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PLOS ONE

RESEARCH ARTICLE

Isolation and characterization of a novel bacteriophage WO from *Allonemobius socius* crickets in Missouri

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Abstract

Wolbachia are endosymbionts of numerous arthropod and some nematode species, are important for their development and if present can cause distinct phenotypes of their hosts. Prophage DNA has been frequently detected in *Wolbachia*, but particles of *Wolbachia* bacteriophages (phage WO) have been only occasionally isolated. Here, we report the characterization and isolation of a phage WO of the southern ground cricket, *Allonemobius socius*, and provided the first whole-genome sequence of phage WO from this arthropod family outside of Asia. We screened *A. socius* abdomen DNA extracts from a cricket population in eastern Missouri by quantitative PCR for *Wolbachia* surface protein and phage WO capsid protein and found a prevalence of 55% and 50%, respectively, with many crickets positive for both. Immunohistochemistry using antibodies against *Wolbachia* surface protein showed many *Wolbachia* clusters in the reproductive system of female crickets. Whole-genome sequencing using Oxford Nanopore MinION and Illumina technology allowed for the assembly of a high-quality, 55 kb phage genome containing 63 open reading frames (ORF) encoding for phage WO structural proteins and host lysis and transcriptional manipulation. Taxonomically important regions of the assembled phage genome were validated by Sanger sequencing of PCR amplicons. Analysis of the nucleotides sequences of the ORFs encoding the large terminase subunit (ORF2) and minor capsid (ORF7) frequently used for phage WO phylogenetics showed highest homology to phage WOAu of *Drosophila simulans* (94.46% identity) and WOCin2USA1 of the cherry fruit fly, *Rhagoletis cingulata* (99.33% identity), respectively. Transmission electron microscopy examination of cricket ovaries showed a high density of phage particles within *Wolbachia* cells. Isolation of phage WO revealed particles characterized by 40–62 nm diameter heads and up to 190 nm long tails. This study provides the first detailed description and genomic characterization of

and analysis, decision to publish or preparation of the manuscript.

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phage WO from North America that is easily accessible in a widely distributed cricket species.

Introduction

It is estimated that 66% of all insect species and the majority of filarial parasites that infect humans are infected/colonized with *Wolbachia*, obligate intracellular bacteria belonging to the order Rickettsiales [1] *Wolbachia* cause phenotypes such as cytoplasmic incompatibility (CI) and feminization in arthropods, or support growth and reproduction in filarial nematodes [2, 3]. Cytoplasmic incompatibility is the most prevalent *Wolbachia*-induced phenotype in insect hosts and presents a form of conditional sterility whereby crosses between infected males and uninfected females produce unviable offspring; infected females may successfully mate with *Wolbachia*-infected or uninfected males, conferring them a selective advantage [4, 5]. *Wolbachia* are divided into several supergroups based on their ftsZ gene sequence, with supergroups A and B found exclusively in arthropods and supergroups C and D found exclusively in nematodes [6]. *Wolbachia* are abundant in male and female germlines and are enriched along the reproductive tract, but also present in somatic structures (e.g., the brain and gastrointestinal tract) of select host species. Transmission is predominantly vertical, from female to offspring, although horizontal transmission has been documented in nature [4, 5, 7]. Active bacteriophages infecting *Wolbachia* (phage WO) were first discovered in 2000 and remain one of few published cases of bacteriophages that infect intracellular bacteria [8]. Phages are estimated to infect most of the *Wolbachia* taxa in the supergroups A and B, but are believed to be absent from *Wolbachia* supergroups C and D [9]. The persistence of the phage despite its documented lytic activity has led to the hypothesis that phage WO provides benefit to its *Wolbachia* or arthropod host [10]. Phage WO may regulate *Wolbachia* density and therefore, affect development and phenotype of its eukaryotic host [11]. Further, phage WO provides *Wolbachia* with accessory genes for cytoplasmic compatibility and male killing [12, 13].

In recent years, an increasing number of *Wolbachia* genomes have been sequenced and phage WO is of interest for being the only known mobile genetic element in *Wolbachia*, and its hypothesized role in generating the high level of diversity seen among *Wolbachia* today [10, 14]. Evidence has been provided for horizontal gene transfer between *Wolbachia* strains mediated by WO phages [15]. For a majority of *Wolbachia* phages, sequence data is limited to the minor capsid protein-coding gene, and there remain entire families and genera of *Wolbachia*-harboring arthropods in which phage has not yet been described [8]. One such example is found in crickets (*Gryllidae*) of the genus *Allonemobius* (ground crickets), whose members include *A*. *socius* (the southern ground cricket) and *A*. *maculatus* (the spotted ground cricket), found throughout North America. *Wolbachia* belonging to supergroup B has been identified in *A*. *socius* (strain: *w*Soc), where it is hypothesized to play a role in lengthening female crickets' spermathecal ducts, thus increasing their control over mate choice. [16–18]. However, a phage WO has neither been identified nor described in *Allonemobius*.

In the present study we identified, for the first time, a phage WO in *Allonemobius* crickets (phage WOSoc) and estimated its prevalence in a local *A*. *socius* population. We characterized the novel phage WOSoc by immunohistochemistry, transmission electron microscopy, and whole genome sequencing, expanding the limited set of fully described bacteriophages of *Wolbachia* by adding this novel bacteriophage for which we provide evidence of phage particle production, and complete genes which may mediate bacterial cell wall lysis and manipulation of host translation.

