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High-Efficiency Thermal Asymmetric Interlaced PCR (hiTAIL-PCR) for Determination of a Highly Degenerated Prophage WO Genome in a *Wolbachia* Strain Infecting a Fig Wasp Species

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Temperate bacteriophage WO is a model system for studying tripartite interactions among viruses, bacteria, and eukaryotes, especially investigations of the genomic stability of obligate intracellular bacteria. Few WO genomes exist because of the difficulty in isolating viral DNA from eukaryotic hosts, and most reports are by-products of *Wolbachia* sequencing. Only one partial genome of a WO phage has been determined directly from isolated particles. We determine the complete genome sequence of prophage WO (WOSol) in *Wolbachia* strain *w*Sol, which infects the fig wasp *Ceratosolen solmsi* (Hymenoptera: Chalcidoidea), by high-efficiency thermal asymmetric interlaced PCR. The genome of WOSol is highly degenerated and disrupted by a large region (14,267 bp) from *Wolbachia*. Consistent with previous molecular studies of multiple WO genomes, the genome of WOSol appears to have evolved by single nucleotide mutations and recombinations.

Wolbachia (*Alphaproteobacteria*), a maternally inherited, endosymbiotic bacterium found in arthropods and filarial nematodes, has the potential to serve as a vector in insect pest control and management (1, 2). However, the bacteria are exceedingly difficult to culture outside their eukaryotic host cells. The DNA of *Wolbachia* comprises a small portion of total host DNA, and this makes the isolation of *Wolbachia* DNA very difficult. Consequently, the sequencing of *Wolbachia* genomes has been plagued by many technical challenges (3). Recent array-based genomic protocols can capture the genomic sequence of *Wolbachia w*VitB (4). A recent protocol for sequencing of the genome of *Plasmodium falciparum* facilitates the efficient enrichment and sequencing of *Wolbachia* and pathogenic DNA (5).

The temperate bacteriophage WO is the only known mobile genetic element that transforms the genome of *Wolbachia* and thus is an ideal object for studying the three-way interactions among viruses, bacteria, and eukaryotes (6, 7). However, it is more difficult to isolate WO than *Wolbachia* DNA from a host and this complicates analyses (1). Virus-like particles in *Wolbachia* were detected by electron microscopy early in 1978 (8), yet the phage-related genes were not identified until 2000 (9). Several WO phage genomes (e.g., WOCauBs, WOPips, WOMels, WORis, and WOVits) are by-products of sequencing of *Wolbachia* genomes (4, 10–13), and only one WO phage partial genome (WOcauB1) has been sequenced independently (7).

Unknown DNA sequences can be identified by virtue of flanking DNA with a known sequence (14). Many PCR-based methods, such as inverse PCR (15–17), adapter ligation-mediated PCR (18–20), hemispecific or one-sided PCR (21, 22), and thermal asymmetric interlaced PCR (TAIL-PCR) (23, 24), have been developed for the amplification of unknown DNA sequences. Among these methods, TAIL-PCR allows the handling of a large number of samples manually or automatically (23). Recent improvements, termed high-efficiency TAIL-PCR (hiTAIL-PCR), increase both the rate of success to >90% and target products sizes to 1 to 3 kb (14).

We used hiTAIL-PCR to determine the WO phage genome located on the genome of *Wolbachia* sp. strain *w*Sol, which infects the fig wasp *Ceratosolen solmsi*. We also evaluated the evolution of WO genomes by comparing ours with those infecting the insects *Cadra cautella*, *Culex pipiens*, *Drosophila melanogaster*, *D. simulans*, and *Nasonia vitripennis* (4, 10–13, 25).

MATERIALS AND METHODS

Insect collection and identification. All wasp samples were collected from different fig trees in Guangdong, Yunnan, and Hainan Provinces, China, in 2011. Mature fig fruits were collected and then dissected in the laboratory to collect wasps before their emergence. Specimens of *C. solmsi* were identified morphologically (see Fig. S1 in the supplemental material) by using Nikon SMZ80 microscopes. All wasps were initially immersed in 95% ethanol and subsequently maintained at −20°C until DNA extraction.

