THESIS

Comparative Genetics of the *Wolbachia* Endosymbiont Associated with Great Salt Lake Brine Flies

Submitted by

Amanda Truong

In partial fulfillment of the requirements for the Thesis in Zoology

Weber State University

Ogden, Utah

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY AMANDA TRUONG ENTITLED

Comparative Genetics of the *Wolbachia* Endosymbiont

Associated with Great Salt Lake Brine Flies

BE ACCEPTED AS FULFILLING PARTIAL REQUIREMENTS FOR THE THESIS IN ZOOLOGY.

THESIS COMMITTEE

Advisor

Department Chair

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INTRODUCTION

The genus *Wolbachia* is a diverse group of endosymbiotic bacteria found in many species of arthropods and nematodes. It was first identified in 1924 by Marshall Hertig and S. Burt Wolbach in *Culex pipiens*, a species of mosquito (Hertig and Wolbach, 1924). There was little interest in this bacterium until 1971, when *Wolbachia* was discovered to cause cytoplasmic incompatibility between *Wolbachia*-infected males and *Wolbachia*-free females (Yen and Barr 1971). Since then, *Wolbachia* has been found to induce numerous effects on the reproductive mechanisms of its host. These observed effects include cytoplasmic incompatibility, parthenogenesis, male killing, and feminization. Although these mechanisms are not fully understood, recent studies have shown that it may affect germline proliferation (Fast et al., 2011). Mutualistic relationships have also been noted in nematodes. Since *Wolbachia* are maternally inherited, these effects on the host result in efficient dispersal of *Wolbachia* into host populations and may also promote rapid speciation. The study of this bacterium has generated much excitement in recent years because of its role in biological, ecological, and evolutionary processes and its potential in controlling insect pests and disease vectors, such as malaria and dengue fever (Walker et al., 2011).

Wolbachia is closely related to the pathogenic Rickettsiales order in the Anaplasmataceae family of gram-negative bacteria. Both *Wolbachia* and Rickettsiales are alphaproteobacteria that live obligately within host cells. The *Wolbachia* genus contains one species, *Wolbachia pipientis*, and is currently divided into ten supergroups (A through K), based on phylogenetic analysis of the 16s ribosomal RNA and other gene sequence information (Doudoumis et al., 2012). In some cases, the phylogeny of the supergroups correlates with the effects on the host. For example, the *Wolbachia* associated with nematodes (supergroups C and D) are strictly mutualistic, whereas *Wolbachia* associated with arthropods (supergroups A and B) are parasitic (Figure 1). Therefore, identifying

the phylogenetic affiliation of *Wolbachia* may provide an indication of host effect, be it parasitism or mutualism (Werren et al., 2008).

Phylogeny, as is used for *Wolbachia* classification, is the study of the evolutionary relatedness among groups of organisms, and it is important in many aspects of biological study. It is particularly useful in studies where other types of evidence are lacking or uninformative. For example, in a study by Boyko et al. (2009), looking at the mitochondrial DNA of various dog breeds from Africa, Puerto Rico, and the US showed that domesticated dogs may not have originated in East Asia, as is currently believed. Also, this study showed that some purely African breeds of dogs, such as the Pharaoh hound and Rhodesian Ridgebacks, do not have African origins.

Phylogeny also has clinical applications. In a study by Harris et al. (2010), the microevolution and population structure of the methicillin-resistant bacterium, *Staphylococcus aureus* (MRSA), was traced using phylogenetic analysis. Determining the relationships between MRSA populations worldwide is informative in understanding its distribution and spread, which could potentially detect new means for infection control.

In another interesting application of phylogeny, Moran and Jarvik (2010) found that horizontal gene transfer from a fungus to a pea aphid was responsible for the insect's red and green body coloration. Analysis of the aphid carotenoid gene showed that it grouped with fungal genes, suggesting that this gene was transferred from a fungus to an aphid ancestor. Although thought to be a relatively rare phenomenon, horizontal gene transfer has also been discovered between *Wolbachia* and some insect and nematode species (Dunning Hotopp et al., 2007). Molecular phylogenetic analysis is especially useful for studying bacteria, like *Wolbachia*, that cannot be cultured in the laboratory.