Materials and methods

Sample collection and DNA extraction

Adult *A*. *socius* crickets (n = 40) were collected in the summer of 2019 from Forest Park, St. Louis, Missouri, USA (N 38.4˚ 38', W 90˚ 17'). Crickets were sexed based on the presence (female) or absence (male) of an ovipositor and ecological data including morphological features and geographical distribution were used to confirm species identification. All insects were euthanized by placement at -20˚ C for 30 minutes before dissection and homogenization of abdomens in 500 μL of phosphate buffered-saline by 15-minute high-intensity beating with a 3.2 mm chrome Disruption Bead (BioSpec Products, Bartlesville, USA) on the Vortex-Genie 2 mixer (Scientific Industries, Inc., Bohemia, USA). The homogenate was spun down, and DNA was prepared from the supernatant using the DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to manufacturer recommendations, with elution into 100 μL sterile water and storage at -20˚C or 4˚C until use.

PCR for phage and *Wolbachia* detection

Conventional PCR reactions with total cricket abdomen genomic DNA template were run using previously validated primers to the conserved *Wolbachia* surface protein (WSP) gene [19] and to the *Wolbachia* phage capsid protein (WPCP) gene [20]. PCR was performed in 25 μL reactions with 0.625 μL of 10 μM forward and reverse primers (250 nm final concentration), 2 μL DNA template (2–5 ng), 12.5 μL Hot Start Taq DNA Polymerase (2X (New England Biolabs, Ipswich, USA), and 9.25 μL sterile water. Following an initial 30 s denaturation at 95˚C, 40 cycles were run with 30 s denaturation at 95˚C, 60 s annealing at 55˚C, 1 min extension at 68˚C, and a single 5 min final extension at 68˚C. For each primer set and reaction, sterile water was run as a non-template control. PCR products were sent to Genewiz (South Plainfield, USA) for Sanger sequencing. Forward and reverse primer sequencing reactions were performed for each region of interest and chromatograms were visually inspected for base call quality.

Real-time PCR prevalence estimates

Primer 3 software [21] was used to create qPCR-optimized WSP and WPCP primers from their respective *w*Soc and WOSoc sequences (Table 1). For each DNA template and primer set, qPCR reactions were performed in duplicate 25 μL reactions with 0.625 μL of 10 μM forward and reverse primers (250 nm final concentration), 2 μL DNA template, 12.5 μL Power SYBR Green Master Mix (Thermo Fisher, Waltham, USA), and 9.25 μL sterile water using the standard Power SYBR Green PCR Master Mix RT-PCR Protocol (Protocol Number 436721) on a QuantStudio 6 Flex Real-Time PCR System (Thermo Fisher). As positive controls for the WSP and WPCP primer sets, we used 2μL Sanger-confirmed WSP- and WPCP-positive

Table 1. List of primers designed and used in the study.

Primer name	Forward primer sequence (5'–>3')	Reverse primer sequence (5'–>3')	Amplicon length (bp)	Description
*w*Soc	AGATAGTGTAACAGCGTTTTCAGGAT	CACCATAAGAACCAAAATAACGAG	60	qPCR detection of *w*Soc in crickets
WOSoc	CCCTGCCTCTGTTGATCG	CCCTGCCTCTGTTGATCG	60	qPCR detection of WOSoc in crickets
WOSoc tail	CAGGTCACACCTTGTGAGTGGCG	GCCAATAATCCAGCGGCTTGTGC	6144	Region containing tail tube protein, tape measure protein, and ankyrin repeat domain
WOSoc capsid	TGACGTTACGGCCAATCAAGA	CTATGTGCTCGCTGTTCCTACTGGAAA	2335	WOSoc major and minor capsid protein genes

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cricket genomic DNA. Sterile water was run as the negative control. A conservative cycle threshold (CT) r value of ≤ 23 for positive determination was set for both primer sets based on melting curve and relative abundance analysis corresponding to three standard deviations below the negative control detection level.

Immunohistology for visualization of *Wolbachia*

For immunohistology, 10 whole *Allonemobius* crickets were fixed in 80% ethanol, embedded in paraffin, and sectioned at 10 μm. Sections were stained with a monoclonal mouse antibody against the *Brugia malayi Wolbachia* surface protein (1:100) for 1 hour at room temperature or overnight at 4˚C using the alkaline phosphatase-anti-alkaline-phosphatase (APAAP) technique according to the manufacturer's protocol (Dako, Carpinteria, CA, USA). Hybridoma supernatant was kindly provided by Dr. Patrick Lammie and the antibody was purified as described previously [22]. All antibodies were diluted in TBS with 0.1% BSA. TBS with 1% albumin was used as a negative control, whereas sections from *B. malayi* worms from previous studies [22] were used as positive controls, respectively. After a 30 min incubation with the secondary rabbit-anti mouse IgG antibody (1:25) (Dako) followed a 30 min incubation step with alkaline-phosphatase-anti-alkaline-phosphatase (1:40) (Millipore Sigma, St. Louis, USA). As substrate, SIGMA*FAST* Fast Red TR/Naphthol AS-MX (Millipore Sigma) Tablets were used, and sections were counterstained with Mayer's hematoxylin (Millipore Sigma). Sections were analyzed using an Olympus-BX40 microscope and photographed with an Olympus DP70 camera.