DNA extraction. Before DNA extraction, each specimen was washed several times with 70% ethanol, followed by sterile water, to remove surface contamination. DNA was isolated from each wasp by using an EasyPure Genomic DNA extraction kit (TransGen, Beijing, China) and following the manufacturer's recommendations. The quality of the DNA templates was confirmed by the amplification of a partial fragment of cytochrome *c* oxidase subunit I (about 700 bp) with primers LCO1490 (5′-CCTGGTTCTTTRATTGGTAATGATC-3′) and HCO2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA-3′) (26). DNA templates of poor quality were discarded. All specimens and DNA vouchers were deposited at the Institute of Zoology, Chinese Academy of Sciences.

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PCR amplification and sequencing. We screened the wasps for *Wolbachia* infection by using three primer pairs, *wsp* 81f and 691r for amplification of the *Wolbachia* surface protein gene *wsp* (27), 16SwolF and 16SwolR for amplification of *Wolbachia* 16S rRNA genes (28), and ftsZF and ftsZR for amplification of *Wolbachia* cell division gene *ftsZ* (29). The PCR program was 5 min at 94°C; 30 cycles of 30 s at 94°C, 45 s at 55°C, and 45 s at 72°C; and then 10 min at 72°C for the final extension step. Amplification of *orf7*, which codes for a minor capsid protein of WO (9), demonstrated the presence of WO. Sequences of *orf7* retrieved from GenBank were used to design *de novo* primers for the amplification of *orf7* as follows: WOSolF, 5′-GTCTGGAAAGCTTACAAAAAG-3′; WOSolR, 5′-TTGCTCTATAAATTCTCCT-3′. The PCR program was 5 min at 94°C; 30 cycles of 30 s at 94°C, 40 s at 52°C, and 25 s at 72°C; and 10 min at 72°C for the final extension step. PCR products were purified with the EasyPure PCR purification kit (TransGen, Beijing, China) and directly sequenced with an ABI 3730 sequencer at Biosune (Beijing, China).

On the basis of *orf7* sequences, we designed hiTAIL-PCR primers (see Table S3 in the supplemental material) to amplify a fragment including the two flanking regions. The PCR conditions used were standard (14). Each putative open reading frame (ORF) was subjected to homology searches in DNA databases. All PCR experiments used negative controls (no DNA template) and positive controls. The positive control used DNA templates from *N. vitripennis* infected with *Wolbachia* and WO (Hangzhou strain; from Gongyin Ye, ZheJiang University) (30). Products of hiTAIL-PCR amplification were purified with the EasyPure Quick Gel PCR purification kit (TransGen, Beijing, China) and cloned with the Peasy-T5 vector (TransGen, Beijing, China); a minimum of three positive clones were sequenced.

Sequence annotation. Sequence editing was performed with BioEdit (31). ORFs were predicted with Glimmer3.0 (http://www.ncbi.nlm.nih.gov/projects/gorf/). ORFs consisting of at least 50 codons and starting with ATG, GTG, or TTG were considered putative genes. Functions of the ORFs were inferred on the basis of (i) the current gene annotation found in NCBI, (ii) the annotation of non-*Wolbachia* homologs identified in a tblastx search of the nr database, and/or (iii) the presence of conserved protein domains. Before the annotation of a pseudogene, PCR amplification and sequencing with specific primers were used to double guarantee the correctness of the sequences.

The tRNAs were identified by using the tRNAscan-SE server (http://lowelab.ucsc.edu/tRNAscan-SE) and ARAGORN (32).

Alignment and tree-based analyses. Homology searches by BLASTp/BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) were performed on the nonredundant protein database/nucleotide collection (nr/nt) at NCBI by using deduced amino acid and/or nucleotide sequences as queries. Output E values of the searches were used as criteria for data parsing. Sequences were aligned with ClustalW in BioEdit (31), followed by manual refinement with SeaView (33). All trees were constructed by the neighbor-joining method in Mega5 (34), with 1,000 bootstrap replicates. For *orf7* (nucleotide and amino acid sequences) and recombinase gene (amino acid sequences), we also carried out maximum-likelihood (ML) analyses with PhyML 3.0 (35). Model selection for the ML analysis was based on the Akaike information criterion in ProtTest 3 (for amino acid sequences) (36) and jModelTest 2 (for nucleotide sequences) (37). ML bootstrap values were generated from 100 bootstrap replicates by using JTT+G for *orf7* and JTT+G+F for recombinase.

