model has been suggested, specifying that while both *cifA* and *cifB* induce CI, only *cifA* is able to rescue the CI phenotype when transgenically expressed in the host's ovaries (Shropshire et al., 2018; Shropshire et al., 2019).

The other widespread phenotype of *Wolbachia* inducing reproductive manipulation is male-killing. During embryogenesis, the development of the male embryo is disturbed by *Wolbachia*, leading to embryonic lethality (Werren et al., 2008). In consequence, the fitness of infected sister embryos is enhanced by higher allocation of resources during ovogenesis and reduced intraspecific competition during juvenile development and adult life (Hurst & Jiggins, 2000; Jaenike et al., 2003). The gene WO-mediated killing (*wmk*) of the *Wolbachia* strain *w*Mel of the fruit fly *Drosophila melanogaster* has been identified to recapitulate this male-killing phenotype when transgenically expressed in *D. melanogaster* flies (Perlmutter et al., 2019). So far, *wmk* homologues have been found in all *Wolbachia* strains associated with male-killing, surprisingly also localized within the eukaryotic association module of phageWO, only a few genes upstream from the CI-inducing genes *cifA* and *cifB* (Perlmutter et al., 2019). There are at least five homologues of the *wmk* gene encoded in the genome of *w*Mel and the function of many of these remain enigmatic as only the transgenic expression of the original *wmk* gene, but not other homologues, caused male-killing (Perlmutter et al., 2020). *Wolbachia* strains causing CI and male-killing phenotypes have been well studied within the insect orders Diptera and Hymenoptera, such as the fruit fly *D. melanogaster* (Perlmutter et al., 2020), the southern house mosquito *Culex quinquefasciatus* (Duron et al., 2005) and the parasitoid wasp *Nasonia vitripennis* (Bordenstein & Werren, 1998, 2007). Although beetles infected with *Wolbachia* have repeatedly been reported in recent years, little is known about the functional consequences of *Wolbachia* infections within the order Coleoptera (Aikawa et al., 2022; Fialho & Stevens, 2000; Heddi et al., 1999; Kajtoch & Kotásková, 2018; Li et al., 2015; Li et al., 2016).

The sawtoothed grain beetle *Oryzaephilus surinamensis* (Coleoptera, Silvanidae) is a worldwide distributed pest of cereals and other stored food (Boyer et al., 2012). It is associated with the bacteriome-localized Bacteroidota endosymbiont *Candidatus* Shikimatogenerans silvanidophilus OSUR (hereafter called *Shikimatogenerans silvanidophilus*; Engl et al., 2018; Hirota et al., 2017; Kiefer et al., 2021; Koch, 1931). The endosymbiont *S. silvanidophilus* provides aromatic amino acid precursors for cuticle synthesis of the host via the shikimate pathway (Kiefer et al., 2021). In addition, *O. surinamensis* is commonly infected with *Wolbachia* (Li et al., 2015; Sharaf et al., 2010). Sharaf et al. (2010) identified a higher *Wolbachia* infection rate in feral populations of *O. surinamensis* compared to adapted silo populations, but also a strong female bias among adults emerging under laboratory conditions, suggesting active reproductive manipulation by these *Wolbachia* strains. Elucidating *Wolbachia*'s capabilities of reproductive manipulation in *O. surinamensis* is therefore relevant in understanding the biology of this agricultural pest as well as a symbiotic model insect.

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In this work, we localized *Wolbachia* in the *O. surinamensis* JKI strain and quantified its growth dynamics across developmental stages. A phylogenetic analysis and functional prediction of the associated *Wolbachia* *w*Sur genome revealed it to be a member of supergroup B, presumably capable of CI as it encodes homologues of the cytoplasmic incompatibility factor genes *cifA* and *cifB*. However, the strain is incapable of inducing male-killing, possibly due to an eight-nucleotide deletion in the identified male-killing gene *wmk* creating a stop codon as well as subsequent reading frame-shift. Finally, we experimentally tested the predicted phenotype of reproductive manipulation—unidirectional CI and no male-killing—using mating assays of beetles with manipulated infection status, where we were able to verify the phenotype of reproductive manipulation via unidirectional CI.

2 | MATERIAL AND METHODS

2.1 | Insect cultures

The initial *Oryzaephilus surinamensis* culture (strain JKI) was obtained from the Julius-Kühn-Institute/Federal Research Centre for Cultivated Plants in 2014 and kept in culture since then. Continuous symbiotic and aposymbiotic (by aposymbiotic we refer in this paper to beetles without both *S. silvanidophilus* and *w*Sur symbionts). *O. surinamensis* cultures (see below) were maintained in 1.8-L plastic containers, filled with 50 g oat flakes, at 28°C, 60% relative humidity and a day and night cycle of 16/8 h.

2.2 | Elimination of *O. surinamensis* symbionts

An *O. surinamensis* sub-population was treated for 12 weeks with tetracycline (150 mg/5 g oat flakes, see for details see Engl et al. (2018)) to eliminate both of their symbionts (*S. silvanidophilus* and *w*Sur) and then kept for several generations on a normal diet to exclude direct effects of tetracycline on the host physiology. A control group was established in parallel with all steps except the addition of tetracycline to account for any unforeseen effects of the handling, population bottlenecks, etc. The apo−/symbiotic status regarding both symbionts of these beetle sub-populations was confirmed before each following experiment. Therefore, female adult beetles were individually separated in single jars with oat flakes to lay eggs. After 4 weeks, the adult generation was removed before their offspring finished metamorphosis, DNA of these parent females was extracted and the symbiont titre was analysed by quantitative PCR (polymerase chain reaction; see below).

2.3 | Quantitative PCR

Absolute titres of *S. silvanidophilus* and *w*Sur during host development and after different treatments from previous publications (Engl et al., 2018, 2020; Kiefer et al., 2021) were determined via quantitative PCR (qPCR) amplifying respective single-copy 16 S rRNA gene fragments. DNA was extracted from individual beetles using the Epicentre MasterPure™ Complete DNA and RNA Purification Kit (Lucigen) and dissolved in 30 µl low TE buffer (1 mM Tris–HCl + 0.1 mM EDTA). qPCRs were carried out in 25-µl reactions using EvaGreen (Solis BioDyne), including 0.5 µM of each primer and 1 µl template DNA. All reagents were mixed, vortexed and centrifugated in 0.1-ml reaction tubes (Biozym). The *Wolbachia*-specific 16 S rRNA gene fragment was amplified with the primers Wolb_16S_qPCR_fwd (5′-TTGCTATTAGATGAGCCTATATTAG-3′) and Wolb_16s_qPCR_rev (5′-GTGTGGCTGATCATCCTCT-3′; Makepeace et al., 2006), and the 16 S rRNA of *S. silvanidophilus* OSUR was amplified with the primers OsurSym_fwd2 (5′-GGCAACTCTGAACTAGCTACGC-3′) and mod. CFB563_rev (5′-GCACCCTTTAAACCCAAT-3′) (Engl et al., 2018; Kiefer et al., 2021). qPCR was carried out on a Rotor-Gene Q thermal cycler (Qiagen). The initial temperature was 95°C for 12 min, followed by 60 cycles of 95°C for 40 s followed by 20 s at 60°C. A melting curve analysis was used to assess the specificity of the qPCR reaction by a gradual increase of temperature from 60 to 95°C, with 0.25°C/s. The qPCR results were analysed using the Rotor Gene Q Software (Qiagen).

Standard curves with defined copy numbers of the 16 S rRNA gene were created by amplifying the fragment first via PCR using the previously mentioned primers, followed by purification via an innuPREP PCRpure (Analytik Jena) and determination of the DNA concentration via a NanoDrop1000 (Peqlab). After determination of the DNA concentration, a standard containing 10^{10} copies/µl was generated and 1:10 serial dilutions down to 10^{1} copies/µl were prepared. One microlitre of each standard was included in a qPCR to standardize all measurements.