DNA extraction, library preparation and whole genome sequencing

High molecular weight (HMW) DNA was purified from a homogenate of a whole single adult female cricket prepared by 15 min beating with a lead bead using the MagAttract HMW DNA Kit (Qiagen) according to manufacturer specifications, eluting in 100 μL sterile water. Presence of HMW was visualized by gel electrophoresis as a dark band (stained with DNA Gel Loading Dye 6X, Thermo Fisher) above the 15 kb DNA ladder limit (1 kb Plus DNA Ladder, Thermo Fisher). Presence of WPCP in HMW DNA was confirmed by qPCR. DNA was then purified further using AMPure XP beads (Beckman Coulter, Brea, USA) at a ratio of 1.8:1 bead to DNA sample. Library was prepared according to Oxford Nanopore's 1D Genomic DNA Ligation Protocol (Version GDE_9063_v109_revA) using the LSK-109 Ligation Sequencing Kit (Oxford Nanopore Technologies, Cambridge, England) with DNA fragments of all sizes purified using the Short Fragment Buffer. 60 μL of library containing 12 μL genomic DNA was loaded as input into the flow cell and the sequencing reaction run for 20 hours using MinKNOW GUI software (Oxford Nanopore Technologies) set to the High Accuracy Flip-Flop Model, generating 6.1 giga base pairs of data (estimated N50: 2.46 kb). Basecalling of Fast5 files into Fastq format was performed using Guppy neural network basecalling software [23]. Base statistics, average quality per read, sequence duplication level, and GC content were assessed using FastQC software (Babraham Institute, Cambridge, UK). In parallel, genomic DNA was extracted from the ovary tissue of a single cricket using Qiagen DNeasy kits as described above and sequenced using a NovaSeq 6000 Sequencing System (Illumina, San Diego, USA) with 2x150 bp output generating 12.2 giga base pairs of data, following qPCR confirmation of phage positivity in the sample

Assembly and annotation of the WOSoc genome

Putative WOSoc reads were extracted by mapping MinION sequences against published phage WO reference genomes using Minimap2 software [24]. Mapped reads were then

mapped against themselves in order to merge overlapping reads. The self-mapping output and the MinION-generated Fastq sequences were input into CANU Single Molecule Sequence Assembler [25] to generate a phage assembly consisting of multiple contigs. Quality trimming and adapter clipping of Illumina reads was performed using Trimmomatic [26]. The PRICE assembly tool [27] was used to extend existing contigs using the Illumina data. Redundans was used collapse redundant contigs, scaffold contigs, and close gaps using both the Oxford Nanopore Technologies (ONT) reads and Illumina reads. ONT reads were error-corrected using FMLRC [28] before feeding them into the Redundans pipeline [29]. We then manually curated the assembly and corrected assembly errors. Finally, Pilon automated genome assembly improvement pipeline [30] was used to polish the assembly and reduce base-call errors. Annotation of the assembled phage genome was performed using the Rapid Annotation Using Subsystem Technology Toolkit (RASTtk) SEED-based prokaryotic genome annotation engine with default presets, which has established validity for annotating phage genomes [31, 32], identifying genomic "features" (protein-coding genes and RNA). RASTk annotations were manually verified by BLASTing amino acid RASTK-identified features against *Wolbachia* phage WO (taxid:112596); features automatically assigned "hypothetical protein" were relabeled with known function if homologous (>80% query cover, >80% identity) to a described phage protein. Genomic features were visualized in scaffolds independently and manually color-coded by function using Gene Graphics visualization application [33].

PCR and Sanger sequencing for genome verification

Primers were manually designed to amplify phage tail and capsid regions based on the MinION reads (Table 1). Conventional PCR reactions were run with these primers and cricket abdomen DNA as described previously with a 60˚C annealing temperature for both primer sets. Amplicons were gel-excised, purified, and 3730 Sanger sequenced.

Phylogenetic analyses

DNA sequences of phage WO open reading frames 2 (ORF2) and 7 (ORF7), respectively coding for the large terminase subunit and minor capsid, are biomarkers known to produce highly congruent phage WO phylogenies [8]. Nucleotide sequences of ORF2 and ORF7 of WOSoc were compared to published gene sequences in NCBI Genbank. Phylogenetic trees were generated based on WOSoc ORF2 and ORF7 identity to the top 4 BLAST hits based on pairwise alignments using the NCBI BLAST Tree View Neighbor-Joining tree method with distances from the node computed by NCBI presets. ORF2 sequence was extracted from Scaffold 1 of the phage assembly, while the entire ORF7 gene was provided by Sanger sequencing of the capsid region as described above.

Phage particle purification

Phage was purified according to the protocol described in [34] with slight modification. Unless otherwise noted, all reagents were purchased from Sigma-Aldrich, St. Louis, USA. Complete mature *A. socius* males and females (N = 70) were euthanized and thoroughly homogenized in 40 mL of SM buffer (50 mM Tris-HCL, pH 7.5, 0.1 M NaCl, 10 mM $MgSO_4 \bullet 7\ H_2O$ and 0.1% w/v gelatin containing 1 μg/mL RNase A). Homogenate was incubated on ice for 1 hour followed by 11,000xg centrifugation for 10 minutes at 4˚C to remove debris. Solid polyethylene glycol (PEG) was added to homogenate to a final concentration of 10% and mixed by manual shaking for 1 minute, followed by an additional 1-hour incubation on ice and 11,000xg centrifugation for 10 minutes at 4˚C. Supernatant was discarded and the remaining pellet was resuspended in 10 mL of SM buffer. To the suspension, an equal volume of chloroform was added

followed by centrifugation at 3,000xg for 15 minutes at 4˚C to remove the PEG. The aqueous layer containing phage was filtered through a 0.22 μM vacuum filter to remove *Wolbachia* and other bacteria. Phage lysate was concentrated using Amicon Ultra-15 100 kDA Centrifugal Units (Millipore, Burlington, USA) according to [35] and reconstituted in a final volume of 1 mL of SM buffer.