Nucleotide sequence accession number. *De novo* nucleotide sequences were deposited in GenBank under accession number KC955252.

RESULTS

Prevalence of WO in *C. solmsi*. We screened 437 *C. solmsi* wasps for the prevalence of *Wolbachia* and prophage WO. In total, 83.3% (364/437) of the wasps were infected with *Wolbachia* (detection of *wsp*, 16S rRNA genes, and *ftsZ* amplification), which was similar to previous reports (38). All *Wolbachia*-infected wasps also harbored prophage WO. Further, four *Wolbachia*-free wasps (based on three gene markers) also harbored WO *orf7* sequences (data not shown), possibly indicating horizontal gene transfers.

TABLE 1 Comparative genomic features of WO

Phage	G+C content (%)	% pseudogenes and fragmented genes per WO genome	Gene density[a]	Predicted no. of functional protein-coding genes	Genome size (bp)
WOsol	36.3	27.6	0.70	21	30,213
WOMelB	35.5	7.0	1.01	53	52,613
WOMelA	37.0	12.5	1.10	28	25,585
WOCauB2	35.3	2.2	1.05	45	43,016
WOCauB3	35.7	0.0	1.00	45	45,078
WOPip1	35.4	3.1	1.14	30	26,252
WOPip2	35.3	4.0	0.92	24	26,050
WOPip3	36.8	0.0	1.38	20	14,500
WOPip4	35.2	2.2	1.08	44	40,861
WOPip5	35.2	0.0	1.14	45	39,598
WORiA	37.2	13.6	0.96	20	20,835
WORiB	35.9	3.00	1.00	32	31,946
WORiC	35.1	16.4	0.73	56	77,261
WOVitA1	34.0	0.0	1.21	51	42,122
WOVitA2	33.3	2.6	0.92	37	40,003
WOVitA4	35.8	3.3	1.36	29	21,272
WOVitB	35.5	10.8	1.10	33	29,969
wHa WO1	36.5	13.9	0.83	31	37,447
wHa WO2	35.6	10.9	0.97	41	42,099
wNoWO1	35.9	6.5	1.18	29	24,635
wNoWO2/3	34.5	16.0	0.80	21	26,141
wNoWO4	35.7	8.0	0.52	23	44,334

[a] Number of functional genes per kilobase.

Genome properties and comparisons. The WO genome (WOSol here) was linear and double stranded with a length of 30,213 bp, a G+C content of 36.3%, and 29 ORFs (Table 1; see Table S1 in the supplemental material). Similar to that of WOMelB1 (11), the prophage genome was separated into two parts because of the insertion of a *Wolbachia* genome fragment (Fig. 1A). A gene encoding a Ser-type recombinase (So0001) putatively involved in phage integration and a gene encoding an SNF2 family helicase (So0025) were located at each end of the longer part (Fig. 1A and B). The "smaller" part of the prophage contained only four genes. In WOSol, we also discovered two unexpected unique genes (see Table S2 in the supplemental material) that were not previously known (39). No tRNA gene was detected in the WOSol genome.

Highly degenerated WOSol genome. Our assembly of the WOSol genome failed to detect the tail module required for assembly and movement. This contrasted with previous reports that a *Wolbachia* genome (e.g., *w*CauB, *w*Pip, *w*Mel, *w*Ri, *w*VitA) with WO always harbored at least an intact WO prophage with a head, a baseplate, a tail, and virulence modules (39).

The WOSol genome had the highest pseudogene ratio (8 out of the 29 ORFs, up to 27.6%) of any reported bacteriophage WO genome. This is higher than the previously reported record of 13.6% (39). The eight pseudogenes were scattered in different modules and regions (Table 1; see Fig. S2 in the supplemental material). Further, the WOSol genome exhibited nearly the lowest gene density; wNoWO4, with the lowest gene density, was an exception because the lengths of the genes for a hypothetical protein and the ankyrin motif protein were more than 9 kbp) (Table 1).

