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Novel host-bacterial symbioses revealed: characterization of *Wolbachia* in arthropods of western North America

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Abstract.—Arthropod-bacterial symbioses are prevalent and play significant roles in ecosystems and the economy, and in some cases, habitat invasion. *Wolbachia* bacteria form symbiotic associations with a wide range of arthropod hosts and can affect both host reproduction and resistance to viral infections. The extent to which *Wolbachia* infects different arthropod species is fundamental not only to host biology, but also to the health of humans, ecosystems, and agriculture. Much of what we know about the effects of *Wolbachia* comes from a few key taxa, such as the model organism *Drosophila melanogaster* and the mosquito vector of human disease, *Aedes aegypti*. The majority of arthropods, even at higher taxonomic levels, have not been tested for infection, with a lack of surveys conducted in western North America. We screened and characterized *Wolbachia* diversity in arthropods in 2 types of collections in western North America: broad surveys and targeted collections of species known to be infected with *Wolbachia.* Our goals were to (1) find new *Wolbachia* strains and hosts, (2) characterize *Wolbachia* in commonly studied taxa to see whether there are different infection frequencies or strain types in this location, and (3) compare new *Wolbachia* strains in western North America to previously characterized strains. PCR screening of broadly sampled arthropods with *Wolbachia*-specific *16S rDNA* (*W16S*) identified 5 novel host species. Three of these are invasive: a ground beetle, *Nebria brevicollis* (Coleoptera: Carabidae); a cereal crop agricultural pest, *Oulema melanopus* (Coleoptera: Chrysomelidae); and a residential nuisance pest, *Raglius alboacuminatus* (Hemiptera: Rhyparochromidae). The crab spider *Philodromus dispar* (Araneae: Philodromidae) is nonnative, though not considered invasive, and the hyaline grass bug *Liorhyssus hyalinus* (Hemiptera: Rhopalidae) is of undetermined origin. To characterize 9 novel *Wolbachia* strains in our collections, we analyzed the 5-gene MultiLocus Sequence Type (MLST) and the *Wolbachia surface protein* gene (*wsp*). We identified 10 novel alleles among 5 MLST genes and 10 novel alleles of the highly variable regions (HVR) of *wsp.* This is the first report of *Wolbachia* hosts and strain identification from the Pacific Northwest and Rocky Mountain regions. These studies contribute to our understanding of the natural history of arthropod hosts, the biogeography of *Wolbachia,* and host-symbiont evolution. Moreover, strain identification is the first step in implementing *Wolbachia*-based biocontrol for conservation and pest mitigation, including control of the invasive *N. brevicollis, O. melanopus,* and *R. alboacuminatus* reported herein.

Resumen.—Las simbiosis artrópodo-bacteria prevalecen y juegan un papel importante en los ecosistemas y en la economía, y en algunos casos, en la invasión del hábitat. Las bacterias *Wolbachia* forman asociaciones simbióticas con una amplia gama de hospederos artrópodos, y pueden afectar la reproducción del hospedero, así como su resistencia a las infecciones virales. El grado en que *Wolbachia* infecta diferentes especies de artrópodos es fundamental no sólo para la biología del hospedero, sino también para la salud de los humanos, los ecosistemas y la agricultura. Gran parte de lo que sabemos sobre los efectos de *Wolbachia* proviene de pocos taxones claves, tales como el organismo modelo *Drosophila melanogaster* y el mosquito vector de la enfermedad humana, *Aedes aegypti*. La mayoría de los artrópodos, incluso en niveles taxonómicos más altos, no han sido sometidos a pruebas de infección, con mayor carencia de muestreos en el oeste de América del Norte. En este estudio, examinamos y caracterizamos la diversidad de *Wolbachia* en artrópodos, en dos tipos de colecciones del oeste de América del Norte. Estos estudios fueron llevados a cabo en especies objetivo que se sabe son infectadas con *Wolbachia.* Nuestros objetivos fueron: (1) encontrar nuevas cepas y huéspedes de *Wolbachia*, (2) caracterizar a *Wolbachia* en taxones comúnmente estudiados, para ver si hay diferentes frecuencias de infección o tipos de cepas en esta ubicación, y (3) comparar nuevas cepas de *Wolbachia* al oeste de América del Norte con cepas previamente caracterizadas. Mediante, la detección por Reacción en Cadena de la Polimerasa (PCR, por sus siglas en inglés) en artrópodos ampliamente muestreados con *Wolbachia* el ADNr específico 16S (W16S) identificó cinco nuevas especies de hospederos, tres de las cuales son invasivas, (1) el escarabajo de tierra *Nebria brevicollis* (Coleoptera: Carabidae), (2) una plaga de cultivo de cereales *Oulema melanopus* (Coleoptera: Chrysomelidae), y (3) una plaga doméstica dañina *Raglius alboacuminatus* (Hemiptera: Rhyparochromidae). La araña cangrejo *Philodromus dispar* (Araneae: Philodromidae) no es nativa y no se considera invasiva, y la chinche *Liorhyssus*

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hyalinus (Hemiptera: Rhopalidae) es de origen la indeterminado. Para caracterizar nueve cepas nuevas de *Wolbachia* en nuestras colecciones, analizamos cinco genes por medio de tipificación multilocus de secuencias (MLST, por sus siglas en inglés) y el gen de la *proteína de superficie de Wolbachia* (*wsp,* por sus siglas en inglés). Identificamos 10 alelos nuevos en cinco genes MLST y 10 alelos nuevos en regiones altamente variables (HVR, por sus siglas en inglés) de *wsp.* Este es el primer registro de hospederos y cepas de *Wolbachia* en el Pacífico Noroeste y en las Montañas Rocallosas (Rocky Mountains). Estos resultados contribuyen a nuestra comprensión de la historia natural de los artrópodos hospederos, la biogeografía de *Wolbachia* y la evolución parásito-hospedero. Además, la identificación de las cepas, es el primer paso para implementar un control biológico basado en *Wolbachia,* para la conservación y mitigación de plagas, incluyendo a los invasores *N. brevicollis, O. melanopus,* y *R. alboacuminatus* reportados en este trabajo.

Arthropod-bacterial symbioses are prevalent and affect host reproduction, resistance to viral infections, and in some cases, habitat invasion. Invasive species and their symbionts play significant roles in ecosystems by impacting the environment and economy (Holway et al. 1998, Goulson 2003, Snyder and Evans 2006, Lu et al. 2016). Bacterial symbioses abound within arthropods and include *Wolbachia, Spiroplasma,* and *Cardinium* (Goodacre et al. 2006, Duron et al. 2008, Zhang et al. 2016). These associations are established through horizontal transmission (via the environment) or vertical transmission (via germ cells) (Baldo et al. 2006, Hurst 2017). Symbiotic associations range from mutualism to parasitism (Correa and Ballard 2016), in which host and symbiont partners intimately affect each other, from gene expression and metabolism to behavior and evolution (Douglas 2014, Shropshire and Bordenstein 2016, Hurst 2017), sometimes resulting in co-evolution. For example, we see parallel evolution between lachnid aphids and their nutritional endosymbionts (*Buchnera*) (Wilson and Duncan 2015, Chen et al. 2017).