Transmission electron microscopy (TEM) for visualization of WOSoc particles

From freshly caught adult female *A*. *socius*, ovaries were dissected and adsorbed to an electron transparent sample support (EM) grid. Tissue was washed in PBS and fixed in 1% glutaraldehyde for 5 minutes at room temperature, followed by two 30-second washes with deionized water. Phage particles were negatively stained in 1% uric acid for 1 minute and wicked gently and placed in a grid box to dry. Phage suspension was processed identically, with 50 μL of the concentrated suspension adsorbed to an EM grid. Samples were observed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc., Peabody, USA) equipped with an AMT 8-megapixel digital camera (Advanced Microscopy Techniques, Woburn, USA)

To confirm the presence of phage in *Wolbachia* by TEM, one half of the ovaries of each of 6 crickets was fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, USA) in 100 mM phosphate buffer, pH 7.2, for 1 hour at room temperature. The other half of the ovary sample was added to 1X PBS for DNA extraction and confirmation of *Wolbachia* presence by PCR. Only samples that were positive by PCR for Wolbachia were further processed for TEM. These samples were washed in phosphate buffer and post-fixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hour. Samples were then rinsed extensively in distilled water prior to staining with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, USA) for 1 hour. Following several rinses in distilled water, samples were dehydrated in a graded series of ethanol and embedded in Eponate 12 resin (Ted Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica Microsystems Inc., Bannockburn, USA), stained with uranyl acetate and lead citrate, and viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.) equipped with an AMT 8-megapixel digital camera (Advanced Microscopy Techniques) [36].

Results

Prevalence of phage WO and *Wolbachia* in *A*. *socius*

DNA encoding WSP was used as a marker for assessing the prevalence of *Wolbachia* in crickets. In order to confirm the DNA sequence of WSP of Missouri crickets, DNA was amplified by conventional PCR using the pre-validated WSP primers. WSP sequence showed 100% identity to WSP of *A*. *socius* from Virginia (Accession: AY705236.1). A 400 bp amplicon of phage DNA was amplified by conventional PCR using pre-validated primers corresponding to nucleotide positions 7353–7761 of phage WO of cricket *Teleogryllys taiwanemma* cricket and showed close homology to the capsid protein genes from phage WO of *Supella longipalpa* (95.50% identity, 100% query coverage, Accession: KR911861.1) and *Cadra cautella* (94.50% identity, 100% query coverage, Accession: AB478515.1). The *A*. *socius* WSP and phage WOSoc WPCP gene sequences were used to design SYBR-based real-time PCR assays for WSP and WPCP, respectively. Using the strict CT cutoff of 23 cycles, we determined that from 40 insects sampled 19 (47.5%) were positive for both WPCP and WSP DNA via qPCR with our optimized primers; three samples (7.5%) were WSP-positive but WPCP-negative (Table 2).

Table 2. Prevalence estimates of *Wolbachia* surface protein (WSP) and phage capsid protein (WPCP) DNA in *Allonemobius socius* crickets from Missouri.

		WSP		
		Positive N (%)	**Negative N (%)**	**Total N (%)**
WPCP	**Positive N (%)**	19 (47.5%)	1 (2.5%)	20 (50%)
	Negative N (%)	3 (7.5%)	17 (42.5%)	20 (50%)
	Total N (%)	22 (55%)	18 (45%)	**40 (100%)**

Estimates are based on a SYBR qPCR assay with a strict cutoff of CT $\leq$ 23 in 40 adult *A. socius* abdomen genomic DNA extracts.

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Confirmation of the *Wolbachia* prevalence results was done using an orthogonal approach, i.e visualization by immunohistology. Endobacteria were found in about 50% of the female crickets. They were detected throughout the abdomen, however density was highest in the reproductive tract (Fig 1). *Wolbachia* were detected in distinct, but varying parts of the panoistic ovarioles. In the apical part of the ovariole, *Wolbachia* were seen in the inner section of the follicle epithelium (Fig 1C), but in more mature eggs, these cells are devoid of *Wolbachia* and endobacteria were concentrated in large numbers in one pole of the egg cell (Fig 1F). The high density of *Wolbachia* in developing eggs ensures transovarial transmission of *Wolbachia* and phage WO [37]. It is expected that in this context, where *Wolbachia* negatively impacts its