To date, *Wolbachia* has been found in more than 65% of insect species, as well as in nematodes and arachnids, but its presence in many invertebrate taxa is unknown. In particular, to date there is no

identification of *Wolbachia* from organisms inhabiting extreme environments, such as Great Salt Lake (GSL), Utah, USA. GSL is the largest saline lake in the western hemisphere and is characterized by salinity levels from 6% to full saturation in some areas. One of the few organisms that can thrive in such conditions is the brine fly (also known as shore fly). Female brine flies release their eggs in salt water, where they hatch as larvae. Brine fly larvae have been known to reach numbers approaching 400 million per mile. As adults, brine flies are important for the ecosystem of GSL because they consume algae and organic matter and are also a food source for migratory birds (http://www.wildlife.utah.gov).

The three species of brine flies examined in this study are *Cirrula hians*, *Ephydra gracilis*, and *Mosillus bidentatus*. As discussed above, since the some *Wolbachia* supergroups correlate with the effects on the host, supergroup identification may provide insights into understanding the nature of the effect that *Wolbachia* may have on their brine fly hosts. Several genes have been used for identifying *Wolbachia*, including *wsp*, *ftsz*, and 16s rRNA. In this study, the 16s rRNA gene was employed for phylogenetic investigation. This gene is useful for phylogenetic studies because it is highly conserved among species and yet exhibits sufficient variation that is taxon-specific. The sequence variation observed is the basis for establishing supergroup affiliations among *Wolbachia* from a variety of hosts (O'Neill, S. et al. 1992) This gene is also useful because there is an abundance of 16S rRNA gene sequence data in Genbank for *Wolbachia*, which is useful for initial sequence comparisons among *Wolbachia* from brine flies.

The aim of this study was to answer the following questions:

i. Is *Wolbachia* found in GSL brine flies? This is determined by detection of the *Wolbachia* 16s rRNA gene in brine fly DNA samples.

ii. What is the phylogenetic affiliation of *Wolbachia* found in brine flies compared to *Wolbachia* found in other invertebrates? This is determined by phylogenetic analysis of *Wolbachia* 16s rRNA gene sequences.

iii. Are all *Wolbachia* sequences from brine flies monophyletic?

MATERIALS AND METHODS

DNA Isolation

Adult brine flies were collected from Antelope Island and GSL Marina of Great Salt Lake in 2010 and 2011. The flies were caught by nets and stored in plastic bags at -20°C. *Cirrula hians*, *Ephydra gracilis*, and *Mosillus bidentatus* were identified and separated based on gross morphology, and each fly was placed into a 1.5 mL microcentrifuge tube. DNA was isolated from the brine flies using the MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies). An individual fly was homogenized in the microcentrifuge tube in a solution of 300 µl Tissue and Cell Lysis Solution and 1µl Proteinase K (stored at -20°C). The sample was then incubated at 65°C for 15 minutes, vortexed every 5 minutes, and then cooled to 37°C. 1 µl of 5µg/µl RNase A (stored at -20°C) was then mixed thoroughly with the sample and incubated at 37°C for 15-20 minutes. The samples were then placed on ice for 3-5 minutes. To precipitate the DNA, 150 µl of MPC Protein Precipitation Reagent was added to the lysed sample and vortexed continuously for 10 seconds. The sample was then placed into a centrifuge for 10 minutes at 10,000 *g* to pellet the debris. The resulting supernatant was transferred to a clean microcentrifuge tube and the pellet was discarded. 500 µl of isopropanol was added to the supernatant and the solution was inverted in the tube 30 times. The mixture was then placed in a centrifuge for 10 minutes at 10,000 *g* to pellet the DNA. The isopropanol was carefully poured out into a sink without dislodging the pellet. The pellet was then rinsed twiced with 500 µl of 70% ethanol. Residual ethanol was removed with a micropipette. The tube was then left open for 15 minutes to allow the pellet to air dry completely. 35 µl of TE Buffer was then added

to the pellet to resuspend the DNA. The DNA sample was stored in a 1.5 mL microcentrifuge tube at -20°C.