Wolbachia is arguably the world's most charismatic bacterial symbiont, infecting a wide range of arthropod hosts, including insects, spiders, and scorpions (Duron et al. 2008). This obligate intracellular symbiont is vertically transmitted through the host egg, with a high fidelity of transmission to 97% of *Drosophila melanogaster* offspring (Hoffmann et al. 1998). *Wolbachia* can also be transmitted horizontally, from parasitoids, wounding, or predator consumption of infected prey (Rowley et al. 2004). *Wolbachia* causes a range of reproductive effects, including feminization, parthenogenesis, and male killing, all of which select for *Wolbachia*-infected female offspring. Additionally, *Wolbachia* causes cytoplasmic incompatibility, which dramatically decreases the number of viable offspring from matings between uninfected females and *Wolbachia*-infected males. Due to these effects, *Wolbachia*-based biocontrol has been developed to reduce vector-borne human disease (e.g., mosquitoes transmitting Dengue and Zika) (Frentiu et al. 2014, Hoffmann et al. 2015, Dutra et al. 2016) and to control agricultural pests (Zhou and Li 2016). Characterizing *Wolbachia* strains in novel hosts not only increases our understanding of the evolution of this widespread symbiosis, but also has implications for pest and disease management.

Surveys identifying *Wolbachia* infections across wild arthropod taxa have largely been conducted outside of the western hemisphere (e.g., Asia—Kittayapong et al. 2000; Australasia—Hariri et al. 1998, Wenseleers et al. 1998; Pacific islands—Bailly-Bechet et al. 2017, Bridgeman et al. 2018; Europe—Ricci et al. 2002, Duron et al. 2008). Only 2 broad arthropod surveys for *Wolbachia* infections have been performed in North America, specifically in the United States of America (USA), in the Midwest and the South (Jeyaprakash and Hoy 2000, Werren and Windsor 2000). Both surveys tested specimens from 13 arthropod orders and found infection rates of 19.3% and 76%, respectively. Targeted surveys of particular species have been performed within the USA in the Midwest, the Northeast, the South, and California. The most commonly studied hosts, mosquitoes (e.g., *Culex pipiens*) and the fruit fly *D. melanogaster*, have only been surveyed at a few locations (Rasgon and Scott 2004, Duron et al. 2005, Verspoor and Haddrill 2011), while other studies examined stalk-eyed flies and butterflies (Hariri et al. 1998, Nice et al. 2009). Although surveys have been useful, neither broad surveys of arthropods nor targeted surveys of *D. melanogaster* and *C. pipiens* have been done in the western USA.

A successful and widely used approach to define and classify different *Wolbachia* strains is MultiLocus Sequence Typing (MLST), a universal genotyping tool designed to characterize *Wolbachia* genetic diversity within and

among arthropod species. The alleles at 5 housekeeping genes (*coxA, ftsZ, fbpA, hcpA,* and *gatB*) are used to determine the sequence type (ST) in the MLST database (Baldo et al. 2006). Sequences of the *Wolbachia surface protein* (*wsp*) gene and its 4 hypervariable regions (HVR 1–4) are also cataloged in this database (Baldo et al. 2005). Analysis of *Wolbachia* MLST data is used for several reasons: (1) to determine taxonomic classification of *Wolbachia* supergroup (Baldo et al. 2006), (2) to assign a specific strain name (ST), and (3) to make comparisons within the MLST database (https://pubmlst.org/wolbachia/), which catalogs hundreds of strains and thousands of isolates (Jolley and Maiden 2010). Based on phylogenetic analyses of MLST genotypes, *Wolbachia* is taxonomically subdivided into supergroups A–Q (Ma et al. 2017), with the arthropod-infecting *Wolbachia* typically assigned to supergroup A or B (Gerth et al. 2014).

Host-*Wolbachia* evolutionary history has been described by using targeted surveys within taxonomic groups and MLST genotyping. A study of funnel-web spiders found 3 distinct *Wolbachia* strains and inferred horizontal transmission across species, possibly through parasitoids (Baldo et al. 2008). In contrast to the horizontal transmission found for many other arthropod-*Wolbachia* associations tested thus far (Russell et al. 2009, Stahlhut et al. 2010, Gerth et al. 2013), the phylogeny for *Wolbachia* in aquatic beetles (Coleoptera: Hydraenidae) revealed genera-specific infections, supporting vertical transmission (Sontowski et al. 2015). Finally, a study of 120 species in 13 families of butterflies and moths delineated widespread MLST gene recombination (Ilinsky and Kosterin 2017). These studies contribute to our understanding of *Wolbachia* transmission across arthropod hosts and reconstruction of *Wolbachia*-host evolution.

Our study had 3 objectives to address the gap in host-*Wolbachia* associations within the western regions of the USA. First, we performed a broad survey by collecting and testing western North American arthropods for *Wolbachia* infection. Second, we performed targeted surveys of wild populations of the fruit fly and model organism (*D. melanogaster*), and 2 mosquito species (*Aedes vexans* and the West Nile Virus vector *C. pipiens*). *Wolbachia* infection rates of these widely known hosts were used for comparison to other studies. Third, we used MLST and *wsp* analyses to characterize 9 infections identified through the broad survey. We report that broadly collected arthropods in data-poor regions yielded novel hosts and novel *Wolbachia* strains, each of which contributes to understanding host-symbiont evolution. Moreover, strain identification is the first step in implementing *Wolbachia*-based biocontrol for conservation or pest mitigation, including control of the invasive species reported herein.

Methods

Broad Survey Field Collections

Field collections consisted of a broad survey and specific targeted surveys. A broad group of 281 specimens from phylum Arthropoda was collected in the western USA from the campuses of Metropolitan State University of Denver, Colorado (MSUD), and Pacific University, Forest Grove, Oregon (PACU) (Table 1). Arthropods were collected by hand, without collection traps, from mostly terrestrial, but sometimes aquatic or subterranean environments, with the goal of collecting a variety of species from different microhabitats. The first specimens encountered were collected. MSUD collections occurred during October of 2011, 2012, and 2013, as well as March 2014. PACU collections occurred during September 2014, February 2015, and April 2016 and 2017. Specimens were immediately placed live into vials with 95% ethanol. They were transferred to individual tubes within 24 h and stored in 95% ethanol at −20 °C, typically for 2–5 d until they were photographed, identified to order, and prepared for DNA extraction.

Targeted Surveys Field Collections

The following arthropod taxa were collected in our targeted surveys: (a) fruit flies, (b) mosquitoes, (c) the ground beetle *N. brevicollis*, and (d) the orb-weaving spider *Zygiella x-notata*. The first 2 groups were selected for in-depth targeted surveys of taxa commonly infected by *Wolbachia* (Kittayapong et al. 2000, Verspoor and Haddrill 2011). *Nebria brevicollis* and *Z. x-notata* were added because preliminary data from broad sampling indicated that they were novel *Wolbachia* hosts. *Nebria brevicollis* specimens were collected by hand from under concrete blocks or fallen tree trunks at 3 sites in Oregon between May 2017

TABLE 1. Sampling sites in Colorado (CO) and Oregon (OR) for broad and targeted arthropod collections.