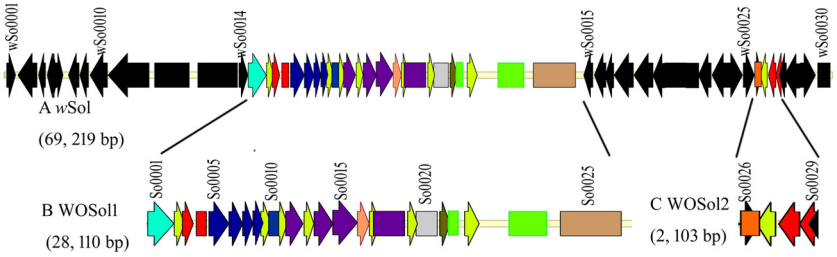

FIG 1 Gene organization in the WOsol genome. Genes colors are based on functional type and homology as follows: cyan, integrase/recombinase; red, ankyrin repeat protein; purple, head module; blue, baseplate module; orange, putative virulence factors; yellow, transposases; olive, Holliday junction resolvasome/endonuclease; gray, DNA methylase; brown, SNF2 helicase; tomato, lysozyme; lime, regulatory protein gene *repA* (replication module); black, *Wolbachia* genes; yellow-green, genes that encode proteins of unknown function.

Comparison of WOSol with WO phages in *D. melanogaster*. The gene content and order of WOSol were comparable to those of WO prophages WOMelA and WOMelB carried by strain *w*Mel from *D. melanogaster*. Of the 29 ORFs of WOSol, 16 were homologous to WOMelA and 17 were homologous to WOMelB. Gene order was only partially conserved between WOSol and WOMelA/B (Fig. 2), which suggested that many inversion/translocation/recombination events occurred in the evolution of these phages (12).

Similar to WOMelB, which harbors a large *Wolbachia* genome region (spanning ORFs WD0611 to WD0633) (11), WOSol had a large insertion of *Wolbachia* sequences (14,267 bp) including ORFs wSo0015 to wSo0026 (Fig. 1 and 3). All 12 inserted ORFs in WOSol were conserved in sequence similarity and gene order in the 5′ region of WOMelB (WD0611 to WD0622). In contrast, the 3′ part of the WOMelB inserted region, including WD0627 to WD0632, was homologous to the 5′-flanking region of WOSol (Fig. 3). This suggested the occurrence of two independent translocation events involving different sequence regions in both *Wolbachia* genomes. Further tree-based analyses of the amino acid sequences of *orf7* (Fig. 4; tree-based analyses of the nucleotide acid sequences of *orf7*; see Fig. S3 in the supplemental material) and 10 other genes commonly found in WO phage sequences (see Fig. S4; see also below) suggested that WOSol was evolutionarily close to nontailed phages of WORiA while being distantly related to WOMelB. Further, *w*Sol was identified as ST19 (38) and *w*Mel was identified as ST1 (11) by multilocus sequence typing with the five standard housekeeping genes *gatB*, *coxA*, *hcpA*, *ftsZ*, and *fbpA* (40); this excluded the possibility that WOSol and WOMelB were descended from a common ancestor with modification. Thus, the insertion in *Wolbachia* happened independently in distantly related WO genomes.

Analyses of recombinase. The tyrosine and serine recombinases are two of many diverse integrases that promote the integration of temperate phages into and their excision from host bacterial genomes (12, 41–43). Both recombinase families have been detected in WO.

Trees constructed from 295 aligned amino acid sites of the recombinase sequences, including genes in WOSol and WO phages in five other insects, distinguished groups I, II, and III. These groups represented three major families of recombinase (39) (see Fig. S5 in the supplemental material).

Diversity of genes in WOSol. In addition to *orf7*, tree-based analyses were performed for 10 other genes of WOSol commonly found in WO phage genomes (see Fig. S4 in the supplemental material). In 9 of the 10 trees, WOSol clustered with WORiA, WOMelA, wHa-WO1, and wHa-WO2 or at least with some of them. These results were similar for ORF7. In five trees, the groups