2.4 | Fluorescence in situ hybridization

Wolbachia was localized in *O. surinamensis* tissues by fluorescence in situ hybridization (FISH) on semithin sections of adult beetles. Therefore, 5-day-old pupae and maximum 2-week-old adult beetles were fixed in tertiary butanol (80%; Roth), paraformaldehyde (37%–40%; Roth) and glacial acetic acid (Sigma-Aldrich) in proportions 6:3:1 for 2 h, followed by post-fixation in alcoholic formaldehyde paraformaldehyde (37%–40%) and tertiary butanol (80% at a proportion of 1:2). After dehydration, the specimens were embedded in Technovit 8100 (Kulzer; Weiss & Kaltenpoth, 2016) and cut into 8-µm sagittal sections using a Leica HistoCore AUTOCUT R microtome (Leica) equipped with glass knives. The obtained sections were mounted on silanized glass slides. Each slide was covered with 100 µl of hybridization mix, consisting of hybridization buffer (0.9 M NaCl, 0.02 M Tris/HCl pH 8.0, 0.01% SDS; Roth) and 0.5 µM of the modified Bacteroidota probe CFB563 (5′-GCACCCTTTAAACCCAAT-3′; Engl et al., 2018; Weller et al., 2000) or the "Eubacteria" probe EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′; Amann et al., 1990) labelled with Cy3, as well as the two *Wolbachia*-specific probes Wolb_W2

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(5′-CTTCTGTGAGTACCGTCATTATC-3′; Heddi et al., 1999) and Wolbachia-Wol3 (5′-TCCTCTATCCTCTTTCAATC-3′; Sanguin et al., 2006) labelled with Cy5. DAPI (0.5 µg/ml) was included as a general counterstain for DNA. Slides were covered with glass cover slips and incubated in a humid chamber at 50°C overnight. After washing and incubating them for 2 h at 50°C in wash buffer (0.1 M NaCl, 0.02 M Tris/HCl, 5 mM EDTA, 0.01% SDS), they were washed in deionized water for 20 min and mounted with Vectashield (Vector Laboratories). The sections were either observed under a Zeiss AxioImager Z2 with Apotome.2 (Zeiss) illuminated by a SOLA Light Engine (Lumencor), or a Leica THUNDER imager Cell Culture 3D (Leica). Images obtained on the Leica microscope were processed with the instant and small volume computational clearing algorithm using standard settings in the Leica Application Suite X software (Leica).

2.5 | Symbiont genome sequencing, assembly and annotation

We combined short- and long-read sequencing technologies to assemble the metagenome of *O. surinamensis* and associated microorganisms. Total DNA for both approaches was isolated from 20 pooled adult abdominal (without wings) tissue of *O. surinamensis* JKI using the Epicentre MasterPureTM Complete DNA and RNA Purification Kit (Illumina) including RNase digestion. Short-read library preparation and sequencing were performed at the Max Planck Genome Centre (SRR12881563–SRR12881566) on a HiSeq2500 Sequencing System (Illumina). Long-read sequencing (SRR12881567–SRR12881568) was performed on a MinION Mk1B Sequencing System (Oxford Nanopore Technologies [ONT]). Detailed methods are described in Kiefer et al. (2021).

Hybrid assembly of MinION and Illumina reads was performed using SPADES (version 3.13.0; Bankevich et al., 2015) with default settings. The resulting contigs were then binned using BUSYBEE WEB (Laczny et al., 2017) and screened for taxonomic identity to α-proteobacteria. The single resulting circular *Wolbachia* contig was extracted, which was then automatically annotated with RAST (Overbeek et al., 2014) using the app *Annotate Microbial Assembly* (RAST_SDK version 0.1.1) on KBase (Arkin et al., 2018). The annotated contig was curated manually and plotted using CIRCOS (version 0.69–6; Krzywinski et al., 2009) for the visualization of gene locations, GC content and coverage. Additionally, the completeness of the obtained genome was assessed with the app *Assess Genome Quality with* CHECKM version 1.0.18 in KBase (Arkin et al., 2018).

2.6 | Phylogeny and comparative genomics of *Wolbachia* strains

A phylogenetic tree for placement of the *Wolbachia* strain of *O. surinamensis* was reconstructed using the KBase app insert set of genomes into species tree version 2.1.10 (SPECIESTREEBUILDER version 0.0.12; Arkin et al., 2018) based on the FASTTREE2 algorithm (Price et al., 2010), including 49 highly conserved clusters of orthologous groups (COG) genes. Therefore, 74 additional publicly available and published genomes of *Wolbachia* endosymbionts were obtained from NCBI (https://www.ncbi.nlm.nih.gov/assembly). The resulting tree was visualized using FIGTREE (version 1.4.4, http://tree.bio.ed.ac.uk/software/figtree/)

2.7 | Identifying genes important for reproductive manipulation

The obtained genome was manually searched for *wmk*, *cifA* and *cifB* genes. For the *wmk* gene, coding sequences (CDSs) annotated as "Transcriptional regulator" were extracted and identified as *wmk* homologues by a BLASTN search of NCBI's nucleotide collection (nr/nt). The nucleotide sequence of all 18 *wmk* homologues of *w*Sur and five phenotypically described *wmk* homologues of *w*Mel (WD0255, WD0508, WD0622, WD0623, WD0626 [*wmk*]; Perlmutter et al., 2020), *w*Bor (MK873001-3), *w*Bif (MK873005), *w*Inn (MK873080-2), *w*No (WP015587820) and *w*VitB (WP010405531) were aligned using MUSCLE (Edgar, 2004) in GENEIOUS PRIME 2019 (version 2019.1.3; https://www.geneious.com). In addition, we reanalysed a set of sequencing libraries from *O. surinamensis* sampled in a grain storage facility and two field sites in Israel (SRX2583549–SRX2583574) (Hong et al., 2020). We assembled reads following the workflow of our own data set and extracted *wmk* homologues from *Wolbachia* contigs by searching for genes annotated as "Transcriptional regulator" as well as mapping *wmk* homologues from the JKI *w*Sur strain against the assemblies and vice versa. All *wmk* homologues from the first analysis were combined with *wmk1* and *wmk12-like* homologues from all these strains and aligned as mentioned above.

Phylogenetic reconstructions of the nucleotide alignment were performed with the MrBayes-plugin (Huelsenbeck & Ronquist, 2001) of GENEIOUS PRIME using the HKY85 substitution model and inv-gamma rate variation as recommended by JMODELTEST 2.1.10 version 20,160,303 (Sullivan et al., 2012). The analysis ran for 1,100,000 generations, with a burn-in of 100,000 generations and trees sampled every 200 generations until the likelihood values stabilized. Protein domains were identified and annotated by running the protein sequences from the NCBI database through SMART (Simple Modular Architecture Research Tool; http://smart.embl-heidelberg.de/).

Additionally, the annotated genome of *w*Sur was manually checked for *cif* genes. The *cif* genes were identified by whole-genome alignment to the genome of *w*Pip and translation alignment with the annotated genes of *w*No (WNO_RS01055/WNO_RS01050) and *w*Meg (CAI20_01650/CAI20_01645). To identify whether the *cif* genes belonged to the same type, we performed a phylogenetic analysis following Lindsey et al. (2018) and Ün et al. (2021). Briefly, the nucleotide sequences were aligned based on translation into a protein sequence and then back-translated into nucleotide sequences as

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implemented in GENEIOUS PRIME 2019 ("translation alignment," version 2019.1.3; https://www.geneious.com). Phylogenetic reconstruction of the alignment was performed with the MRBAYES-plugin (Huelsenbeck & Ronquist, 2001) of GENEIOUS PRIME using the GTR substitution model and gamma rate variation as predicted by JMODELTEST 2.1.10 v20160303 (Sullivan et al., 2012) using the same parameters as above. According to Ün et al. (2021), potential protein domains of the Cif genes were searched using HHPRED's version 3.2.0 web server (https://toolkit.tuebingen.mpg.de/tools/hhpred; Zimmermann et al., 2018) with default parameters and the following databases: SCOPe70 version 2.07, COG/KOG version 1.0, Pfam-A version 32.0 and SMART version 6.0 (Ün et al., 2021). The seven phage WO regions in the *w*Sur genome were compared and visualized using CLINKER (Gilchrist & Chooi, 2021).