Site name	Site code	State	Latitude	Longitude	Study
Banner Lakes Wetlands	BANN	CO	40°04′35.62″	−104°33′46.77″	Targeted[M]
Denver Residence	DENV	CO	39°44′7.16″	−104°55′44.05″	Targeted[M]
Chatfield Reservoir Wetlands	CHAT	CO	39°31′32.56″	−105°04′54.50″	Targeted[M]
Metropolitan State University of Denver	MSUD	CO	39°44′41.97″	−105°00′05.08″	Broad
Wadsworth and Yale Green Belt	WYGB	CO	39°40′5.07″	−105°05′11.36″	Targeted[M]
Purple Park	PUPA	CO	39°56′33.26″	−105°09′37.47″	Targeted[M]
Apolloni Vineyards	APOL	OR	45°37′23.74″	−123°13′2.14″	Targeted[FF]
Cornelius Residence	CORN	OR	45°30′54.49″	−123°03′10.53″	Targeted[FF]
David Hill Vineyards	DAHI	OR	45°32′51.33″	−123°09′26.10″	Targeted[FF]
Forest Grove 1 Residence	FGO1	OR	45°30′40.73″	−123°05′58.74″	Targeted[FF, NB]
Forest Grove 2 Residence	FGO2	OR	45°31′01.43″	−123°06′16.18″	Targeted[FF]
Forest Grove 3 Residence	FGO3	OR	45°31′03.06″	−123°06′10.40″	Targeted[FF]
Forest Grove 4 Residence	FGO4	OR	45°31′48.69″	−123°06′34.59″	Targeted[FF]
Nehalem Residence	NEHA	OR	45°48′04.92″	−123°47′27.28″	Targeted[FF]
Pacific University	PACU	OR	45°31′15.89″	−123°06′33.82″	Broad, Targeted[NB, ZX]
Plum Hill Vineyards	PLHI	OR	45°28′26.11″	−123°08′32.24″	Targeted[FF]
Portland Residence	PORT	OR	45°29′21.74″	−122°42′24.45″	Targeted[FF]
VanderZanden Farms	HBRO	OR	45°33′48″	−122°58′17″	Targeted[NB]

[M]Mosquitoes
[FF]Fruit flies
[NB]*Nebria brevicollis*
[ZX]*Zygiella x-notata*

TABLE 2. Numbers of *Wolbachia*-infected and total collected (in parentheses) *Nebria brevicollis, Zygiella x-notata,* and *Drosophila melanogaster* during targeted sampling in Oregon from 2014 to 2019. Collection sites correspond to Table 1. A dash indicates that specimens were not collected. Sex was not determined for individuals of *N. brevicollis* or *Z. x-notata.*

			D. melanogaster	
Collection site	*N. brevicollis*	*Z. x-notata*	♀	♂
APOL	—	—	8 (18)	3 (7)
CORN	—	—	9 (15)	1 (1)
DAHI	—	—	18 (32)	6 (10)
FGO1	15 (15)	—	21 (28)	7 (13)
FGO2	—	—	2 (2)	3 (4)
FGO3	—	—	19 (35)	2 (8)
FGO4	—	—	2 (3)	—
HBRO	1 (1)	—	—	—
NEHA	—	—	5 (10)	—
PACU	9 (10)	14 (33)	20 (30)	6 (7)
PLHI	—	—	13 (25)	8 (13)

and June 2018. *Zygiella x-notata* specimens were collected from buildings by hand at one site in Oregon in September 2018 and April 2019 (Table 2). All specimens were immediately placed live into 95% ethanol. They were transferred to individual tubes within 24 h and stored in 95% ethanol at −20 °C for up to 4 months before DNA extraction.

In targeted mosquito surveys, *C. pipiens* and *A. vexans* were collected by aspirating adults or by dip-netting larvae at urban, suburban, and rural sites in Colorado during September and October of 2009 and 2010 (Table 1). Adult field-collected mosquitoes were transferred live into individual test tubes with 95% ethanol and stored in a cooler with ice for up to 12 h. Specimens were stored at −20 °C until DNA extraction. Collected larvae were reared to adults at room temperature. Within 24 h of eclosion, adult mosquitoes were aspirated and immediately transferred live into individual vials with 95% ethanol. Colorado Mosquito Control (Broomfield, CO) staff performed species identification based on morphology.

In targeted fruit fly surveys, *D. melanogaster* specimens were collected using polyethylene

plastic containers baited with overly ripe fruit, water, and yeast. Collections were made over the course of 5 d at private residences and 3 wineries in western Oregon during September 2014, July and October 2015, and July, September, and October 2016 (Table 1). Traps with lids askew were placed at the locations and set up indoors for all sites except FGO4. At FGO4, adults were aspirated directly from the field site. Following collection, live fruit flies were anaesthetized using carbon dioxide. A dissecting microscope was used to confirm fruit fly species. Visual examination of genitalia and presence of male-specific sex combs (*D. melanogaster*) were used for sex determination. Male and female specimens were placed live into 95% ethanol, with one fly per tube, and stored at −20 °C until DNA extraction, which was performed within 2 weeks. Voucher specimens from the same locality were saved for targeted surveys.

DNA Extraction, PCR Amplification, Sequence Analysis, and Strain Typing

DNA was extracted from individual specimens using Qiagen DNeasy Blood and Tissue (Valencia, CA) kits according to manufacturer specifications. For the broad survey collection, small specimens such as fruit flies or mosquitoes were used whole, whereas for larger specimens (such as wasps or beetles), an approximately 2-mm by 3-mm section of the upper abdomen was used. For targeted collections of *N. brevicollis* and *Z. x-notata*, DNA was extracted only from legs to avoid false positives of *Wolbachia*-infected prey. All DNA extractions were stored at −20 °C. *Wolbachia* infection status was determined via co-PCR of *Wolbachia* (*16S rRNA; W16S*, 438 bp) and arthropods (*cytochrome oxidase subunit I, COI,* 708 bp) by using previously described methods (Folmer et al. 1994, Werren and Windsor 2000). Each PCR reaction was carried out in a total reaction volume of 25 μL with one PCR-ready bead (GE Healthcare, Chicago, IL), forward and reverse primers at 0.4 μM each, and 2 μL of DNA template. Reaction programs followed a thermocycler profile of initial denaturation at 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min, and a final extension at 72 °C for 10 min. PCR products were visualized with 1.5% agarose gel electrophoresis to determine *Wolbachia* infection status. Controls used in parallel were a known *Wolbachia*-infected *D. melanogaster* and a water-only negative control. The arthropod *COI* amplicon was used as an internal positive control for DNA quality for each specimen. Specimens with amplification of *W16S* and a robust *COI* band were scored as *Wolbachia* infected, whereas specimens yielding only a robust *COI* band were scored as uninfected. Weak or absent *COI* bands indicated a poor PCR reaction and were omitted from the broad survey (10.68%). *Wolbachia* infection status was confirmed for most specimens by sequencing *W16S* (Eurofins; Louisville, KY).