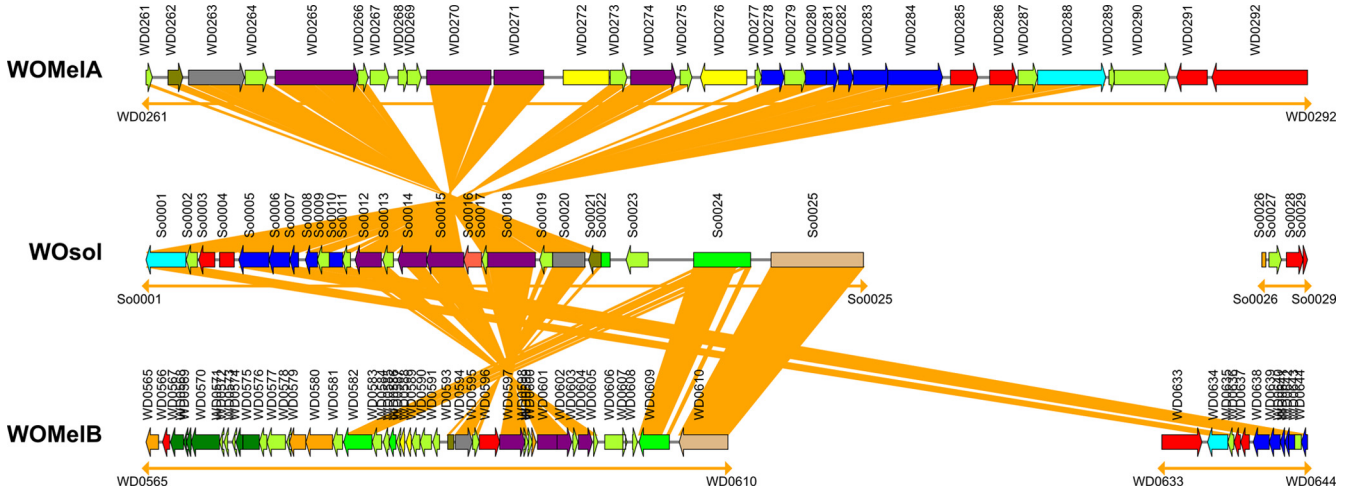

FIG 2 Gene order comparisons among WOMelA, WOSol, and WOMelB. Orange lines connect matched ORFs with E values of <1e−15. Orange bidirectional arrows in WOMelA and WOMelB lines represent regions of wMel WO-A and WO-B assigned by Wu et al. (11). Colors of ORFs are the same as in Fig. 1.

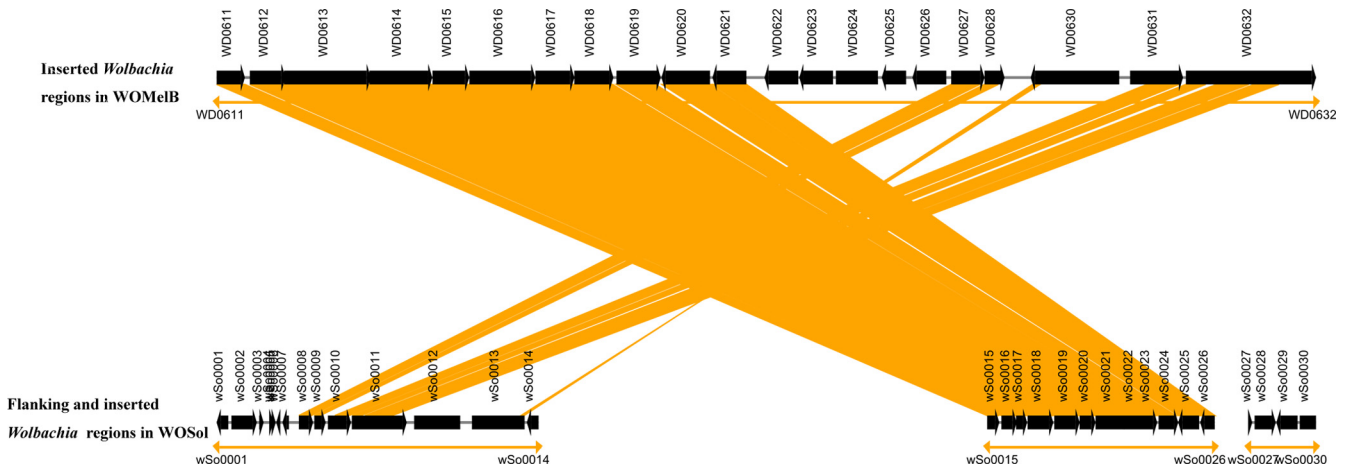

FIG 3 Gene order comparisons between the inserted *Wolbachia* regions in WOMelB and flanking and inserted *Wolbachia* regions in WOSol. Orange lines connect matched ORFs with E values of <1e−15. The orange bidirectional arrow in the *Wolbachia* region in WOMelB indicates inserted *Wolbachia* regions in WOMelB assigned by Wu et al. (11).

were supported by bootstrap values of >90%. However, one exception (So0010) indicated that these genes might have a high level of genetic diversity. Mosaic evolution of WO phages (39) was probably mediated by the dynamic gene flux among WO phages through coinfection, lateral gene transfer, and/or genetic recombination (44).