2.8 | Bioassays for reproductive manipulation

By mating experiments with differentially infected individuals, we tested whether *Wolbachia w*Sur is causing reproductive manipulation in *O. surinamensis*. To ensure the virginity of the female and male individuals and prevent unwanted crossbreeding, pupae, and 5th instar larvae of aposymbiotic (*S. silvanidophilus* and *w*Sur uninfected) and symbiotic (*S. silvanidophilus* and *w*Sur infected) *O. surinamensis* were isolated into 24-well TC plates (Sarstedt AG), closed with Adhesive Foil (Kisker-Biotech) with several needle punctures to allow for air exchange and maintained under general rearing conditions (see above). The isolated individuals were observed until hatching, and the sex of the individual insect was determined by the presence (males) or absence (females) of spikes on the third femur (Halstead, 1963). Males and females were combined into mating pairs at an age of 7–10 days. In total, 30 mating pairs were prepared, 10 for each group: The first group consisted of mating pairs where both partners, female and male, were aposymbiotic, whereas the second group was made up of crossings with two symbiotic partners. The third group contained symbiotic males of *O. surinamensis* paired with aposymbiotic females. The mating pairs were given one microspatula scoop of ground oat previously filtered through a 0.6-mm sieve. Furthermore, to prevent the specimens escaping the setup, the edge of each individual well was coated with a polytetrafluoroethylene 60 wt% dispersion in H_2O (PTFE-dispersion; Sigma-Aldrich). For the first 2 weeks of the experiment all pairs were left undisturbed. In the following 6-week period, the number of laid eggs and hatched larvae were counted twice weekly, and the adults were placed one well further down in the 24-well plate. In addition, we quantified the sex ratio of 100 randomly picked individuals in both symbiotic and aposymbiotic stock cultures to test for a sex bias induced by male-killing.

2.9 | Statistical procedure for qPCR results and differences in hatching rate and sex ratio

The influence of glyphosate and tetracycline on the symbiont titre of the adult beetles (Figure S1) was analysed using Dunn's test from the package "FSA" in RSTUDIO (R version 4.1.1) with two-sided post hoc tests corrected for multiple testing using the Benjamini–Hochberg (BH) method (Benjamini & Hochberg, 1995; Dunn, 1964). A compact letter display (CLD; Piepho & Piepho, 2009) was generated with the package "rcompanion" (Mangiafico, 2017). Comparison between hatching rates was performed with Wilcoxon rank sum tests including correction for false discovery rates (FDRs) by repeated testing following the BH procedure (Benjamini & Hochberg, 1995), implemented in the R package "stats." Plots were visualized using "ggplot2" (Wickham, 2016). Sex ratio in beetle cultures was analysed using a manually calculated χ^2 test of homogeneity.

3 | RESULTS

3.1 | Localization and infection dynamics in *Oryzaephilus surinamensis*

qPCR quantification of *Wolbachia* titres in 106 control samples of multiple experiments indicated a *w*Sur prevalence of 100% within laboratory cultures of the *O. surinamensis* JKI strain. Based on FISH, *Wolbachia* is localized throughout the entire body of *O. surinamensis* (Figure 1a). *Wolbachia*-induced CI has been linked to sperm modification during spermatogenesis (Veneti et al., 2003), but *Wolbachia* must also be present in the female reproductive tissues for successful transmission. A close inspection of the reproductive organs of female and male *O. surinamensis* confirmed a high abundance of *Wolbachia* in both testes and ovaries (Figure 1b,c).

Further, we compared infection titres of the two bacterial endosymbionts in *O. surinamensis* during all life stages of *O. surinamensis* via qPCR (Figure 2). The population of *Wolbachia* reached its maximum as early as in the pupa during early metamorphosis (early pupa: 5.9×10^6 median copies; late pupae: 3.9×10^6 median copies; Figure 2, left), while we observed in the same sample set a peak of *S. silvanidophilus* only within the first week after metamorphosis (male 6.7×10^7 median copies and female 6.7×10^7 median copies; Figure 2, right).

After our findings on *S. silvanidophilus* conferring enhanced cuticle synthesis and higher fitness under biotic and abiotic stresses (Engl et al., 2018, 2020; Kiefer et al., 2021), we assessed whether *Wolbachia* could have contributed to the previously reported cuticular phenotypes in *O. surinamensis*. Therefore, we also quantified *w*Sur titres (in addition to *S. silvanidophilus*) in *O. surinamensis* samples from different previous treatments (Kiefer et al., 2021, Figure S1). While strict tetracycline treatment eliminated both *S. silvanidophilus* (Engl et al., 2018; Kiefer et al., 2021) and *w*Sur (Kruskal–Wallis $\chi^2 = 52.605$, $df = 7$, $p = .000000004437$, Dunn's test: $p < .05$; Figure S1), resulting in dual aposymbiotic (hereafter aposymbiotic) beetles, the herbicide glyphosate had a differential effect: *S. silvanidophilus* was drastically reduced, yet still present in low amounts while *w*Sur was not negatively affected (Kruskal–Wallis $\chi^2 = 52.605$, $df = 7$, $p = .000000004437$, but Dunn's test: $p > .05$; see Table S1 for pairwise comparisons; Figure S1).

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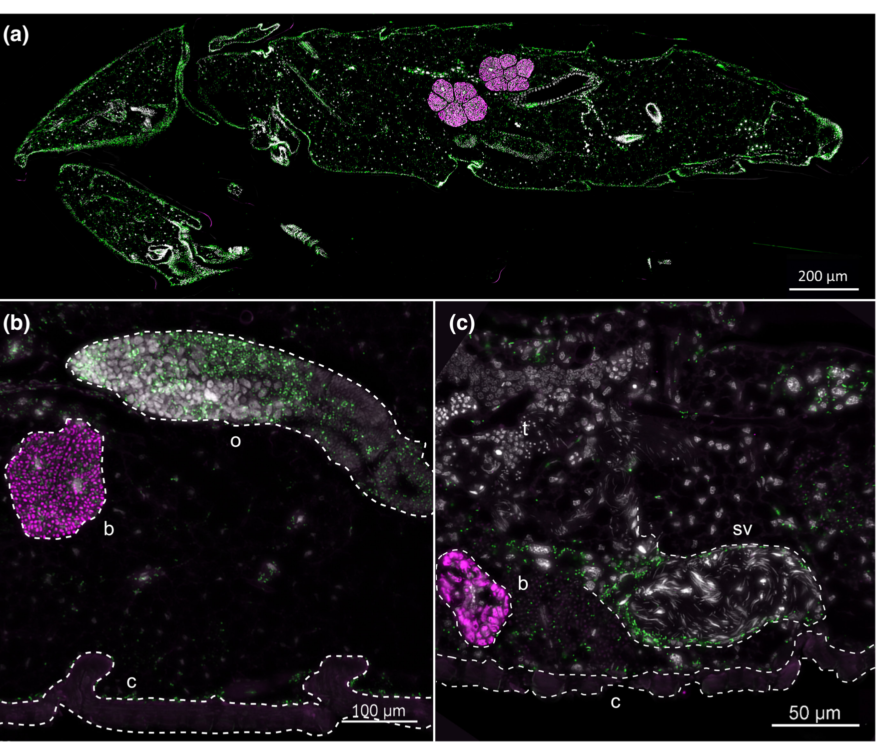

FIGURE 1 Fluorescence in situ hybridization micrographs of *Wolbachia* (green) and *Shikimatogenerans* (magenta) in sagittal sections of (a) a 5-day-old *Oryzaephilus surinamensis* pupa stained with a Bacteroidota-specific probe highlighting *Shikimatogenerans silvanidophilus* (CFB563mod-Cy3, magenta), and in the gonads of (b) an adult female and (c) an adult male stained with a eubacteria-specific probe highlighting *S. silvanidophilus* (EUB338-Cy3, magenta), *Wolbachia*-specific probes (Wol-W3-Cy5 and Wolb-2-Cy5, green) and DAPI targeting DNA in general (white). b = bacteriomes, c = cuticle, o = ovariole, t = testes, sv = seminal vesicle. Image (a) was originally published without the *Wolbachia* channel in Kiefer et al. (2021).