In the *N. brevicollis*-targeted collections (n = 31 specimens), 16.13% had weak or no amplification of *COI* and were removed from the study. Similarly, in targeted collections of *D. melanogaster* (n = 261), *C. pipiens* (n = 126), *A. vexans* (n = 27), and *Z. x-notata* (n = 33), we omitted poor PCR reactions (2.98%, 5.26%, 10.00%, and 0%, respectively). These reactions were subjected to secondary screening by doubling the DNA and decreasing DNA by half, for which *D. melanogaster*, *C. pipiens*, and *A. vexans* were then scored (8.17%, 0.75%, and 0% of total screened, respectively).

PCR amplification and sequencing were used to characterize the *Wolbachia* strain within targeted collections of *N. brevicollis*, *D. melanogaster*, and *C. pipiens*, as well as from select host specimens from the broad survey. All 5 loci of the MLST system (Baldo et al. 2006) and *wsp* were amplified (Zhou et al. 1998).

MLST genes and *wsp* (including the HVR regions) were individually amplified using previously published, gene-specific forward and reverse primers (Baldo et al. 2006). Each 25-μL volume PCR reaction included 0.8 μM primers (or 0.4 μM for *wsp*), 1 PCR ready bead, and 2 μL of DNA template. Thermocycling included an initial denaturation of 94 °C for 2 min, 36 cycles of 94 °C for 30 s, annealing temperature (*ftsZ*, 54 °C; *hcpA*, 53 °C; *gatB*, 54 °C; *coxA*, 55 °C; *fbpA*, 59 °C; *wsp*, 59 °C) for 45 s, and 72 °C for 1.5 min and final extension of 70 °C for 10 min. Products were visualized on 1.5% agarose gels for expected length and then Sanger-sequenced using PCR primers by Eurofins (Louisville, KY). Forward and reverse sequences were checked and assembled for each gene across individuals using Geneious R9 (www.geneious.com; Kearse et al. 2012). *COI* was also sequenced for host confirmation using mitochondrial barcoding (Hebert et al. 2003).

MLST allele numbers for each locus were determined by sequence query of the MLST database (https://pubmlst.org/wolbachia/). Identical sequences of standardized length were assigned the same allele number. Sequences differing by one or more nucleotides from those in the database were submitted to the MLST database curator and assigned a unique allele number. Novel combinations of alleles for all 5 MLST genes were submitted and designated with a unique ST (sequence type) integer. Sequences shorter than the required MLST length were submitted to GenBank (https://www.ncbi.nlm.nih.gov/nucleotide/) (Table 3). Novel strains with unique MLST or *wsp* allele sequences or combinations of alleles were named using the standard naming scheme of a lowercase "*w*" followed by 3–4 letters referring to host taxonomy (e.g., genus and species).

Phylogenetic Analyses

We estimated the phylogenetic relationships of novel *Wolbachia* strains (Table 3) with an MLST data set including 109 different strains (Supplementary Material 1) to (1) assign *Wolbachia* supergroup and (2) place novel *Wolbachia* strains relative to previously characterized STs. Representative sequences from supergroups A, B, D, F, and H were included, similar to previous study designs (Montagna et al. 2014, Ali et al. 2018a, 2018b). Furthermore, we included select STs from the same host order as our novel strains: Araneae, Coleoptera, Hemiptera, and Isopoda. STs identified as closest matches to each of our novel strains (i.e., results from the "search by combination of alleles" and "exact or nearest match" functions of the MLST database) were included. Published phylogenetic analyses were used to limit the number of STs to one *Wolbachia* strain per major clade for hosts from targeted studies of Araneae (Baldo et al. 2008, Yun et al. 2011) and Hemiptera (Watanabe et al. 2012, Guidolin and Cônsoli 2013, Bing et al. 2014). All 25 STs from coleopteran hosts in the MLST database (accessed 21 January 2019) and GenBank sequences corresponding to 14 aquatic coleopteran *Wolbachia* strains (Sontowski et al. 2015) were included. Because exploratory trees identified a lepidopteran ST as most closely related to the *Wolbachia* strain infecting *N. brevicollis*, the data set was expanded to include additional STs from lepidopteran hosts. One *Wolbachia* strain per clade was included from a previously published targeted lepidopteran study (Ilinsky and Kosterin 2017).

Locus-specific alignments of the 5 MLST genes, as well as concatenated alignment, were done using ClustalW (Larkin et al. 2007) within Geneious. We identified the open reading frames to verify alignments. Alignment of sequences for each gene was straightforward, with only 26 sequences in the *fbpA* gene containing a 6 nucleotide indel. The data matrix was partitioned by gene, with 2 partitions per gene (codon positions 1 + 2, and codon position 3). We estimated the best-fit nucleotide substitution model for each partition using MrModeltest v2 (Nylander et al. 2004) within PAUP* version 4.0b (Swofford 2002) (Supplementary Material 2).

Bayesian estimates of the phylogeny for the partitioned, concatenated alignment, including all 5 MLST genes, were conducted in MrBayes version 3.2.7 (Ronquist and Huelsenbeck 2003, Ronquist et al. 2012). Identical haplotypes were collapsed to single representatives to reduce run time. The final 2 runs each consisted of 4 chains of 50 million generations sampled every 5000 generations. Mixing within runs and convergence between runs were checked in Tracer version 1.6 (Rambaut et al. 2018). The posterior distribution of trees was summarized on a consensus tree after the first 25% of our samples were discarded as burn-in.

RESULTS

Wolbachia Infection in Broadly Collected Arthropods

Of the arthropods collected from the campuses of MSUD and PACU, 251 total individuals were from 5 taxonomic classes and 11 orders (Table 4). *Wolbachia* infections were detected in 5 orders, of which 10 infected taxa were identified to genus or species. Sample sizes were small for many specimens identified to species ($n < 6$). Based on preliminary findings (Table 1), *N. brevicollis* and *Z. x-notata* were selected for targeted surveys. Targeted collections of *N. brevicollis* beetles from 3 sites in Oregon resulted in a 96.15% *Wolbachia* infection rate ($n = 26$). From one site in Oregon, 42.42% of *Z. x-notata* spiders were *Wolbachia* infected ($n = 33$, Table 2).

TABLE 3. MLST-derived *Wolbachia* supergroups and allele profiles in host specimens from broad and targeted collections in Colorado and Oregon.