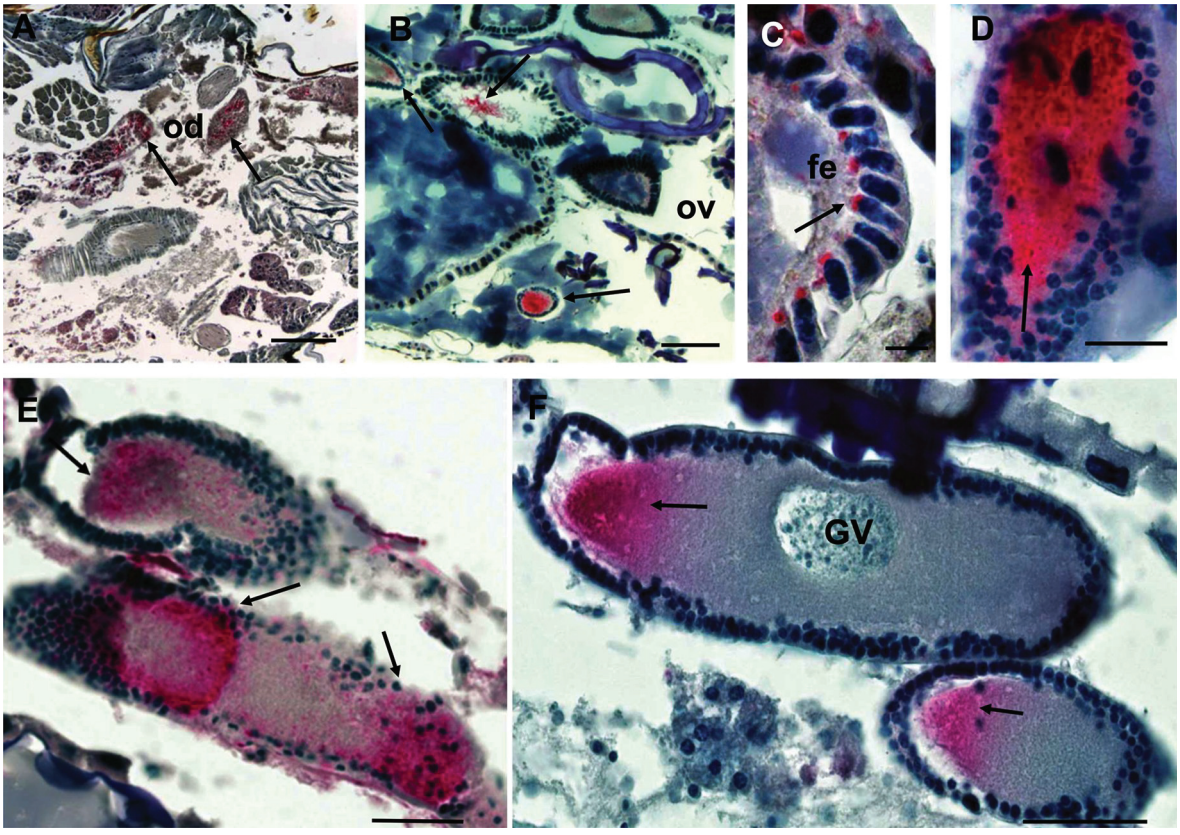

Fig 1. Immunohistological localization of *w*Soc. Black arrows indicate *Wolbachia* (red). **A.** Posterior abdomen containing intestinal tissue and oviduct containing *Wolbachia* (200μm). **B.** Ovary tissue showing dense clusters of *Wolbachia* at the site of maturing oocytes (200μm). **C.** *Wolbachia* localized to the follicle epithelium. **D** (50 μm)**, E,** and **F.** Close-up of oocytes in the female cricket oviduct showing *Wolbachia* cells in studding follicles. The nucleus (GV) is visible in the upper oocyte in **F.** (20 μm) Abbreviations: FE = follicle epithelium; od = oviduct; ov = ovaries; GV = germinal vesicle. Scale bar: 10 μm.

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host's fitness, host selection will act to limit or eliminate the endosymbiont, which may explain the less than ubiquitous *w*Soc prevalence. At the same time, high phage density favors the insect host in a parasitic *Wolbachia* context, which benefits from the reduction in *Wolbachia* density resulting from phage-mediated lysis or transcriptional regulation, which could promote phage abundance to the high levels seen in *w*Soc-infected insects [6].

Isolation and visualization of phage WO of *A. socius*

Although we detected DNA encoding capsid protein of phage WO in most *Wolbachia*-positive *A. socius* samples, it was theoretically possible that this was exclusively prophage DNA integrated into the genome of *Wolbachia* and that no phage particles were formed. Therefore, we used TEM to visualize particles of phage WO of *A. socius*. Several intracellular *Wolbachia*-containing stereotypical hexagonal phage particles were detected in ovarian tissue (Fig 2). Small clusters of *Wolbachia* cells that contained up to 30 complete phage particles per cells were obverted to mature egg cells (Fig 2A, 2B, 2D). TEM examination of the filtrate from phage precipitation revealed numerous phage WO particles. Measurement of 10 particles showed an average diameter of the icosahedral head structure of 55 nm (±7 nm SD) and 155 nm (± 20 nm SD) long, striated tails (Fig 2E, 2F).

The WOSoc genome indicates potential for lysis and transcriptional manipulation of the host

Following the detection of phage DNA in WSP-positive crickets and the demonstration of distinct phage particles, we set out to genomically characterize the novel phage WO to gain insight into its lytic potential and its similarity to known WO phages. Using the well-characterized genome of WOVitA1 (a *Wolbachia* bacteriophage found in the parasitic wasp, *Nasonia vitripennis*) as a reference genome, we identified 511 homologous WOSoc reads from the MinION run of whole-cricket homogenate HMW DNA with an average quality per read (Phred Score) of 23, corresponding to an overall base call accuracy exceeding 99%. From these reads, we assembled 12 contigs totaling 53,916 bp at an average depth of 14.6X and a GC content of 35%. After confirming and extending these contigs with Illumina reads and removing low quality reads and reads derived from the *Wolbachia* genome, the WOSoc genome was captured in 4 high-quality scaffolds totaling 55,288 bp (Fig 3). To further validate our assembly, we Sanger-sequenced PCR-amplified phage sequences from taxonomically important phage regions using primers generated from the scaffolds. These sequences collectively represented nearly one-eighth of the assembly including a continuous 6,144 bp contig containing complete open reading frames for tail morphogenesis proteins and a 2,289 bp region encoding the major and minor capsid proteins and head decoration protein (all sequence data are available in S1 File and the assembly is available in GenBank under the accession IDs MD788653-MW788656). RASTtk annotation identified 63 features which included 33 described and 30 hypothetical or unidentified ORFs based on similarity and bidirectional best hit computation. An additional 10 ORFs were manually assigned a function based on high homology to described phage elements [38]; in total, 43 described and 20 hypothetical proteins comprised the final WOSoc genome annotation (see S2 File for a complete list of these features including full-length protein and gene sequences). Of the 43 described ORFs, 17 (39.5%) encoded structural features including phage tail (N = 9), head (N = 5), and baseplate (N = 3). We also identified genes necessary for phage replication (DNA repair and transcription); a glycosyl transferase which may protect phage DNA from host nucleases or, alternatively, is used by lysogenic phages to modify host serotype [39], and a PAAR (Proline-Alanine-Alanine-aRginine)-domain-containing protein which has been hypothesized to sharpen the phage contractile tail facilitating translocation of phage DNA across the bacterial lipid membrane [40]. WOSoc was found to encode two putative lysis proteins: N-acetylmuramoyl-l-alanine amidase (NAMLAA), a