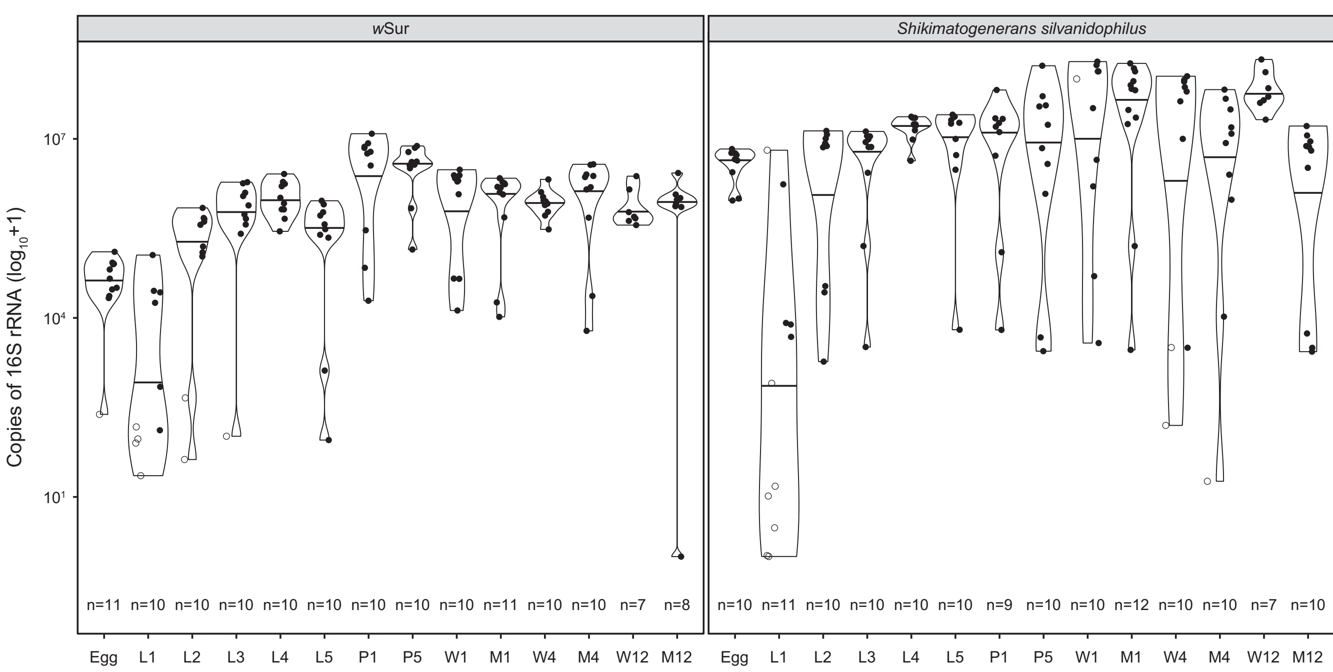

FIGURE 2 Symbiont titres in different life stages of *O. surinamensis* from the JKI stock line. Titres of *Wolbachia* *w*Sur (left) and *S. silvanidophilus* (right) were measured as 16S rDNA copies by quantitative PCR in single individuals. Juvenile life stages (eggs, larvae and pupae) contained mixed sexes, adults were separated by sex. Larvae stages 1–5 (L); 1- and 5-day-old pupae (P1 and P5); female adults 1, 4 and 12 weeks (W1–12) and male adults 1, 4 and 12 weeks (M1–12) after metamorphosis. The data distribution is visualized with violin plots and an additional horizontal line depicting the mean. The scales of the vertical axes are logarithmic. Filled circles represent specific target amplification, and empty circles off-target amplification during late qPCR cycles, identified by melting curve analysis.

3.2 | Genomics and phylogeny of the *Wolbachia* strain

We previously sequenced the metagenome of *O. surinamensis* combining short- and long-read technologies (Illumina and ONT) into a hybrid assembly. Besides the Bacteroidota endosymbiont *S. silvanidophilus* (Kiefer et al., 2021) we also extracted the full genome of a *Wolbachia* strain in a single, circular contig in the assembly (Figure 3). The circular genome is 1,728,764 bp in length with an average GC content of 34.1% and a coverage of 186× with short-read sequences

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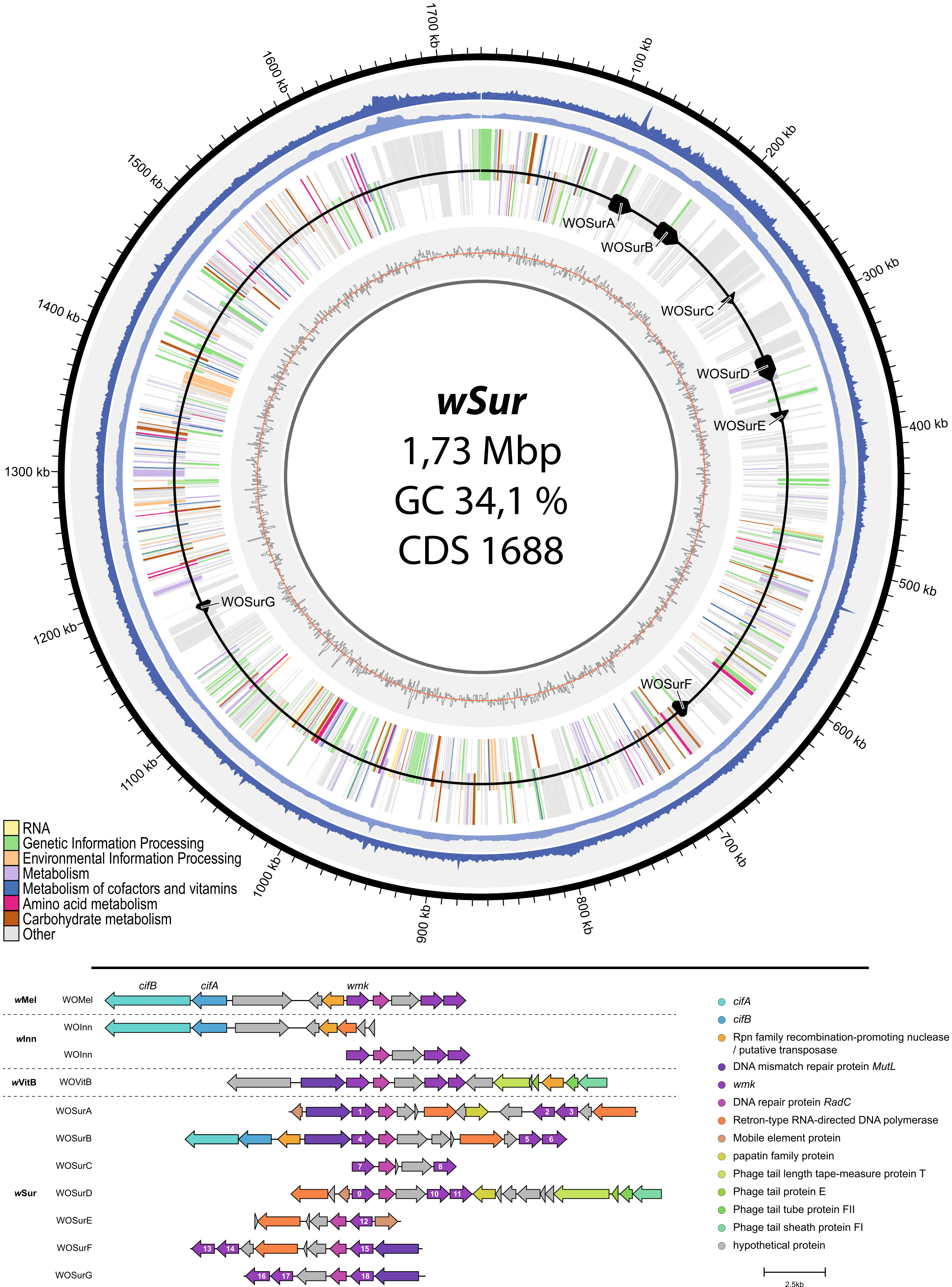