Host species	Strain[a]	n[b]	Study: site(s)[c]	Super-group[d]	ST: ID[e]	Alleles[f]									
						gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*	*wsp*	HVR1	HVR2	HVR3	HVR4
Zygiella x-notata	***w*Zxno**	3	Broad: PACU	A	**485:** 1849 1850	**277**	**268**	**299**	**236**	**442**	MH759011	**257**	**290**	**286**	—
Philodromus dispar	***w*Pdis**	1	Broad: PACU	—[g]	— 1878	—	—	—	—	—	MK449428	**256**	**289**	**285**	—
Porcellio spp.	***w*Porc**	2	Broad: PACU	B	— 1862	MK449432	13	MK456487	**237**	401	—	—	—	—	—
Armadillidium vulgare	***w*Vul3**	4	Broad: PACU	B	— 1863	13	13	—	9	13	MK449429	—	12	21	245
Nebria brevicollis	***w*NBre**	4 4	Targeted: PACU FGO1	A	**502:** 1864	84	**270**	196	54	64	MK449431	**255**	175	204	165
Oulema melanopus	***w*OMe1**	1	Broad: PACU	A	— 1860	—	**269**	MH759010	**238**	36	—	—	—	—	—
Culex pipiens	*w*Pip	1 1 1	Targeted: WYGB DENV PUPA	B	9	4	3	3	22	4	10	10	8	10	8
Drosophila melanogaster	*w*Mel	1 1 1	Targeted: FGO1 NEHA CORN	A	1	1	1	1	1	1	31	1	12	21	24
Drosophila melanogaster	*w*Mel	5 3	Targeted: APOL DAHI	—	—	—	—	—	1	—	31	1	12	21	24
Liorhyssus hyalinus	***w*LHya**	1	Broad: MSUD	B	— 1865	**278**	14	MH759009	22	9	MK449427	69	17	**288**	—
Raglius alboacuminatus	***w*RAlb**	1	Broad: PACU	B	— 1859	188	—	—	20	25	MK449430	125	**291**	**287**	—
Polistes dominula	***w*Pdom2**	1	Broad: MSUD	B	— 1861	9	9	MK456485	MK456486	10	63	19	17	24	33

[a]Novel hosts, strains, and alleles are in bold.
[b]Number of specimens (n) sequenced.
[c]Study sites correspond to Table 1. *Broad* indicates broadly collected samples. *Targeted* indicates that taxa were specifically targeted for collection.
[d]Supergroup was determined by phylogenetic analyses (Fig. 1).
[e]ST (Sequence Type) and ID (isolate) are MLST database identifiers.
[f]Numbers in *gatB*, *coxA*, *hcpA*, *ftsZ*, *fbpA*, *wsp* and HVR columns are MLST database allele numbers; MH or MK preceding numbers indicates GenBank Accession numbers.
[g]Indicates not identified.

TABLE 4. Numbers of *Wolbachia*-infected arthropods and total collected arthropods (in parentheses) broadly sampled from the campuses of Metropolitan State University of Denver, Colorado (MSUD 2011–2014), and Pacific University, Oregon (PACU 2014–2017). A dash indicates that specimens were not collected.

Class	Order	Classification	Collection site	
			MSUD	PACU
Arachnida	Araneae	*Zygiella x-notata*	—	3 (5)
		Philodromus dispar	—	1 (1)
		Tenuiphantes tenuis	—	0 (1)
		Unidentified	0 (14)	0 (7)
Chilopoda	Unidentified	Unidentified	0 (3)	2 (3)
Crustacea	Isopoda	*Armadillidium vulgare*	—	4 (5)
		Porcellio spp.	—	2 (2)
		Unidentified	2 (3)	8 (12)
Diplopoda	Julida	*Cylindroiulus* spp.	—	0 (1)
	Unidentified	Unidentified	0 (1)	0 (6)
Insecta	Coleoptera	*Nebria brevicollis*	—	5 (5)
		Oulema melanopus	—	1 (1)
		Coccinella septempunctata	—	0 (2)
		Unidentified	0 (13)	0 (14)
	Dermaptera	Unidentified	0 (1)	—
	Diptera	*Brilla* spp.	—	0 (1)
		Family Chironomidae	—	0 (1)
		Unidentified	0 (8)	0 (3)
	Hemiptera	*Liorhyssus hyalinus*	1 (1)	—
		Raglius alboacuminatus	—	1 (1)
		Boisea rubrolineata	—	0 (4)
		Unidentified	1 (8)	0 (31)
	Hymenoptera	*Polistes dominula*	1 (1)	—
		Formica spp.	—	1 (1)
		Prenolepis imparis	—	0 (3)
		Tetramorium spp.	—	0 (2)
		Unidentified	0 (16)	0 (54)
	Lepidoptera	*Noctua pronuba*	—	0 (1)
		Platyedra subcinerea	—	0 (1)
		Unidentified	0 (2)	—
	Orthoptera	Unidentified	0 (8)	—
	Trichoptera	Unidentified	0 (2)	—
	Unidentified	Unidentified	0 (2)	—

TABLE 5. Numbers of *Wolbachia*-infected mosquitoes and total collected mosquitoes (in parentheses) collected in Colorado from 2009 to 2010. Collection sites correspond to Table 1. A dash indicates that specimens were not collected.

Collection site	*Culex pipiens*		*Aedes vexans*	
	♀	♂	♀	♂
BANN	1 (1)	—	0 (22)	—
CHAT	NC	—	0 (5)	—
PUPA	22 (22)	11 (11)	—	—
DENV	29 (29)	23 (23)	—	—
WYGB	23 (23)	17 (17)	—	—

Wolbachia Infection Rates in the Targeted Fruit Flies and Mosquitoes

In *D. melanogaster*, the total *Wolbachia* infection rate compiled from individuals collected from a variety of sites in western Oregon was 58.62% (153 of 261 individuals). Total infection rates of males and females per site among the 7 sites for which our sample size was >10 ranged from 44.00% to 70.27%. Among 3 sampled sites with fewer than 10 sampled individuals, infection rates were similar (50.00% to 83.33%).

A total of 126 *C. pipiens* and 27 *A. vexans* were collected from the greater metropolitan Denver, Colorado, area (Table 5). In *C. pipiens*, all individuals were infected at all collection sites. *Wolbachia* infection was not detected in *A. vexans*.

Strain Typing of *Wolbachia* from Broad and Targeted Surveys

We selected specimens from both broad and targeted collections for strain typing. From the broad collections, we amplified and sequenced MLST and *wsp* genes from 14 *Wolbachia*-infected individuals from 8 different

host species (Table 3). These individuals were randomly selected from the 35 *Wolbachia*-infected specimens in the broad collections (Table 4). From specimens in targeted surveys, we added 3 more species: *N. brevicollis*, *D. melanogaster*, and *C. pipiens*. We identified 5 novel *Wolbachia* hosts, 10 novel *Wolbachia* alleles among the 5 MLST loci, and 10 novel hypervariable region (HVR) alleles of *wsp*. Nine novel strains were characterized (*w*Zxno–ST485, *w*Porc, *w*NBre–ST502, *w*OMel, *w*LHya, *w*RAlb, *w*Pdis, *w*Vul3, *w*Pdom2) (Table 3).

Analysis of 3 *C. pipiens* individuals confirmed previous *Wolbachia* strain analyses of ST9, *wsp* allele 10. Three *D. melanogaster* individuals analyzed confirmed previous *Wolbachia* strain analyses of ST1, *wsp* allele 31 (Kittayapong et al. 2000, Baldo et al. 2006, Verspoor and Haddrill 2011). An additional 8 *D. melanogaster* specimens analyzed across 2 wineries in Oregon confirmed *wsp* allele 31 and *fts*Z allele 1 (Table 3).