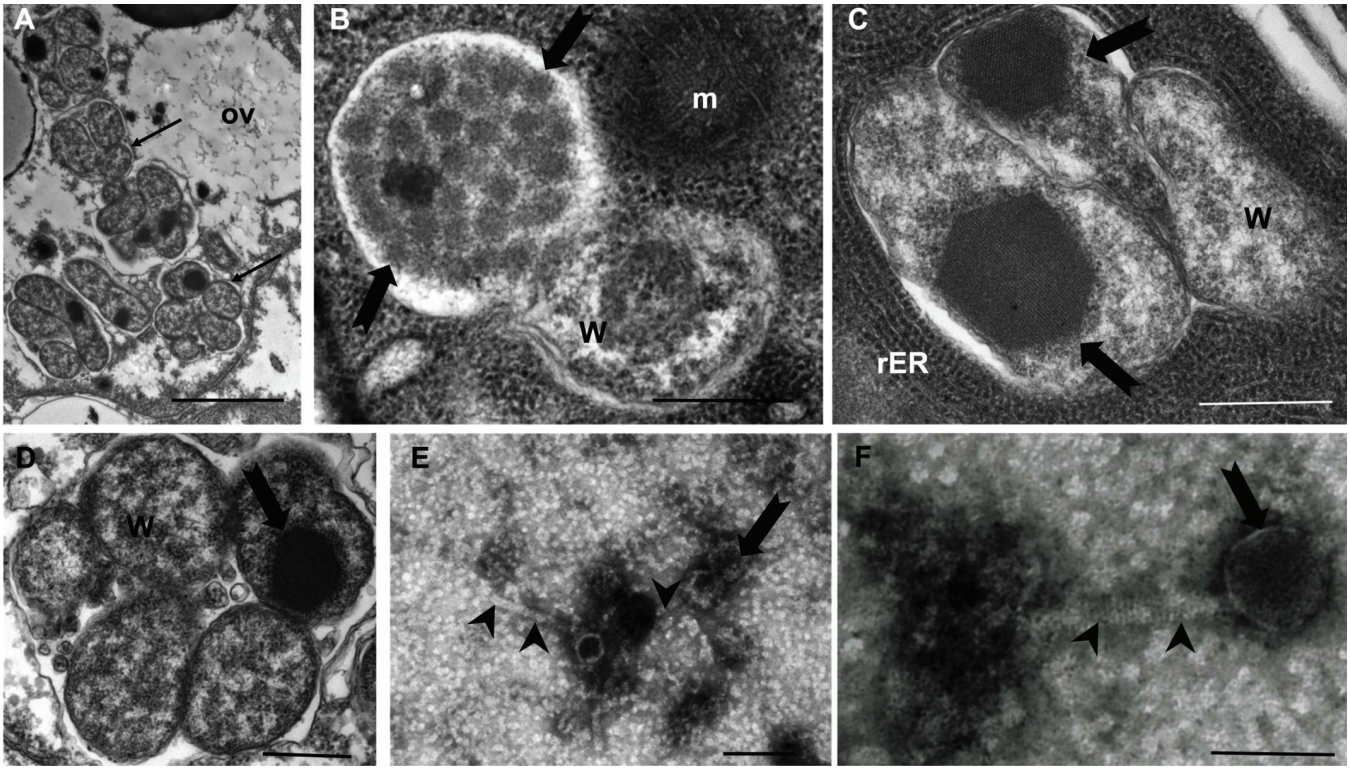

Fig 2. Transmission electron microscopy (TEM) of WOSoc particles. A. Clusters of intracellular *Wolbachia w*Soc (arrows) in the ovary of *A. socius* (scale bar 2 μm). **B.** Densely packed WOSoc phage (arrows) inside a *Wolbachia* endobacterium (scale bar 500 nm). **C.** and **D.** Compact, electron dense hexagonal arrays of WOSoc (arrows) in *Wolbachia* (scale bar 500 nm). **E.** and **F.** Complete, purified phage particles with 47 to 62 nm capsids (arrow) and 175 to 130 nm tails (arrow head, scale bar 100 nm). Abbreviations: ov, ovaries; W, *Wolbachia*, rER, rough endoplasmic reticulum; m, mitochondrion.

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powerful and highly species-specific bacterial cell wall lysin, and a patatin-family phospholipase proposed to mediate entry or exit from *Wolbachia* cells [41, 42]. Multiple WOSoc elements (site-specific resolvases and transposases) are associated with catalysis of site-specific integration into the bacterial genome [43]. We discovered four putative helix-turn-helix domains, DNA-binding motifs which regulate bacterial transcription, allowing viral transcriptional regulation [44]. We identified two virulence factors, including an NAD-dependent epimerase, which has been shown to alter cell surface properties and mediate virulence of gram-negative bacteria [45]. Over one-sixth (9,982 bp) of the WOSoc genome is comprised of ankyrin repeats, consistent with other WO phages; while the function of these repeats in phage WO is unknown, ankyrin is known to mediate protein-protein interactions in multiple domains of life, and its high abundance in the *Wolbachia* genome relative to other bacteria may be the result of genomic flux imposed by phage WO [46]. Collectively, these features suggest that WOSoc is an active, particle-forming phage containing genes, which may regulate host transcription, site-specific integration, and *Wolbachia* cell lysis, reflecting an intimate interaction with its bacterial host.

Phylogenetic analysis of WOSoc suggests a close relationship with WO phages of flies

In order to compare phage WOSoc to a larger number of phage WO for which the complete genome sequence is not available, we performed pairwise comparison with published ORF2 and ORF7 phage WO sequences. Phage WOSoc ORF2 showed the highest homology to phage WOAu of *Drosophila simulans* (94.46% nucleotide identity, 100% query coverage, Accession:

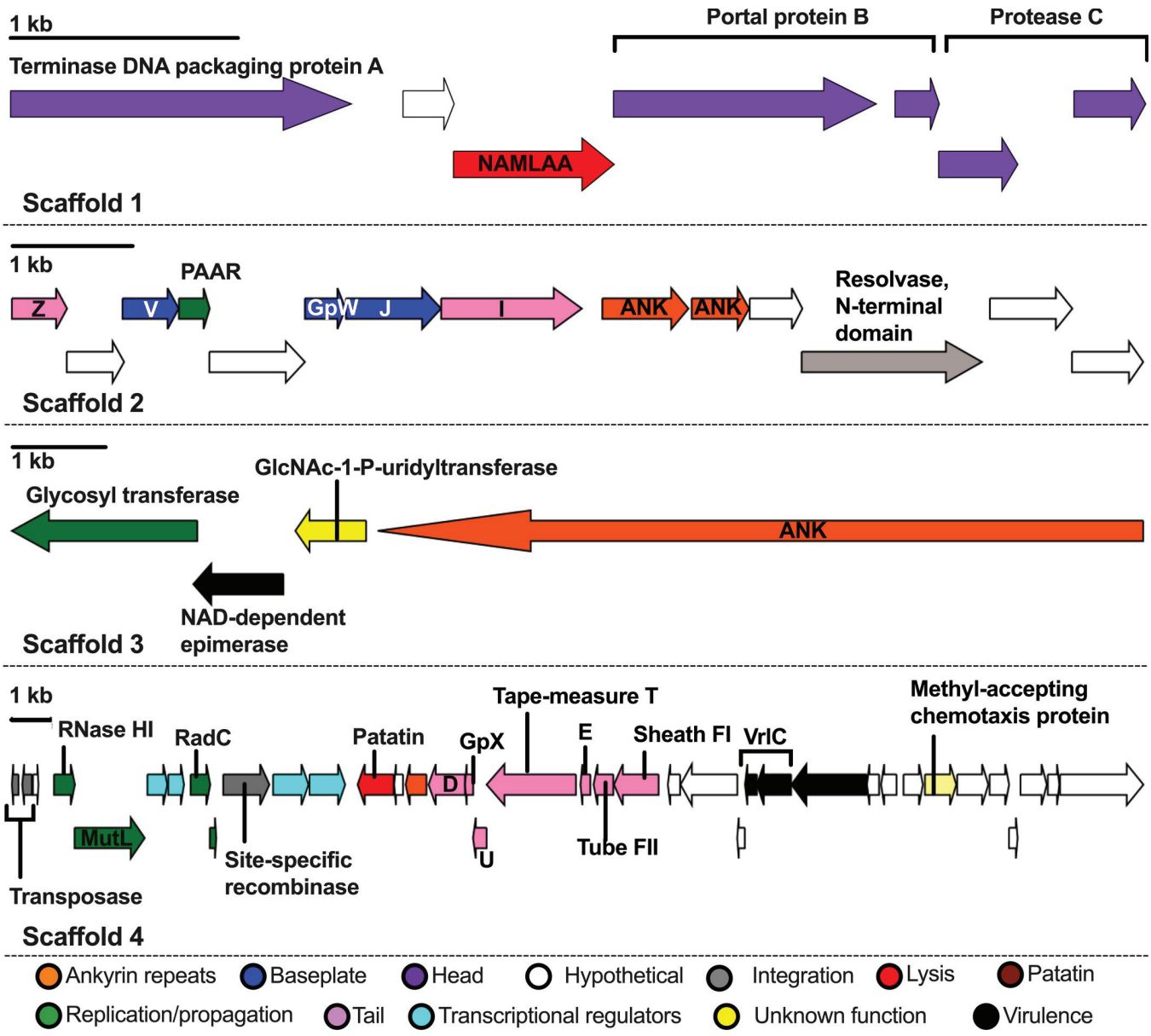

Fig 3. Annotation of the WOSoc genome. 63 features from the RASTk annotation of the 4-scaffold WOSoc assembly are displayed: ankyrin repeats (N = 4), baseplate assembly (N = 3), phage head (N = 5), integration into *Wolbachia*'s genome (N = 4), lysis of *Wolbachia* cells (N = 2), propagation (DNA replication and mismatch repair, injection machinery, protection from host endonucleases) (N = 6), tail formation (N = 9), transcriptional regulation (N = 4), virulence (N = 4), function in phage undescribed (N = 2), hypothetical proteins (N = 20). Abbreviations: NAMLAA = N-acetylmuramoyl-L-alanine amidase; ANK = ankyrin. Scale bars: 1 kb within their respective scaffolds.

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LK055284.1), while phage WOSoc ORF7 was most similar to WOCin2USA1 of cherry fruit fly, *Rhagoletis cingulata* (99.33% nucleotide identity, 100% query cover, Accession: CP072012.1.1), both insects of the order Diptera. (Fig 4).

Discussion

The present study identified for the first time a particle-forming phage WO in North American crickets and provided the whole genome sequence of phage WOSoc. About half of female