FIGURE 3 Legend on next page

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FIGURE 3 Top: Circular representation of the genome of *Wolbachia w*Sur. The outer blue circles denote coverage with short and long reads, respectively (dark blue: Illumina, light blue: ONT), and intermediate circles indicate open reading frames with KEGG functional annotations separated by the direction of transcription (see key for depicted categories). The inner grey circle denotes relative GC content and the average GC content of 34.1% indicated by the red line. Phage WO modules are highlighted in black. Bottom: Comparison of the prophage WO modules in *w*Sur and the well-studied strains of *Wolbachia w*Mel, *w*Inn and *w*VitB. Prophage WO gene regions containing *wmk*, *wmk-like* homologues, and CI genes *cifA* and *cifB* are listed by *Wolbachia* strain in bold and then the corresponding prophage module. At least one *wmk* homologue is associated with each *Wolbachia*-inducing male-killing strain.

and 94× with long-read sequences. The phylogenetic reconstruction based on 49 conserved COG genes classified *w*Sur as a member of supergroup B, closely related to the *Wolbachia* endosymbiont *w*Ecas of the common brassy ringlet *Erebia cassioides*, but also within a clade with *w*Pip of the southern house mosquito *Culex quinquefasciatus* and *w*VitB of the parasitoid wasp *Nasonia vitripennis* (Figure 4).

The genome of the *Wolbachia* strain *w*Sur of *O. surinamensis* coded for 1688 protein-coding sequences, 34 tRNAs and 50 ribosomal proteins (20 small and 30 large subunit proteins, Table 1). Besides general genetic information processing including DNA replication and repair, transcription, and translation, the genome also contained a full glycolysis pathway to process glucose-6-phosphate to erythrose 4-phosphate (E4P) and phosphoenolpyruvate (PEP). Further, it contained a full riboflavin pathway and the pathways to synthesize the amino acids lysine, glutamine, threonine, glycine, and serine but no single gene of the shikimate pathway to synthesize aromatic amino acids, explaining its insensitivity to glyphosate (Fischer et al., 1986; Gresshoff, 1979; Steinrücken & Amrhein, 1980).

3.3 | Analysis of male-killing gene candidates

The genome of *w*Sur contained seven regions with phage WO-associated genes (WOSurA–WOSurG) in total, each with two to three homologues of the *wmk* gene (Figure 3). Overall, the genome coded for 18 *wmk* homologues which were numbered from *wmk1* to *wmk18*. As these copies may share the ability to induce male-killing, we compared these *wmk* homologues of *w*Sur with the functionally described *wmk* homologues in the *w*Mel strain as well as other known male-killing strains. Phylogenetic analysis identified homologues *wmk1* and *wmk12* in the phage region WOSurB as the most likely candidates to confer male-killing due to their high sequence similarity with the functional homologue *wmk* in *w*Mel (for *wmk12*), as well as *w*Inn and *w*Bor (for *wmk1*; Figure 5, top). A closer inspection of the CDS revealed that *wmk12* experienced an eight-nucleotide deletion that resulted in a stop codon and subsequent shift of the reading frame which led to the loss of the second XRE-family HTH DNA-binding region (Figure 5, bottom). We also screened different sequence read archives from an Israeli grain storage and two feral (field) populations of *O. surinamensis* individuals for *wmk1*/*wmk12-like* homologues. We found all individuals from feral populations encoded complete *wmk12-like* homologues clustering together in their own clade, while all individuals from the grain storage facility contained the deletion and frame shift mutation and clustered with the *wmk12* gene from *w*Sur JKI (Figure S2). In addition, we only found *wmk1-like* homologues in individuals from the grain storage population, but in no individual from the feral populations.

We tested for symbiont-mediated male-killing phenotype in the JKI strain of *O. surinamensis* by quantifying the sex ratio in symbiotic and aposymbiotic beetle cultures. We found a uniform frequency of both sexes in both cultures (SYM 50 W + 50 M, APO 52 W + 48 M; χ^2 test of homogeneity: $\chi^2 = 0.080$, $p = .888$). In addition, the male-killing phenotype should also result in a reduced hatching rate of around 50% in mating pairs with symbiotic females and males in comparison with mating pairs with aposymbiotic individuals. However, we did not observe such differences (BH-corrected Wilcoxon rank sum test, $p = .84$; Figure 6, right).

3.4 | Cytoplasmic incompatibility (CI)

Single homologues of both previously identified CI factor genes *cifA* and *cifB* were encoded in the *Wolbachia* prophage region WOSurB. Bayesian phylogenetic inference identified both *cifA* and *cifB* as type II following the classification scheme of Lindsey et al. (2018) (Figure 7). The *cifA* gene found in *w*Sur was closely related to those found in the *Wolbachia* strain *w*Ri of the fruit fly *Drosophila simulans* and *w*Suzi of the spotted wing drosophila *Drosophila suzukii*, while *cifB* did not cluster closely with any previously described genes from other *Wolbachia* strains. Although the *cifA* gene showed no homology to known domains (Figure 7, bottom), putative domains (PD-[D/E]XK nuclease/DpnII-MboI) were found in the *cifB* gene.

We tested the ability of *Wolbachia* infection to cause cytoplasmatic incompatibility by mating experiments with differential *w*Sur infection. First, the impact of *Wolbachia* infection on the number of laid eggs was determined. As expected, infection with *w*Sur had no effect on the number of laid eggs (Kruskal Wallis test: $\chi^2 = 0.29$, $df = 2$, $p = .86$; Figure 6, left). Following further development, we observed overall differences between the three groups' hatching rates (Kruskal Wallis test: $\chi^2 = 10.85$, $df = 2$, $p = .004397$; Figure 6, right). While the hatching rate between the control groups did not differ (aposymbiotic females and males, as well as symbiotic females and males: BH-corrected Wilcoxon rank sum test: $p = .84$; Figure 6, right), the hatching rate in the CI cross with aposymbiotic females and symbiotic males right was reduced by 43%–47% in comparison to both control groups (BH-corrected Wilcoxon rank sum test, $p = .04$ and .0018; Figure 6).