Phylogenetic Analyses: Supergroup Determination

The concatenated alignment for the 5 MLST genes was 2079 bp in length. The numbers of variable sites and parsimony informative sites for each gene were as follows: *gatB*–142 variable sites and 101 parsimony informative sites; *coxA*–127 variable sites and 102 parsimony informative sites; *hcpA*–214 variable sites and 141 parsimony informative sites; *ftsZ*–138 variable sites and 109 parsimony informative sites; and *flbpA*–165 variable sites and 128 parsimony informative sites. Monophyly of supergroup A and supergroup B were unambiguously supported in Bayesian phylogenetic analysis of the concatenated MLST data set (posterior probabilities, PP = 1). We assigned 8 of our 9 novel *Wolbachia* strains to either supergroup A or B (Fig. 1, Table 3). The ninth novel strain, *w*Pdis, was not included in the analysis because high-quality sequence was only obtained for *wsp*. This locus by itself is unreliable in assigning supergroup (Baldo and Werren 2007). On the concatenated tree, *w*NBre shows strong support to form a clade with lepidopteran strain ST351 (Fig. 1).

Sequence Analyses: Shared *Wolbachia* Alleles Across Coleopteran and Lepidopteran Lineages

Query of the MLST database with GenBank *Wolbachia* sequences from aquatic beetle

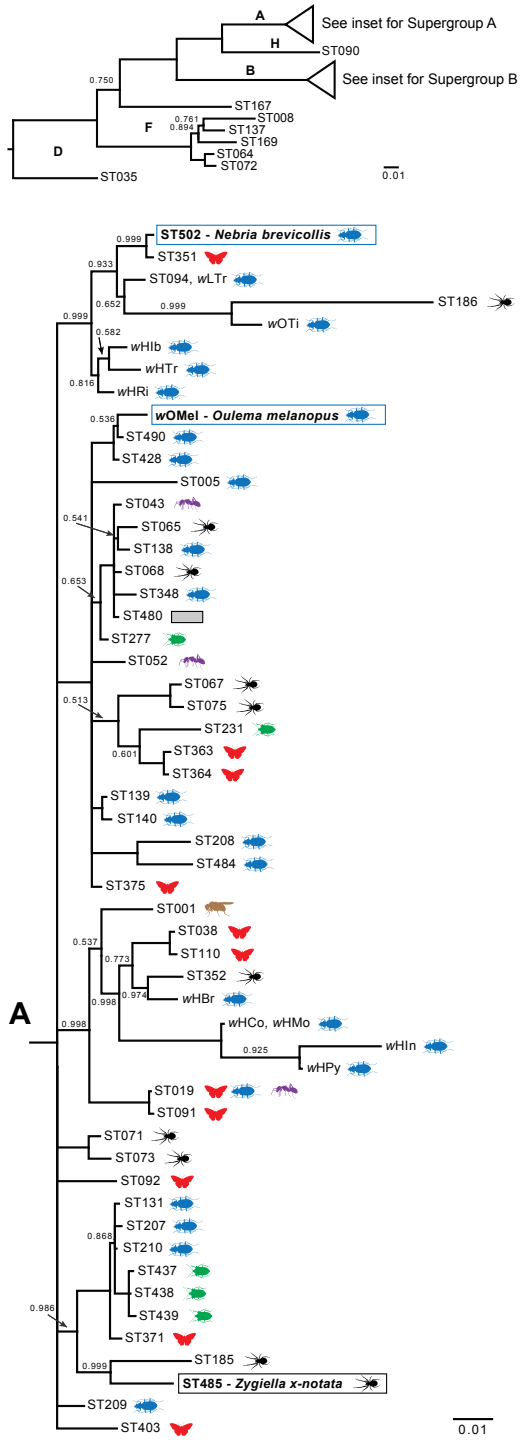

Fig. 1. Phylogeny of *Wolbachia* strains based on Bayesian phylogenetic analysis of MLST 5-gene alignment. **Top left panel (p. 542):** Reduced phylogeny indicating supergroups A, B, D, F and H. **Lower left panel (p. 542):** Supergroup A clade.

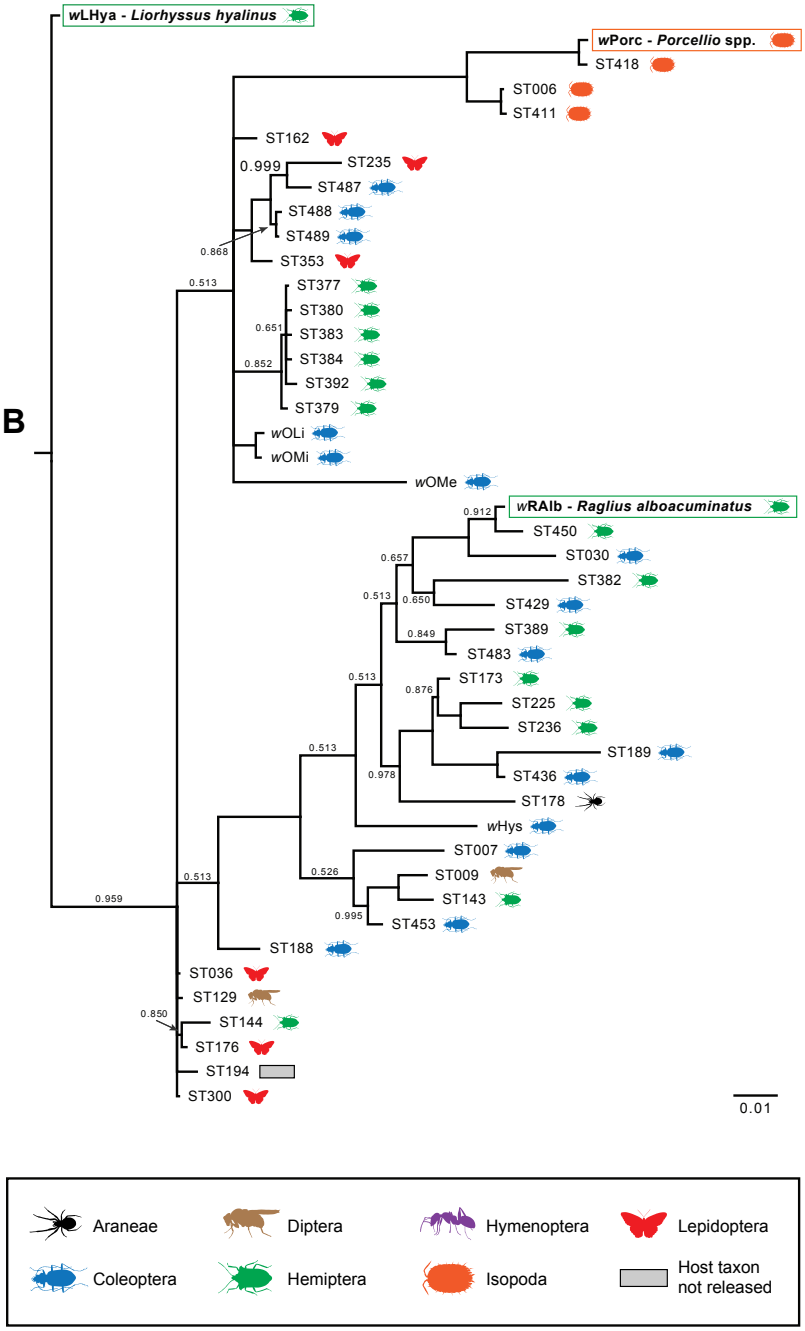

Fig 1. Continued. **Upper right panel (p. 543):** Supergroup B clade. All groups have a posterior probability value of 1, unless otherwise noted. Six novel *Wolbachia* strains are outlined with colored boxes. *Wolbachia* strains are indicated by "ST" followed by a numerical value or "*w*" followed by 3–4 letters. Host taxa and strain sequence source are included in Supplementary Material 1. The scale bar within each panel indicates the distance in substitutions per site. **Lower right panel (p. 543):** Symbol legend indicating host taxomomic order(s) or unreleased host identity (accessed 21 January 2019).