PCR Amplification

For each species, *Wolbachia* DNA was detected using polymerase chain reaction (PCR) with primers specific for *Wolbachia* 16s rRNA. Control primers for mitochondrial gene COI and nuclear ITS-1 interspace region were also used to confirm the brine fly species molecularly and to confirm the presence of brine fly DNA, respectively. Primer sequences are as follows:

16s rRNA (forward): 5'-TTGTAGCCTGCTATGGTATAACT

16s rRNA (reverse): 5'-GAATAGGTATGATTTTCATGT

COI (LepR1): 5'-TAAACTTCTGGATGTCCAAAAAATCA

COI (LepF1): 5'-ATTCAACCAATCATAAAGATATTGG

ITS-1 (7246): 5'-GCTGCGTTCTTCATCGAC

ITS-1 (7247): 5'-CGTAACAAGGTTTCCGTAGG

Each PCR sample contained a mixture of 15.375 µl of pure water, 1.0 µl of 5 mM dNTPS, 1.25 µl of 5 µM forward primer, 1.25 µl of 5 µM reverse primer, 2.5 µl of 10X buffer, 2.5µl of 25 mM $MgCl_2$, 0.125 µl of 5U/µl Taq polymerase, and 1.0 µl of DNA sample in a mini-microcentrifuge tube. The buffer, $MgCl_2$, and Taq were made by New England BioLabs, inc. One drop of mineral oil was added to each sample to prevent evaporation of the sample. PCR amplification was accomplished by preheated block of 94°C, then went through 40 cycles of: 94°C (1:00), 50°C (1:00), 72°C (1:00), and ended at 72°C (4:00).

DNA Cloning

PCR products were visualized by electrophoresis using 1.0% agarose gels with ethidium bromide added to the gel before setting. 10 µl of PCR product was pipetted onto a Dye Dots™ (Bioexpress) dry gel loading dye with bromophenol blue. The PCR products mixed with dye was then loaded onto the gel along with Hyperladder I size standard (Bioline™). Electrophoresis ran at approximately 120-130 V for 30-45 minutes. The gel was then photographed under UV light. Bands at approximately 900 bp represented the presence of the *Wolbachia* 16s rRNA gene (Figure 2). Once a strong band was achieved, the DNA was then cloned using the chemical transformation technique in the TOPO Cloning Kit (Invitrogen). The cloning mixture included 0.5 to 4.0 µl PCR product (less than 24 hours old; based on strength of band), 1 µl salt solution, 1 µl TOPO cloning vector, and distilled water to reach a final volume of 6 µl. The mixture was then incubated at room temperature for 5 minutes. After incubation, the tubes were stored at -20°C.

To perform a transformation on the cloned DNA samples, 2 µl of cloning product was added to vials containing Invitrogen OneShot© cells and incubated on ice for 20-30 minutes. The cells were then heat-shocked at 42°C for 30 seconds and immediately placed back on ice. 250 µl of S.O.C. medium was then added to the cells and shaken horizontally at 37°C at 200 rpm for one hour. Two culture plates were prepared for each cell vial. The plates were prepared with LB, a bacteriological medium containing 10 g tryptone, 5 g yeast, 10 g NaCl, and 15 g agar per 1 L of LB, set to a pH of 7.0 by adding NaOH. One hundred micrograms of ampicillin was added per 1 mL of LB. Each plate was brought to room temperature and covered with 30 µl IPTG and 30 µl X-gal prior to adding the transformation sample. For each incubated sample, 100 µl was spread on one plate and 200 µl was spread on another plate. The plates were then incubated at 37°C overnight.

DNA Sequencing

Two-mL cultures were then inoculated with individual colonies from the plates. An individual white colony was picked off and put into a 2 mL medium containing LB and ampicillin (100 µg/mL). Cultures were then grown overnight in a 37°C incubating shaker.