Efficiency of hiTAIL-PCR. By using hiTAIL-PCR, we successfully amplified 25 of 29 WOSol genes (30,213 bp, 29 ORFs). In the flanking *Wolbachia* genome regions wSo0001 to wSo0014 and wSo0015 to wSo0030, we filled gaps by using PCR and high-throughput sequencing data for the fig wasp species (unpublished data). The success rate of hiTAIL-PCR was 87% with an average sequence length of 1,353 bp (see Fig. S6 in the supplemental material for an electrophoresis image in one hiTAIL-PCR experiment). Our hiTAIL-PCR results were confirmed by long PCR targeting some specific fragments across the prophage genome (see Fig. S7).

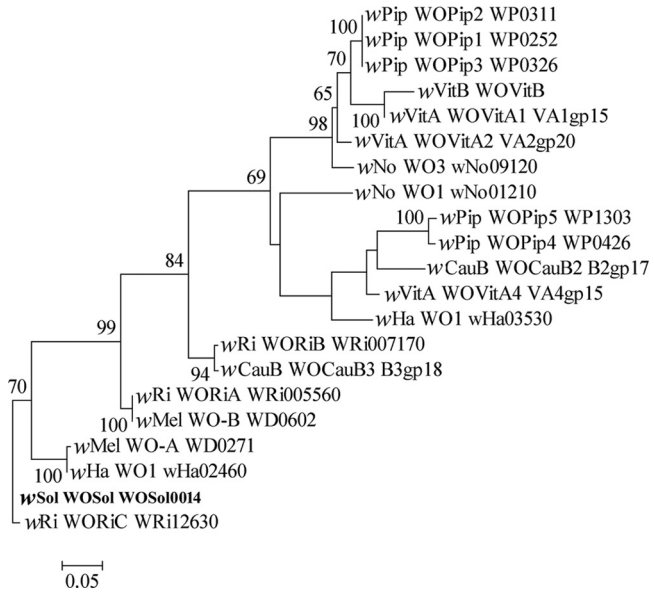

FIG 4 Relationship of WOSol to other sequenced WO phages on the basis of ORF7 sequences. Twenty ORF7 sequences from five insect species were retrieved from databases. An ML inference method inferred from 227 aligned amino acid sites was used. Bootstrap values higher than 50% are shown. The name of each sequence is a combination of the abbreviation of the *Wolbachia* strain, the WO name, and the accession number of the gene. The WOSol sequence is in bold.

DISCUSSION

HiTAIL-PCR can lead to the successful sequencing of the WO phage genome. Its high specificity captures the WO phage genome even given the heterogeneous mixture of eukaryotic, bacterial, and viral DNAs. The method is cost efficient by circumventing the need to construct a genomic library, a requirement for whole-genome sequencing by next-generation technologies. By precluding the need to concentrate bacteria or phage particles, very little DNA template is required; total DNA from two minute wasps is sufficient for results.

An apparent disadvantage of hiTAIL-PCR is that when multiple WO haplotypes in the same genome consist of similar or identical homologs, amplification and assembly of sequences are difficult (13, 39). However, only one WO phage occurs in the *w*Sol genome. Our direct sequencing of *orf7* amplicons results in a single read, which suggests the presence of only one WO phage in the genome of *w*Sol, although taken alone, this result may be misleading (13). Our sequencing of clones from each hiTAIL-PCR product obtains identical sequences from multiple clones. Thus, the results of our hiTAIL-PCR seem to be valid. Our unpublished high-throughput sequencing data for the host wasp also indicate the presence of only one WO phage in the *w*Sol genome (data not shown). Further, Southern blotting experiments will also support the existence of only one WO phage in the genome. Considering that >40% of *Wolbachia* genomes harbor only one type of WO (45), this technique remains broadly applicable. Nonetheless, repetitive sequences such as that of the gene for the ankyrin motif protein in the WO genome (4, 12) require the design of specific primers to validate hiTAIL-PCR results because of possible mismatches.

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