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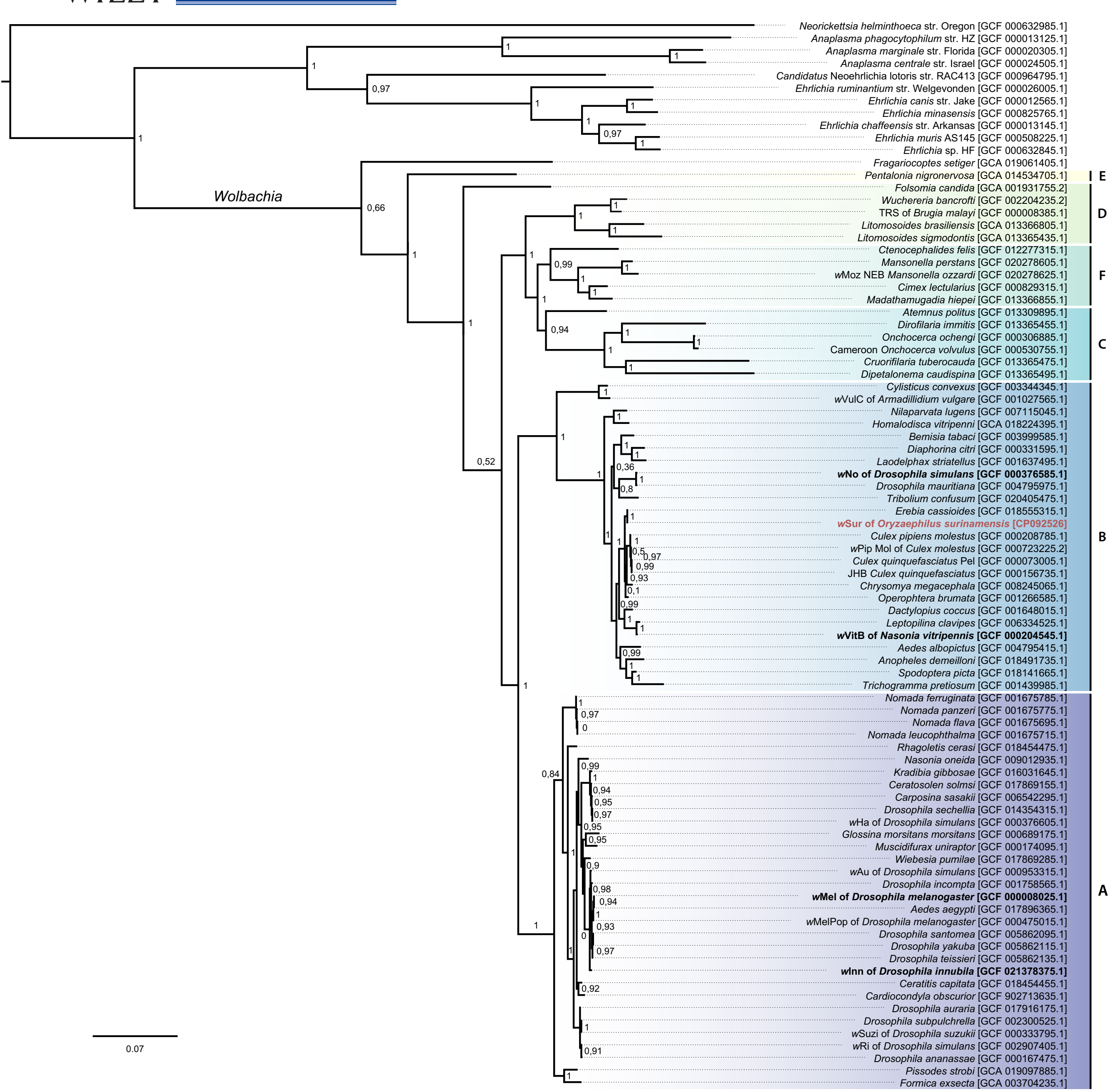

FIGURE 4 Phylogenetic relationship of *w*Sur and other *Wolbachia* strains deposited in sequence databases. The phylogeny was reconstructed based on a defined set of 49 orthologous genes using the KBase app *insert set of genomes into species tree* version 2.2.0 (Arkin et al., 2018), based on the FASTTREE2 algorithm (Price et al., 2010). Node numbers represent local support values. RefSeq assembly accession numbers are given in square brackets. The supergroups are colour-coded and indicated on the right (Baldo et al., 2006; Bandi et al., 1998; Casiraghi et al., 2005; Werren et al., 1995). The *Wolbachia* endosymbiont of *Oryzaephilus surinamensis* (*w*Sur, highlighted in red font) belongs to supergroup B. *Wolbachia* strain genomes highlighted in bold font were utilized for subsequent phylogenetic analyses of *wmk* (Figure 5) and *cif* genes (Figure 7).

4 | DISCUSSION

The sawtoothed grain beetle *Oryzaephilus surinamensis* harbours not only the nutritional Bacteroidota endosymbiont *S. silvanidophilus* but is also infected by a pervasive *Wolbachia* strain. Phylogenetic analysis of the *w*Sur core genome classified it as a member of supergroup B. *Wolbachia* strains of supergroup B together with supergroup A primarily infect arthropod hosts and are generally capable of reproductive manipulation, particularly by causing male-killing and CI (Werren et al., 2008).

The genome of the *Wolbachia* strain *w*Sur of *O. surinamensis* codes for 18 *wmk* homologues, all of which contain two helix–turn–helix (HTH) DNA-binding domains that are important for their function as a transcriptional regulator. The genomic distribution of some of the *wmk* homologues inside the phageWO is comparable to homologues found in other *Wolbachia* strains such as *w*Mel of

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TABLE 1 Genomic characteristics of *Wolbachia* *w*Sur in comparison to other strains

***Wolbachia* strain**	***w*Sur**	***w*No**	***w*VitB**	***w*Con**	***w*Mel**	***w*Inn**	***w*Ri**
Host	*Oryzaephilus surinamensis*	*Drosophila simulans*	*Nasonia vitripennis*	*Tribolium confusum*	*Drosophila melanogaster*	*Drosophila innubila*	*Drosophila simulans*
Accession	CP092526	GCF_00376585.1	GCF_000204545.1	GCF_020405475.1	GCF_000008025.1	GCF_021378375.1	GCF_002907405.1
Supergroup	B	B	B	B	A	A	A
Genome size (bp)	1,728,764	1,301,823	1,107,643	1,418,452	1,267,782	1,290,587	1,117,694
GC content (%)	34.10	34	33.91	34.17	35.20	35.28	35.04
Predicted proteins	1688	1191	1101	1294	1245	1346	1049

Drosophila melanogaster which were proposed as candidate genes responsible for the induction of *Wolbachia*'s male-killing phenotype (Perlmutter et al., 2019, 2020).

Phylogenetic comparison of their nucleotide sequences with previously characterized *wmk* homologues of *w*Mel and *wmk* homologues of known male-killing strains predicted the homologue *wmk1* and *wmk12* of *w*Sur as the most likely candidates to cause male-killing. However, further analysis revealed an eight-nucleotide deletion leading to a stop-codon midsequence and subsequent reading frame-shift of *wmk12*. The loss of half of the encoded protein and one of the two HTH DNA-binding domains presumably abolishes its ability to interfere with transcriptional regulation and the male-killing phenotype of *wmk12* and *w*Sur, while *wmk1* could still be functional. We found no indication of symbiont-mediated male-killing in the context of the JKI strain as no differences in hatching rate could be observed between symbiotic and aposymbiotic (free of both symbionts) mating pairs. Further, JKI stock cultures of both symbiotic and aposymbiotic beetles exhibited homogenous distributions of both sexes. However, Sharaf et al. (2010) compared a feral population of *O. surinamensis* from the field with a population adapted to a grain storage facility. They observed a strong female bias and reduced larval survival among offspring from this feral population emerging under laboratory conditions in contrast to a balanced sex ratio and higher larval survival in the population collected from the grain storage facility. They also observed incomplete *Wolbachia* infection of both populations: an 84% infection rate in the feral and 66% in the storage population. In combination, these data suggest active sex ratio distortion in the feral populations, probably by *Wolbachia*, but not in the population adapted to grain storage.