A boiling mini-prep was performed to isolate the plasmid DNA from the cell culture. 1.5 mL of culture was transferred to a 1.5 mL microcentrifuge tube. Cells were pelleted at 10,000 *g* for 2 minutes. The supernatant was removed with a vacuum system and the cells were resuspended in 300 µl STET (8 g sucrose, 0.5 mL Triton X, 10 mL 0.5 M EDTA, 1 mL 1 M Tris (pH 8.0), distilled water to a final volume of 100 mL). Twenty-five µl of lysozyme (10 mg/ml in 25 mM Tris, pH 8.0) was added and gently mixed by inverting the tube. The tube was then placed in a 100°C heating block for 1 minute, and spun at 10,000 *g* for 6 minutes. Three hundred fifty µl of isopropanol was added to the tube. The mixture was vortex and centrifuged at 12,000 *g* for 5 minutes. The liquid was decanted, and the pellet was washed with 350 µl of 70% ethanol. The ethanol was then decanted and the pellet was left to air dry. Once completely dry, the pellet was resuspended in 20 µl of distilled water.

Restriction enzyme analysis was performed on this DNA to verify the presence of the *Wolbachia* 16s rRNA insert in the bacterial plasmid. Two µl plasmid DNA from the boiling mini-prep, 1 µl 10X buffer, 0.1 µl EcoR1 restriction enzyme, and 6.9 µl distilled water were mixed in a microcentrifuge tube and incubated in a 37°C water bath for at least two hours. Each sample was then analyzed for the proper insert using 1% agarose gel electrophoresis, BPB loading buffer, and ethidium bromide, similar to the gel used for PCR analysis. For samples containing *Wolbachia* 16s rRNA inserts, verified by the presence of 900 bp bands under UV light, 10 µl from the initial cell cultured inoculations were added to a fresh 2 mL culture medium of LB and ampicillin. The culture was incubated at 37°C and shaken at 250 RPM overnight.

The DNA from these reinoculated cultures was purified using the Wizard© miniprep kit. 1.5 mL of the culture was placed into a 1.5 mL microcentrifuge tube. The cells were pelleted in a centrifuge for 2 minutes at 10,000 *g*. The supernatant was removed using a vacuum system, and the pellet was resuspended in 300 µl Cell Resuspension Solution, followed by 300 µl Cell Lysis Solution. The tube was mixed by inverting it 4 times. Then, 300 µl of Neutralization Solution was added and mixed by inverting the tube several times. Tubes were then centrifuged for 5 minutes at 10,000 *g*. Using the manifold vacuum assembly, 1 mL resin was added to each syringe/mini-column setup along with the supernatant that was just centrifuged. The solution was then vacuumed through the syringe. Two mL of ethanol was then added to the column and vacuumed for an additional 60 seconds after no more ethanol was visible in order to assure that the resin was completely dry. The mini-column was then placed on top of a microcentrifuge tube and centrifuged for 2 minutes at 10,000 *g*. The mini-column was transferred to a new microcentrifuged tube where 25 µl of distilled water was added. The tube sat at room temperature for 1 minute and then centrifuged for 20 seconds at 10,000 *g*. The distilled water containing the purified DNA was then stored at -20°C. DNA sequences were determined by Genewiz (www.genewiz.com) using T7 and SP6 primers.

Phylogenetic Analysis

The *Wolbachia* 16s rRNA sequences from the brine flies obtained from this study and other taxa obtained from GenBank were aligned using Clustal W software (www.clustal.org). The alignment was used to construct a phylogenetic tree using MEGA software (Figure 3). Both parsimony and distance methods were used. Branching was evaluated using bootstrapping, a statistical resampling technique.

RESULTS

To examine the phylogenetic signal of the 16SrRNA sequence, forty-one *Wolbachia* 16s rRNA sequences from various hosts representing different supergroups were included in a preliminary

phylogenetic analysis (Figure 4). The supergroup topology was consistent with trees obtained in other studies, indicating the utility of the 16s rRNA gene for examining brine fly *Wolbachia* phylogeny (Werren et al., 2008).