Table 6. Shared *Wolbachia* MLST alleles across select host species in two Arthropod orders, Lepidoptera and Coleoptera. A dash (—) indicates that the strain or allele information is not available.

Host classification	Taxonomic order	Strain/supergroup	Database identifier[a]	Alleles[b]				
				gatB	*coxA*	*hcpA*	*ftsZ*	*fbpA*
Nebria brevicollis	Coleoptera	ST502/A	ID 1864*	84	270	196	54	64
Aphantopus hyperanthus	Lepidoptera	ST351/A	ID 477*	84	57	196	153	240
Ochthebius tivelunus	Coleoptera	*w*OTi/A	&	84	57	Short (Near 245)	153	240
Hydraena riberai	Coleoptera	*w*HRi/A	&	Unique (Near 84)	Unique (Near 57)	Unique (Near 188)	Unique (Near 54)	Unique (Near 36)
Hydraena truncata	Coleoptera	*w*HTr/A	&	Unique (Near 84)	Unique (Near 62)	Unique) (Near 196)	Unique (Near 54)	Unique (Near 36)
Limnebius truncatellus	Coleoptera	*w*LTr	&	60	62	65	54	64
Rove beetle Family Staphylinidae	Coleoptera	ST094/A	ID 77*	60	62	65	54	64
Agabus bipustulatus	Coleoptera	—/A	KT199169&	—	—	—	54	—
Lycaeides melissa	Lepidoptera	ST162/B	ID 73*	108	73	40	80	9
Ochthebius minimus	Coleoptera	*w*OMi/B	&	Unique (Near 9)	73	Short (Near 143)	7	Unique (Near 208)
Ochthebius lividipennis	Coleoptera	*w*OLi/B	&	Unique (Near 186)	73	Short (Near 143)	7	Unique (Near 208)
Ochthebius meridionalis	Coleoptera	*w*OMe/B	&	Unique (Near 186)	73	Short (Near 196)	7	64
Anthaxia anatolica	Coleoptera	—/B	KT199201&	—	—	—	80	—

[a]Asterisks (*) indicate isolates (ID) in the *Wolbachia* MLST database. Ampersands (&) indicate previously reported (Sontowski et al. 2015) nucleotide sequences, which are deposited in GenBank (Supplementary Material 1).

[b]Numbers in *gatB, coxA, hcpA, ftsZ,* and *fbpA* columns correspond to allele numbers derived from MLST analyses. Each allele number is a unique nucleotide sequence. Identical full-length alleles have matching colors.
Purple: alleles matching *N. brevicollis*.
Green: alleles matching ST094 (rove beetle, not identified to species) and unique from *N. brevicollis*.
Orange: alleles matching *A. hyperanthus* and unique from *N. brevicollis*.
Blue: alleles matching *L. melissa*. Pink and yellow each designate matching alleles.
Unique (Near): no identical match in the database on 21 January 2019 with nearest matching allele number—typically 1–3 nucleotides but up to 6 nucleotides differing from the closest match.
Short (Near): sequences shorter than the standard MLST with the nearest matching allele number, as determined by an MLST database query.

coleopteran species (Sontowski et al. 2015) identified shared *Wolbachia* alleles across coleopteran and lepidopteran hosts (Table 6). For example, within supergroup A, *Wolbachia* from *N. brevicollis* (Carabidae), the rove beetle (Staphylinidae), the minute moss beetle *w*LTr (Hydraenidae), and the diving beetle *Agabus bipustulatus* (Dytiscidae) all share the same *ftsZ* allele. Within supergroup B, shared *Wolbachia* alleles were identified between lepidopteran *Lycaeides melissa* (Lycaenidae) and a variety of different aquatic beetle alleles within our data set (Table 6).

DISCUSSION

In general, we find that species previously unscreened for *Wolbachia* have unique strains, indicating that more surveys of nonmodel taxa are warranted to better characterize arthropod-*Wolbachia* symbiosis. Furthermore, our results have 3 main implications for our understanding and further study of the evolutionary and natural history of this symbiosis in the Pacific Northwest and Rocky Mountains. Our study implies (1) that frequent horizontal transfer of coleopteran *Wolbachia* alleles happens within and between taxonomic orders, (2) that *Wolbachia* may be acquired during habitat invasion as hosts encounter new opportunities for horizontal transmission, and (3) that increased sample size and more sensitive screening methodology would yield higher *Wolbachia* infection rates in the western USA.

Wolbachia Alleles Shared Within and Between Taxonomic Orders

Our study identifies intraorder *Wolbachia* allele sharing for coleopteran hosts, as well as interorder allele sharing between Lepidoptera and Coleoptera. This extends previous work on lepidopteran hosts which observed intraorder and interorder *Wolbachia* allele sharing, including with dipteran or hymenopteran hosts (Ahmed et al. 2016). Our broad survey and inclusion of strains in phylogenetic analyses from taxonomically diverse arthropod hosts suggest more complicated host-symbiont infection dynamics than previously described within Coleoptera host taxa and between Coleoptera and Lepidoptera host taxa. The *N. brevicollis* (Coleoptera: Carabidae) strain is particularly interesting because it shares alleles at 2 loci with a strain from a lepidopteran host (ST351, Lepidoptera: Nymphalidae) and with alleles at 2 other loci with 1 strain (ST94) found in both a rove beetle (Coleoptera: Staphylinidae) and the aquatic beetle *Limnebius truncatellus* (Coleoptera: Hydraenidae). Thus, strain ST94 exhibits intraorder allele sharing of all 5 MLST alleles across 2 families, and 2 of these alleles (*ftsZ* 54 and *fbpA* 64) are shared with Carabidae. This combination of shared alleles may result from mutation and evolutionary convergence, though convergence at multiple loci is improbable. Alternatively, because *N. brevicollis* is a predator, its *Wolbachia* strain could have been initially acquired through consumption of *Wolbachia*-infected prey (Le Clec'h et al. 2013) and subsequent recombination between co-infecting strains (Correa and Ballard 2016, Ilinsky and Kosterin 2017).