Individuals from the same collection sites were used for genome sequencing by Hong et al. (2020) with a focus on host genomes. Our analysis of *Wolbachia* encoded *wmk1* and *wmk12*-like homologues in these sequence read archives revealed the absence of *wmk1* together with intact *wmk12* in the two feral populations. In contrast, individuals of the Israeli population collected from the grain storage facility did encode *wmk1* homologues as well as the truncated *wmk12* version. Thus, we hypothesize that the intact *wmk12* represents the ancestral state that mediates *w*Sur male-killing in feral populations in Israel, while *w*Sur from populations adapted to grain storage facilities acquired a gene duplication of *wmk12* in the WOSurB region (=*wmk1*) and a deletion in the original *wmk12* gene as well as a loss of the male-killing phenotype at least in this host genetic background. These changes probably occurred recently, possibly in the process of invasion and adaptation to stored grains within co-adapted hosts that evolved probably under isolation from feral populations, and facilitated by repeated strong population bottlenecks during invasion of novel stored grain facilities or batches, as well as relaxed selection pressure on *w*Sur in completely infected host populations. However, whether the changes in the *wmk12* genes are causative for the loss of the male-killing phenotype remains elusive. Additional factors such as host evolution of resistance to male-killing effectors could also play a role in the loss of male-killing (Hornett et al., 2022).

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FIGURE 5 Top: Bayesian phylogeny of *wmk* homologues based on a nucleotide alignment. Consensus support values are shown at the branches. Bottom: Schematic of *w*Mel and *w*Sur *wmk* native nucleotide sequences. The blue tick marks indicate nonsynonymous nucleotide substitutions. The red tick mark indicates an eight-nucleotide deletion resulting in frame-shift mutation with a stop-codon at the deletion site. The two loci (helix–turn–helix [HTH] protein domain) of *wmk* are highlighted in grey. The hatched area indicates the region of *wmk12* that is predicted to be not translated based on the stop codon (red tick).

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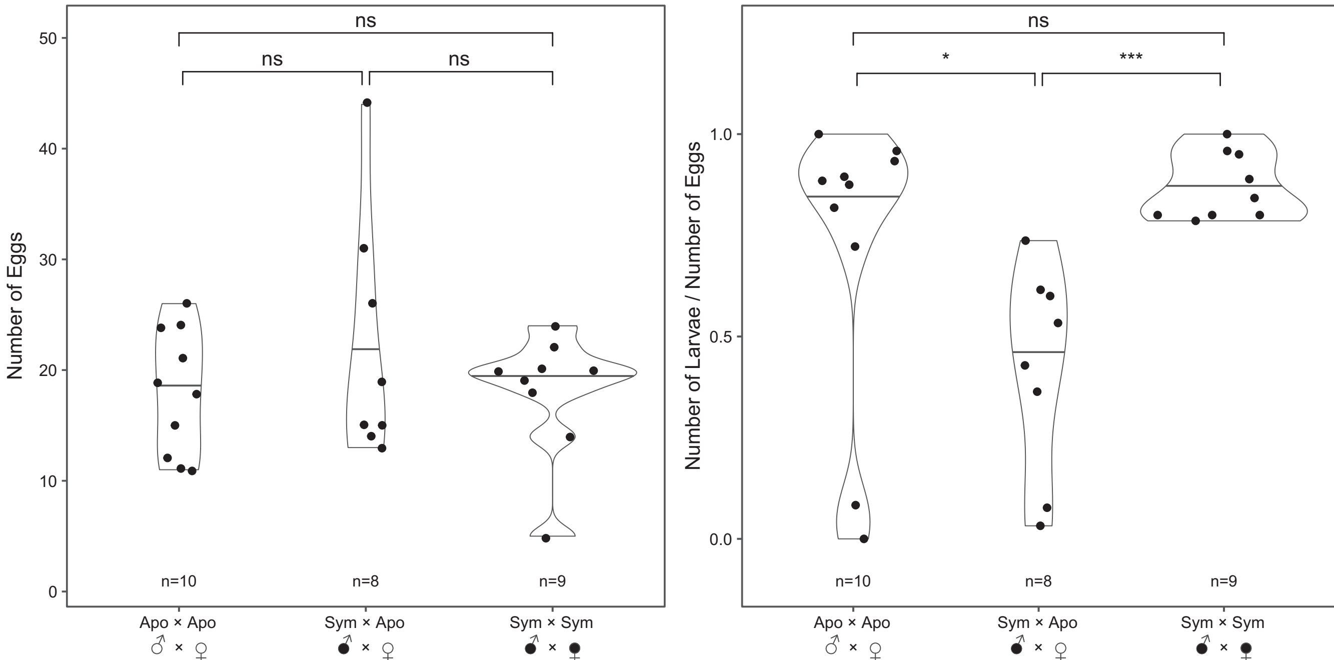

FIGURE 6 Influence of *w*Sur on embryo development of *Oryzaephilus surinamensis*. Number of laid eggs (left) and hatching rate (right) in the three mating groups. The data distribution is visualized with violin plots and an additional horizontal line depicting the mean. A filled sex sign indicates a symbiotic specimen, meaning infected with *w*Sur and *S. silvanidophilus*, whereas an empty sign indicates these specimens are aposymbiotic (regarding both symbionts). n is the number of *O. surinamensis* mating pairs. Statistical significance between the groups is based on Benjamini–Hochberg corrected Wilcoxon rank sum tests (ns, not significant, *$p<.05$, ***$p<.005$).

Multiple homologues of *wmk* have been described in other *Wolbachia* strains, although all except one did not induce male-killing when transgenically expressed in *D. melanogaster* (Perlmutter et al., 2020). Currently, the function of the additional *wmk* homologues in *w*Sur, as well as *w*Mel, remains unknown. *w*Sur and other strains might be multipotent and capable of inducing male-killing under specific conditions, or when infecting other hosts, such as the *Wolbachia* strain *w*Rec inducing CI in its main host *Drosophila recens* but causing male-killing when transferred to the closely related species *Drosophila subiquinaria* (Jaenike, 2007). In addition, the *Wolbachia* strains might manipulate the host in different ways beyond reproductive manipulation, for example by affecting pheromone biosynthesis, perception or behaviour (Bi & Wang, 2020; Engl & Kaltenpoth, 2018; Farahani et al., 2021; Schneider et al., 2019). The *wmk12/1* duplication in *w*Sur at least suggests that it is beneficial to retain a functional *wmk* gene, although possibly in a different context. Additional experiments, utilizing both feral and storage-adapted *O. surinamensis* populations with hybrid crosses, or transgenic expression of different *wmk* genes in aposymbiotic hosts might help to shed light on their function.

CI induced by *Wolbachia* occurs when the sperm of infected males is expressing the *cif* genes, which leads to infertile embryos in uninfected females, while in infected females the rescue factor *cifA* can reverse this effect (Beckmann et al., 2019; Shropshire et al., 2019; Shropshire & Bordenstein, 2019). The genome of *w*Sur encodes homologues for both CI-inducing genes *cifA* and *cifB* in one of the phage WO regions. *cifA* and *cifB* gene products are classified based on the similarity of their expressed amnio acid sequence as type I to type V (Bing et al., 2020; LePage et al., 2017; Lindsey et al., 2018). The CI phenotype was demonstrated in *cif* genes of type I, II and IV (LePage et al., 2017). Our analysis classified the *cifA* of *w*Sur as a type II homologue, while *cifB* clustered between type I and II homologues. Our experimental data indicate *w*Sur to be a reproductive manipulator by causing unidirectional CI to its host. Crossing *Wolbachia*-infected males with uninfected females resulted in a hatching rate that was reduced by 45% compared to crossings between infected males and females or uninfected males and females, respectively. Findings in *Drosophila simulans* showed a strong induction of CI leading to a hatching rate reduction of up to 95% (Sinkins et al., 1995), while data from *D. melanogaster* showed weak induction of CI resulting in a hatching rate reduced by 15%–30% (Hoffmann et al., 1994), depending on environmental conditions (Hague et al., 2020) as well as individual life history (Shropshire et al., 2021). As we have so far not been able to manipulate *S. silvanidophilus* and *Wolbachia* presence in *O. surinamensis* individually, symbiont-mediated phenotypes have to be considered with great care in dual symbiont-depleted experiments. However, with the addition of genomic and ecological information, we confidently attribute the here reported CI to *Wolbachia*. While *S. silvanidophilus* presence mirrored *Wolbachia* in the present experiments on CI and