Wolbachia from three different species of brine flies from Great Salt Lake were examined including three samples from *Cirrula hians*, seven samples from *Ephydra gracilis*, and four samples from *Mosillus bidentatus*. All 14 brine fly samples were positive for *Wolbachia* infection, as verified from PCR and sequence analysis of the *Wolbachia* 16s rRNA gene. The relative size of the *Wolbachia* 16s rRNA gene for each sample was approximately 900 bp, consistent with the expected size. When the nucleotide sequences of the 16S rRNA PCR fragments are compared, the maximum degree of sequence diversity among the brine fly *Wolbachia* sequences was 6.6%.

When two *Wolbachia* sequences from each species of brine fly were included in the phylogenetic analysis, supergroup B is shown to be paraphyletic. The *Wolbachia* sequences from brine flies are affiliated with a particular group within supergroup B, which includes *Wolbachia* from three ant species (*Formica cinerea, Formica polyctena,* and *Formica aquilonia*), a weevil (*Curculio okumai),* a species of honeybee *(Aphis mellifera*), and a fly (*Cacoxenus indagator*). This clade is slightly more well-supported (84/71) as that of the original supergroup B (68/61). The other supergroup B sequences (*Nasonia giraulti, Culex pipiens, Tribolium confusum, Gryllus rubens, Gryllus pennsylvanicus, Trichogramma cordubensis, Strophosoma capitatum, Cnaphalocrocis medinalis* and *Gryllus integer*) form a distinct clade that is also well-supported (bootstrap values of 84/79) (Figure 5). It is worth noting that, when the brine fly sequences are included, the bootstrap support for supergroup B (68/61) is diminished to below 50% (see asterisk, Fig. 5).

This result was further supported when all 14 *Wolbachia* brine fly sequences were included in the phylogenetic analysis (Figure 6). Again, the phylogeny of this putative new supergroup is well-supported (bootstrap values of 87/60) and the support for the previous supergroup B falls to below

50% (see asterisk). Thus, these analyses indicate that supergroup B is paraphyletic and that the *Wolbachia* isolated from brine flies may constitute a previously unrecognized supergroup that includes *Wolbachia* isolated from ants, weevils, honeybees, and at least one species of flies.

DISCUSSION

This study represents the first identification of *Wolbachia* associated with a host that lives in an extreme environment. While there are no studies that have examined the distribution of *Wolbachia* in regard to host ecology, this finding does expand the host diversity of *Wolbachia* to include shore flies. Our findings further support claims that *Wolbachia* is the most common and diverse endosymbiont, found in a broad ecdyzozoan host range.

The phylogenetic analysis of *Wolbachia* presented here suggests that the *Wolbachia* found in brine flies are part of a previously unidentified supergroup, which includes other insect hosts from supergroup B. This result is not particularly surprising because supergroup B includes a diverse group of insect hosts, including ants, crickets, wasps, and beetles. Because other supergroups, such as supergroups C and D (nematodes) and supergroup E (springtails) are represented by a single host taxa, supergroup B may have been destined to be split up once additional sampling has been done. However, it is worth noting that *Wolbachia* from *Drosophila*, from a family of Diptera closely related to the Ephydridae, are confined to supergroup A. This could simply reflect lack of extensive sampling of drosophilids or other Diptera, or it could indicate that there are multiple origins of *Wolbachia* in different taxonomic groups.

Compared to other taxa, the brine fly *Wolbachia* sequences are diverse, showing a nucleotide divergence of over 6%. Because there are no studies of nucleotide diversity within species it is difficult to assess the significance of the divergence in brine flies. It could simply be due to the extensive sampling done here compared to other studies, which only obtained one or two sequences from each host. However, there are at least two other explanations. The first is that the

diversity is due to the transfer of endosymbiont DNA from one host (here, one brine fly) to another. Since GSL brine flies live in close proximity with each other, it is possible that the bacterium has been transferred from one host to another. This is supported by the phylogeny of brine fly *Wolbachia* sequences (Figure 7), in which the sequences from one species are not necessarily monophyletic. Such transfer among species has been described in at least one other study (Vavre et al. 1999).