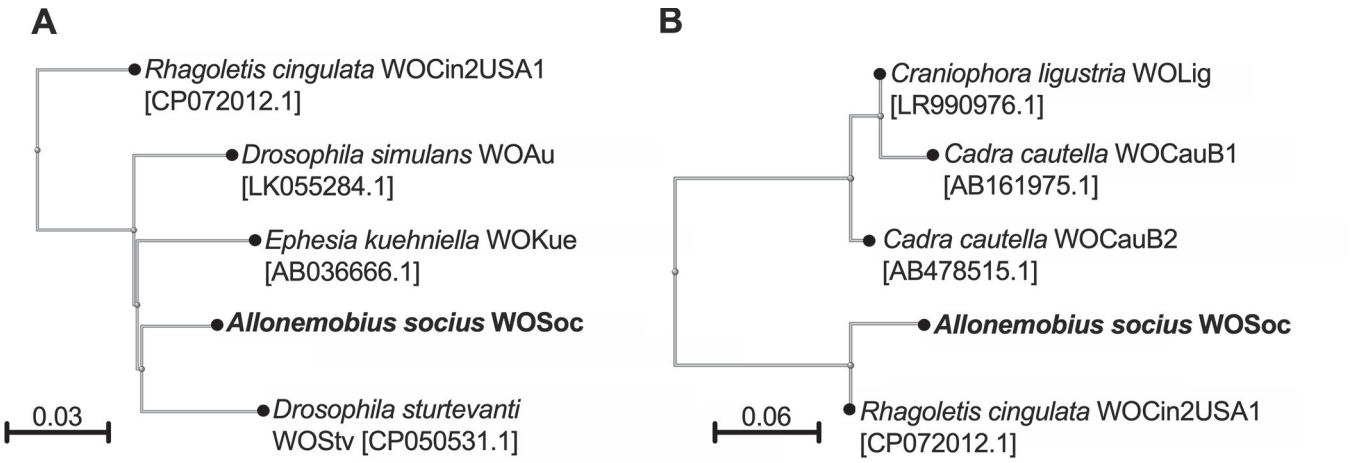

Fig 4. Phylogenetic comparison of WOSoc with published phage sequences. Neighbor-joining trees generated from published phage WO nucleotide sequences aligned to WOSoc **A.** Large terminase subunit (ORF2), showing homology to WOAu of *Drosophila simulans* and **B.** minor capsid protein (ORF7), showing high homology to WOCin2USA1 of the cherry fruit flu, *Rhagoletis cingulata*. Scale bars denote distance from the node as calculated by the NCBI Tree View software.

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A. socius crickets screened by PCR contained *Wolbachia*. Within arthropod populations, *Wolbachia* and phage WO infection prevalence closely resembled that seen in other supergroup B infected species [47–49]. More than 85% of *Wolbachia*-positive crickets were also positive for phage WO DNA, indicating co-transmission of *Wolbachia* and phage WOSoc. *Wolbachia* phage particles may be present in only a subset of *Wolbachia* infecting an individual; together with the potential for high variation of *Wolbachia* density within an insect population, it is possible we failed to pick up small amounts of phage DNA in specimens with low, but detectable *Wolbachia* density. In a DNA extract of one cricket, we detected phage WO DNA, but not *Wolbachia* DNA, which may have resulted from contamination with DNA from a phage-positive sample.

Immunohistological detection of *Wolbachia* in *A. socius* showed high densities of endobacteria in maturing egg cells. TEM examination of ovaries of *A. socius* revealed numerous phage WO particles arranging in varying structures within the *Wolbachia* cells. Occasionally, intracellular, electron-dense, hexagonal arrays where detected that could be the product of phage WOSoc self-assembly into ordered nanoarrays as seen in other bacteriophages [50]. Little information is available that describes the ultrastructure of assembled phage WO particles within *Wolbachia*, however the observed morphology of isolated phage WOSoc particles is similar to other isolated phage WO particles [51–53].

Genomic evidence showed the potential of complete phage WOSoc particle formation and validated the morphology results. Previous reports link the presence of prophage WO DNA with host phenotypes [54, 55]. However, our study showed not only the presence of prophage WO DNA, but also demonstrated particle formation and active propagation of phage WOSoc. Phage WO contains several insertions sequences (IS); generally, these are transposase-encoding genes flanked by short repeat sequences. As lateral transfer of phage WO between *Wolbachia* strains occurs, these IS may introduce insertions, deletions, and inversions in the host genome, potentially driving the high level of diversity seen among *Wolbachia* today [10, 15, 56]. Phages are considered to be relatively host-specific, but potential host species can be predicted based on sequences of annotated receptor-binding proteins [57]. Unfortunately, these sequences are not always available and further experimental studies have to elucidate the host range of phage WOSoc and its potential to genetically manipulate *Wolbachia*. We have

identified resolvases and transposases, which may recognize and bind host DNA to mediate site-specific integration, providing a mechanism for lysogeny [58], as well as proteins which may mediate *Wolbachia* cell penetration and lysis. The isolation of phage WOSoc offers exciting possibilities for understanding the evolutionary and current role of *Wolbachia*'s only known mobile genetic element and an active regulator of *Wolbachia* density on the endosymbiont-induced characteristics such as cytoplasmic incompatibility and reproductive support. Future studies may show whether phage WOSoc plays a role in the spermathecal duct shortening which is a well-documented effect of *Wolbachia* in *Allonemobius* genus crickets [16].

So far, there are only a handful of complete phage WO genome sequences available in the public databases, and this study has expanded the list by adding a validated 55 kilobase genome of phage WOSoc. Like closely related active phage WO of *Cadra cautella*, WOSoc contains intact open reading frames encoding proteins essential to phage particle formation, including tail morphogenesis and DNA packaging, which are absent in inactive, prophages of *Wolbachia* [59].

Wolbachia are considered as targets for alternative chemotherapy of human filariasis, caused by parasitic nematodes [60] and as alternative tools for vector control [61]. Traditional techniques to control vector-borne diseases (particularly mosquito-transmitted diseases including dengue, malaria, yellow fever, and filariasis) have relied often on the use of larvicides and insecticides, incurring technical and financial challenges while risking toxicity and off-target environmental effects [62, 63]. Transfecting mosquitos with *Wolbachia* has shown promise for reducing vector population size (by nature of cytoplasmic compatibility) and vector competence [64–66]. A better understanding of the role of phage WO in regulating *Wolbachia* populations is important to optimize these intervention strategies, which are limited by *Wolbachia*'s host specificity and phenotypic effects. Future studies are needed to show whether phage WOSoc can be utilized to manipulate *Wolbachia* in *A. socius* or other host species infected by *Wolbachia*.

Supporting information

S1 File. Assembled nucleotide sequences of phage WOSoc. Sequences in FASTA format of the assembled scaffolds 1–4, the head decoration protein, major capsid protein, and minor capsid protein.
(DOCX)

S2 File. Detailed annotation of the WOSoc genome. Genome annotation showing start and stop of 63 open reading frames.
(XLS)

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