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Type I

wRi [WP_012673191.1]
wSuzi [WP_044471237.1]
wRec [WP_038198916.1]
wMel [WP_010962721.1]
wMelPop [WP_010962721.1]
wVitA [ONI58213.1]
wHa [WP_015588933.1]
wSol [AGK87106.1]
wPipJHB [WP_007302988.1]
wBol1b [WP_019236549.1]
wPipPel [WP_012481787.1]

Type III

wNo [WP_015587806.1]
wAlbB [WP_006014162.1]

Type IV

wBol1b [WP_019236479.1]
wAlbB [WP_006012794.1]
wPipPel [WP_007302980.1]
wPipJHB [WP_007302980.1]

Type II

wSur
wSuzi [WP_044471252.1]
wRi [WP_012673228.1]

100 100 100 100 100 66 100

0.2

Type I

wVitB [NZ_AERW01000072]
wSuzi [WP_044471243.1]
wMel [WP_010962722.1]
wMelPop [WP_038228284.1]
wVitA [ONI58212.1]
wBol1b [WP_019236548.1]
wPipPel [WP_012481788.1]
wHa [WP_015588932.1]
wSol [AGK87078.1]

Type III

wNo [WP_015587805.1]
wAlbB [WP_006014164.1]
wVitA [WP_077188282.1]
wUni [WP_077188282.1]

Type II

wSur
wSuzi [WP_044471251.1]
wRi [WP_012673227.1]

100 100 97 100 100 99 100 100

0.2

cifA

0 200 400

cifB

0 200 400 600

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FIGURE 7 Phylogeny and domain structure of *cif* genes. Top: Bayesian phylogenies based on a nucleotide alignment of *cifA* (top) and *cifB* (middle) genes. Consensus support values are shown at the branches. Coloured shapes around branches designate monophyletic "types." bottom: Domain structure for the *cif* genes of *w*Sur. The two loci (PD-[D/E]XK nuclease/DpnII-MboI protein domain) of *cifB* are shown and indicated with orange bars.

male-killing, we have no indication for the presence of known CI factors encoded in the highly reduced *S. silvanidophilus* genome (Kiefer et al., 2021), while *w*Sur clearly contains homologues of both so far identified cytoplasmatic incompatibility factors. Thus, the *Wolbachia* strain *w*Sur is probably able to influence its fitness by increasing its transmission in partially infected populations, which is reflected by its high, observed prevalence in laboratory conditions.

Whether *Wolbachia* influences *O. surinamensis* beyond reproductive manipulation remains unclear. Previously reported cuticle supplementation of *O. surinamensis* is probably solely caused by *S. silvanidophilus*, because only the Bacteroidota endosymbiont has the ability to synthesize aromatic amino acid precursors via the shikimate pathway to support the host's cuticle synthesis, while *w*Sur and *Wolbachia*, in general, lack the entire pathway (Kiefer et al., 2021 and this study). Further, cuticle deficiencies (reduced thickness and melanization) were not only reported in dual aposymbiotic individuals after strict tetracycline treatment (deficient of both *S. silvanidophilus* and *w*Sur), but also after glyphosate treatment, which only reduced *S. silvanidophilus*, but not *w*Sur titres (Kiefer et al., 2021; Figure S1). Thus, *S. silvanidophilus* is responsible for supplementation of cuticle synthesis as well as ecological consequences in terms of elevated resistance to abiotic desiccation stress, pathogen and predation pressure (Kanyile et al., 2022), but also costs of symbiont infection on reproduction (Engl et al., 2020).

Certain *Wolbachia* strains were previously reported to supplement the hosts' diet with limited nutrients (especially B-vitamins; Hosokawa et al., 2010) or provide pathogen defence (Moreira et al., 2009). The *Wolbachia* strain *w*Sur of *O. surinamensis* also encodes pathways to synthesize the amino acids lysine, glutamine, threonine, glycine and serine as well as the vitamin riboflavin. While riboflavin does not seem to be limited on cereal-based diets (Škrovánková & Sikorová, 2010), lysine is (Torbatinejad et al., 2005). It remains unclear whether *Wolbachia* might synthesize lysine only for its own benefit, or also contribute it to its host's metabolism. Similarly, it is unclear whether *Wolbachia* infection inflicts additional costs beyond unidirectional CI which is only relevant in populations with incomplete *Wolbachia* infection (Hoffmann et al., 1996; Perrot-Minnot et al., 2002; Vala et al., 2000).

In combination with our previous work on the Bacteroidota symbiont *S. silvanidophilus* (Engl et al., 2018, 2020; Kanyile et al., 2022; Kiefer et al., 2021), we demonstrate that *O. surinamensis* harbours two notable symbionts. Both impact the host's physiology, ecology, and thereby also its and each other's evolution. Based on the high prevalence of both, nutritional symbionts (Douglas, 2009; Douglas, 2014) and reproductive manipulators (Duron et al., 2008; Kajtoch & Kotásková, 2018) in coleoptera and insects in general, dual infections are not uncommon and probably underestimated (Alam et al., 2011; Gómez-Valero et al., 2004; Heddi et al., 1999). However, currently both symbioses are usually studied by experimental approaches in isolation, or from a descriptive perspective on the prevalence and genomic potential. Thereby, we miss out on potential higher levels of ecological interactions of both types of symbioses, mediated either via the host's physiology, or even directly between different symbionts. Future work should thus try to integrate multipartite, symbiotic relationships. Available tools include selective removal or inhibition of individual symbionts, such as by targeting specific, obligate biosynthetic pathways of symbionts. The glyphosate utilized here, inhibiting the Shikimate pathway responsible for the synthesis of aromatic amino acids (Steinrücken & Amrhein, 1980), but, for example, also inhibitors of the diaminopimelate pathway responsible for synthesizing lysine are prominent agents suggested for the manipulation of specific biosynthetic capabilities or organisms encoding them (Hutton et al., 2003). Alternatively, expression of target symbiont genes in suitable host systems are a powerful tool to address gene function in insect symbionts that are elusive to genetic manipulation themselves (Perlmutter et al., 2019; Perlmutter et al., 2020; Shropshire & Bordenstein, 2019). Finally, the example of *O. surinamensis* highlights again the importance of identifying systems with interesting combinations of symbionts and a certain amenability for experimental manipulation and observation to understand more complex eco-evolutionary dynamics of multipartite symbioses.

AUTHOR CONTRIBUTIONS

J.S.T.K. and T.E. designed the project, J.S.T.K., G.S. and R.K. performed experiments, J.S.T.K., G.S., R.K. and T.E. analysed the data, J.S.T.K. and T.E. wrote the initial manuscript, and all authors read and commented on the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

Genetic data: Raw sequence reads are deposited in the SRA (SRR12881563–SRR12881566; SRR12881567–SRR12881568; BioProject PRJNA670819). The annotated *w*Sur genome is available on GenBank (CP092526). Bioassay data are available on the data repository of the Max-Planck-Society Edmond (Engl et al., 2022).

BENEFIT-SHARING STATEMENT

All specimens utilized in this work were obtained from a long-standing laboratory culture (before 2014). Thus, the Nagoya Protocol is not applicable and no benefits are reported.

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