An alternative explanation for the diversity of brine fly *Wolbachia* sequences is that *Wolbachia* DNA may have become integrated into the host genome. Kondo et al. (2002) described a case in which *Wolbachia* genome fragments were found in the X chromosome of the adzuki bean beetle, *Callosobruchus chinensis*. Following integration, these fragments would evolve independently of sequences from the *Wolbachia* genome. Since this was PCR-based survey, the amplification of *Wolbachia* genes occurs regardless of whether the genes are from an intact organisms or are from genes that have been transferred to the host. Without additional experimentation, it is difficult to determine if this is the case with brine fly *Wolbachia*.

Since *Wolbachia* exhibit a wide range of symbiotic associations with its hosts, including parasitic relationships, the bacterium must have evolved a wide range of immune defense mechanisms to ensure its survival and transmission. The host must also have evolved a mechanism to regulate this *Wolbachia* population within its cells to avoid fitness costs. The great diversity among *Wolbachia* and horizontal gene transfer events also suggests that *Wolbachia* has been evolving with its host for hundreds of millions of years. Because of this broad range of host diversity, it is not surprising that *Wolbachia* have evolved different mechanisms to thrive within their hosts and therefore could explain the sequence diversity observed in phylogenetic studies that classifies *Wolbachia* into designated supergroups.

Greater resolution of *Wolbachia* phylogeny can come from further PCR analysis and sequencing of multiple genes, both protein coding and ribosomal RNA. This approach is the basis for multi-locus sequence typing (MLST), which constitutes the definitive genotyping tool for use in *Wolbachia* phylogeny (Baldo et al. 2006). Also more extensive sampling from underrepresented groups, such as Supergroups E and F, and of individuals of a certain lineage would also give greater confidence in the phylogeny of *Wolbachia* and the identification of a new supergroup containing brine fly *Wolbachia*. Nevertheless, this study demonstrates the power of using phylogenetic analysis to explore the interaction of obligate endosymbionts with their hosts.

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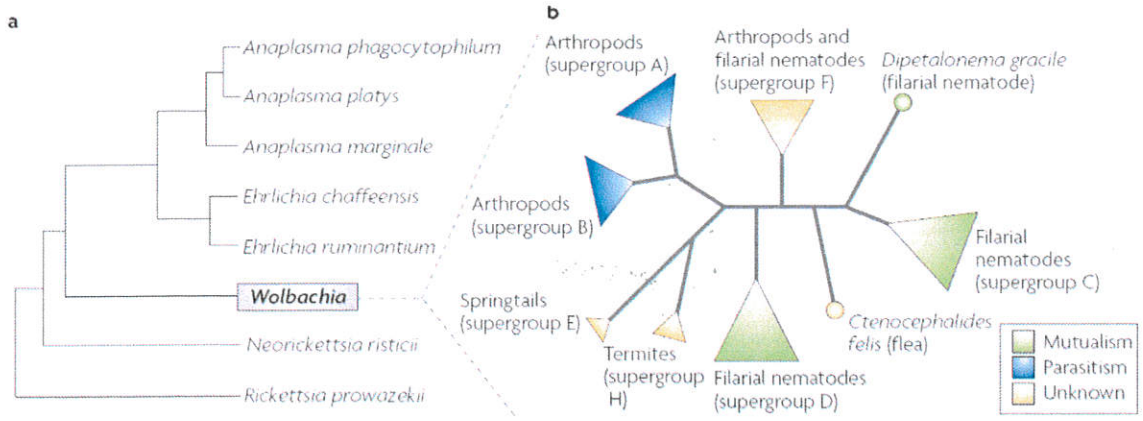

Figure 1. Phylogeny of *Wolbachia* (Werren et al., 2008). a. *Wolbachia* is closely related to other gram-negative alphaproteobacteria, including other endosymbiotic bacteria such as *Rickettsia prowazekii*. b. DNA sequences comparison within *Wolbachia* have identified at least eight different supergroups. The phylogeny of *Wolbachia* is sometimes correlated with their relationship with their host.