Interorder and intraorder horizontal transmission of *Wolbachia* may occur among divergent hosts, with subsequent vertical transmission and horizontal transmission within host clades (Ahmed et al. 2016, Ilinsky and Kosterin 2017). How often horizontal transmission occurs between divergent taxa is an open question, though the mapping of hosts on the evolutionary relationships among strains (Fig. 1) suggests it may happen frequently. For example, the strain in *N. brevicollis* may reflect a long-standing symbiosis, as is typical for *Wolbachia* infections. A study of over 1000 host species estimated that a given species cycles through *Wolbachia*-infected and uninfected phases approximately every 7 million and 9 million years (Bailly-Bechet et al. 2017). In contrast, infection dynamics can change rapidly. *Wolbachia* swept through wild populations of *Drosophila simulans* across 700 km in just over 10 years (Weeks et al. 2007). These results call for greater surveys of *Wolbachia* in arthropods. By combining strain data for diverse host taxa, we see that there are not simply order-specific *Wolbachia* strains. To understand the transmission and evolution of *Wolbachia*, we must consider multiple hosts. Future studies investigating transmission dynamics should include deeper within-taxon sampling (e.g., multiple species within a host order, tribe, family, etc.) alongside taxonomically broad host sampling. Our broad survey and phylogenetic analyses of evolutionarily diverse host taxa allow us to identify potential instances of horizontal gene transfer worthy of further investigation.

Implications of *Wolbachia* in Invasive and Nonnative Hosts

A large number of the infected host species detected among the 281 host specimens screened are nonnative and invasive, suggesting a causative relationship between *Wolbachia* infections and establishment of nonnative species in the western USA. Our sample sizes are low for many arthropods ($n \leq 4$), and thus we cannot confidently classify any host species as uninfected (Hilgenboecker et al. 2008). We focus instead on *Wolbachia*-infected hosts that are nonnative or invasive. These hosts are taxonomically diverse and originate from some shared but often different regions. The European gazelle beetle, *N. brevicollis* (Coleoptera: Carabidae), and the tuxedo bug, *R. alboacuminatus* (Hemiptera: Rhyparochromidae), are recent invaders to the western USA, respectively originating from Europe or Palearctic regions (Henry 2004, Kavanaugh and Labonte 2008). The cereal leaf beetle, *O. melanopus* (Coleoptera: Chrysomelidae), is native to Europe and Asia (Morrill 1995). The hyaline grass bug, *Liorhyssus hyalinus* (Hemiptera: Rhopalidae), is of unknown origin and hypothesized to have been introduced from Palearctic regions (Hradil et al. 2007, Wheeler 2016). Finally, the philodromid crab spider *P. dispar* (Araneae: Philodromidae) is not invasive, but is native to Europe (Dondale and Redner 1969) (Table 4).

Wolbachia's effects are both host specific and dynamic (Weeks et al. 2007); thus, the relationship between *Wolbachia* infection and host habitat invasion may be specific to the biology and ecology of each host. *Wolbachia* can result in both direct and indirect host benefits, including host resistance to RNA viral infections (Hedges et al. 2008, Teixeira et al. 2008, Brownlie and Johnson 2009, Frentiu et al. 2014), and an established *Wolbachia* host infection may confer host benefits that facilitate habitat invasion. Alternatively, because *Wolbachia* can have parasitic effects (Correa and Ballard 2016), habitat invasion might instead result from the loss of symbiotic *Wolbachia*. For example, when *Wolbachia* was absent in a major pest of maize (the western corn rootworm, *Diabrotica virgifera virgifera* [Coleoptera: Chrysomelidae]), plant defense-related genes were indirectly upregulated (Barr et al. 2010). One way to test the contribution of *Wolbachia* in hosts would be to compare the fitness of infected versus uninfected subsets of a host species.

Habitat invasion by an uninfected host could result in *Wolbachia* acquisition. This may occur as hosts invade a new ecosystem, encounter new prey or parasitoids, and establish *Wolbachia* symbiosis through mechanisms of horizontal transmission (Rowley et al. 2004). As an example, the invasive *N. brevicollis* may have acquired *Wolbachia* after it invaded the Pacific Northwest, or this symbiosis may have been previously established. *Wolbachia* surveys within targeted collections of *N. brevicollis* in Europe, its native prey, and prey across its path of invasion would address whether this strain of *Wolbachia* was recently acquired. To test the causative relationship between invasion and *Wolbachia* infection, this approach should be extended to the other invasive and nonnative *Wolbachia* hosts reported in this study.

Wolbachia Survey Methodology and Implications

Our 13.15% infection rate of individuals in the broad survey is at the low end of previous broad surveys reporting infection rates from 16.9% to 76% (Werren et al. 1995, Jeyaprakash and Hoy 2000). This result has several implications regarding methodology and how prevalence is reported. First, limitations to our collection methodology resulted in small sample sizes for many host taxa (e.g., $n = 1$–5). We also have lower confidence in the results of the infection status for species with smaller sample size, especially those that were uninfected with <10 individuals (e.g., *Boisea rubrolineata*).

Our infection rate is similar to studies using comparable methods and calculating infection based on individuals rather than on species (Werren et al. 1995, Werren and Windsor 2000, Duron et al. 2008). The wide range of infection rates in previous studies is attributed primarily to screening methodology (Werren et al. 1995, Jeyaprakash and Hoy 2000). Our PCR method utilized well-established loci (*W16S* and *COI*) to estimate infection rates among arthropods screened in the broad survey (Folmer et al. 1994, Werren et al. 1995). The *W16S* amplification we used clearly identifies *Wolbachia*, with few false positives, but is also likely to include false negatives when used in broad surveys.

Overall, we expect that increased sample sizes and more sensitive screening methodology would yield higher *Wolbachia* infection rates, reported either as individuals or species infected. For example, one broad survey found that 17.8% of individuals and 22.8% of species were infected. However, statistical models correcting for very small and large sample sizes of individuals tested per species estimated that 40% of these species were infected (Duron et al. 2008, Zug and Hammerstein 2012). These analyses are based on a prevalence model in which either a high or a low proportion of individuals are infected within a species (Hilgenboecker et al. 2008). Thus, our small sample sizes bias our estimate of the number of infected individuals downward. In our broad study, only a subset of our specimens were identified to species ($n = 33$) and *Wolbachia*-infected specimens were prioritized. Because of this, while our methodology to identify novel *Wolbachia* hosts and strains was effective, our species infection rate is not comparable to previous studies. However, *Wolbachia* infection rates of individuals from our study can be taken as a conservative estimate of the potential hidden diversity of *Wolbachia* hosts and strains. The identification of novel *Wolbachia* hosts and strains provides foundational data for *Wolbachia* infections in western North American arthropods. Because *Wolbachia* can have substantial consequences for host population biology as well as conservation and biocontrol, understanding the extent and prevalence of *Wolbachia* has broad significance. Furthermore, the genetic characterization of novel *Wolbachia* strains offers insight into *Wolbachia* diversity, with implications for future studies investigating their acquisition, transmission, and evolution. Such studies will shed light on processes underlying host-symbiont evolution, a fundamental part of the biology of arthropod species worldwide.

SUPPLEMENTARY MATERIAL

Two online-only supplementary files accompany this article (https://scholarsarchive.byu.edu/wnan/vol79/iss4/7).

SUPPLEMENTARY MATERIAL 1. Accession numbers and allele numbers for all sequences employed for *Wolbachia* MLST phylogeny. MLST database: https://pubmlst.org/ wolbachia

SUPPLEMENTARY MATERIAL 2. Nucleotide substitution models. Phylogenetic analyses were partitioned by codon position, applying the best-fit nucleotide substitution models from MrModeltest v2 (Nylander et al. 2004). We parameterized with 2 partitions for each gene—one including positions 1 + 2 and the other including only third positions.

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