TTGTAGCCTGCTATGGTATAACTTAGTGGCAGACGGGTGAGTTATATATAGGAATCTACC

TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCCTACGGGGGAAAAATT

TGTGCTATTAGATGAGCCTATATTAGATAAGCTAGTTGGTGGAGTAATAGCCTACCAAGGCAATG

ATCAATAGTGATCTGAGAGGATGATCAGCCACACTGGAACCGAGATACGGTCCAGACTCCTACGG

GAGGCAGCAGTGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGAGTGAAGA

ATGCCTTTGGGTTGTAAAGCTCTTTTAGTGAGGAAGATAATGATGGTACTCACAGAAGGAGTGCT

GGCTTACTCCGTGCCAGCAGCCGCGGTAATACGGAGAGGGCTAGCGTTATTCGGAATTATTGGGC

GTAAAGGGCGCGTAGGCTGGTTAGTAAGTTAAAAGTGAAATTCCGAGGCTTAACCTTGGAACTGC

TTTTAAAACTGCTAACCTAGAGATTGAAAGAGGATAAAGGAATTCCTAGTATAGAGGTGAAATTC

GTAAATATTAGGAGGAACACCAGTGGCGAAGGCGTCTATCTGGTTCAAATCTGACGCTGAGGCGC

GAAGGCGTGGGGAGCAACCAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAATGTTAA

ATATGGGAAGTTTACTTTCTGTATTACAGCTAACGCGTTAAACATTCCGCCTGGGGACTACNGTC

GCAAGATTAAAACTCAAAGGAATTGACGTGGANCCGCACAAGCNGGTTGAGCATGTGNTTTGATT

CGATGCNACGCGAAAAACCTTACCACTCCTTGA**CNTGAAATCATACCTATTC**

Figure 2. Example of *Wolbachia* 16s rRNA sequence from *Mosillus bidentatus.* Nucleotides in bold represent primer sequences, used to amplify a portion of the gene using PCR. The total number of nucleotides is approximately 900.

```
E. gracilis22       TAGTAGTACGGAATAATTGTTGGAAACGGCAGCTAATACCGTATACGCCC
M. bidentatus1      TAGTAGTACGGAATAATTGTTGGAAACGGTAGCTAATACCGTATACGCCC
E. gracilis9        TAGTAGTACGGAATAATTGTTGGAAACGGCAGCTAATACCGTATACGCCC
E. gracilis17       TAGTAGTACGGAATAATTGTTGGAAACGGCAGCTAATACCGTATACGCCC
M. bidentatus16     TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
M. bidentatus12     TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
F. cinerea          TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
F. polyctena        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
F. aquilonia        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
E. gracilis1        -ACTACTCNGGAATAATTGTTGGAAACGGCAAATAATNCCGTATACGCCC
E. gracilis12       -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
C. hians1           -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
C. hians7           -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
E. gracilis13       -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
C. hians4           -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
E. gracilis7        -ACTACTCCGGAATAATTGTTGGAAACGGCAAATAATACCGTATACGCCC
C. okumai           TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
A. mellifera        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
C. indagator        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATAAGCCC
N. giraulti2        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATAGTCCC
N. giraulti1        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATAGTCCC
N. vitripennis      TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
C. pipiens          TAGTAGTACGGAATAATTGTTGGAAACGACAACTAATACCGTATACGCCC
T. confusum         TAGTAGTACGGGATAATTGTTGGAAACGTCAACTAATACCGTATACGCCC
G. rubens           TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
G. pennsylvanicus   TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
T. cordubensis      TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
S. capitatum        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
C. medinalis        TAGTAGTATGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
G. integer          TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
D. sechellia        TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
D. melanogaster     TAGTAGTACGGAATAATTGTTGGAAACGGCAACTAATACCGTATACGCCC
```

Figure 3. Portion of Wolbachia 16s rRNA gene alignment showing 50 positions. This represents an alignment of only 50 nucleotide positions. The complete dataset includes 900 nucleotides from each of 55 taxa. Dashes (-) identify positions where a nucleotide is missing due to a mutational event. All *C. hians*, *E. gracilis, and M. bidentatus* sequences were determined in this study. The remaining sequences were obtained from GenBank. A total of 14 brine flies and 41 other hosts were used for phylogenetic analysis.

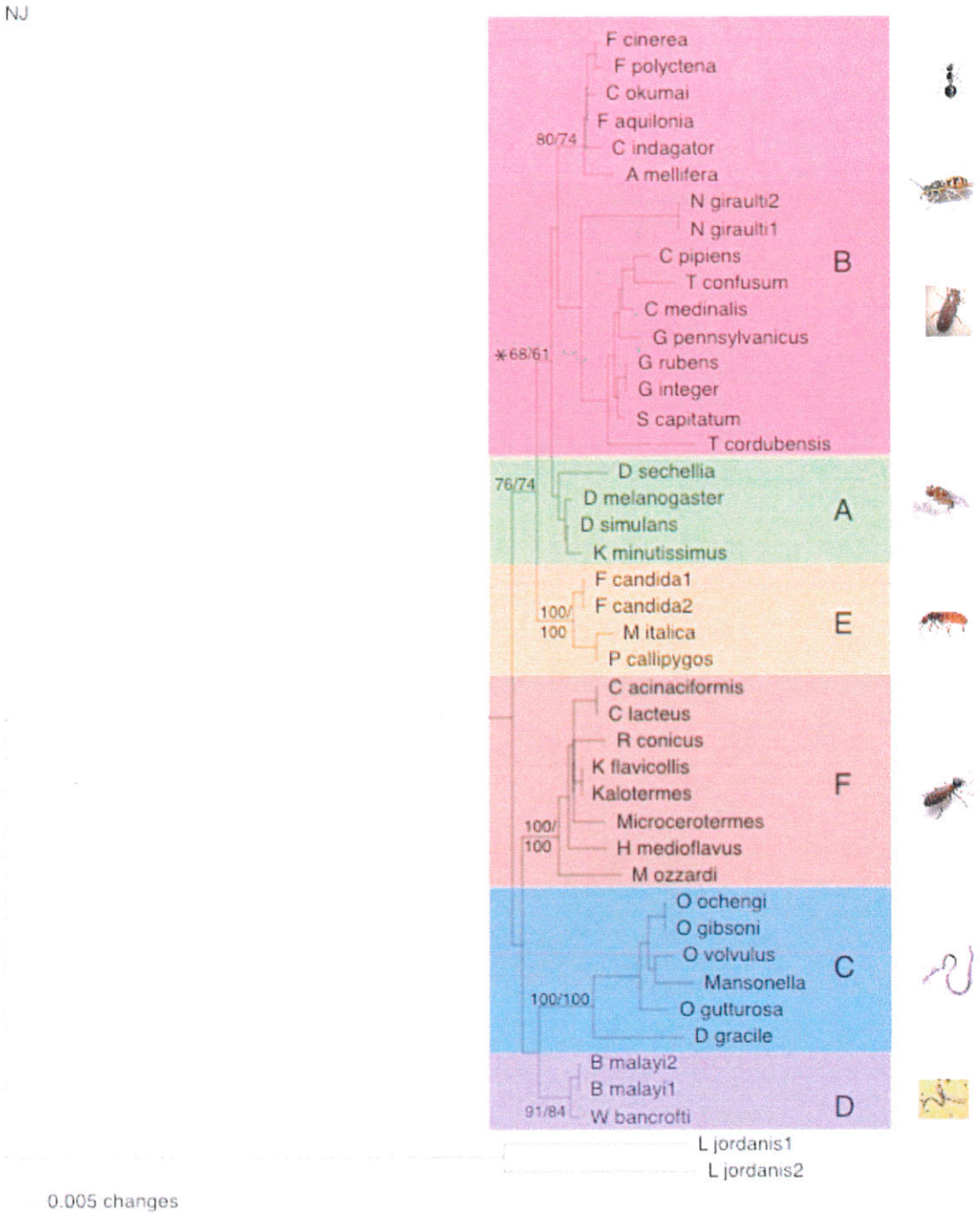

Figure 4. *Wolbachia* phylogeny of 16s rRNA sequences from hosts identified in previous studies. With the exception of supergroup A, major groups are well supported, as indicated by the bootstrap values. Supergroups are separated by colored boxes. An asterisk denotes the supergroup B clade.

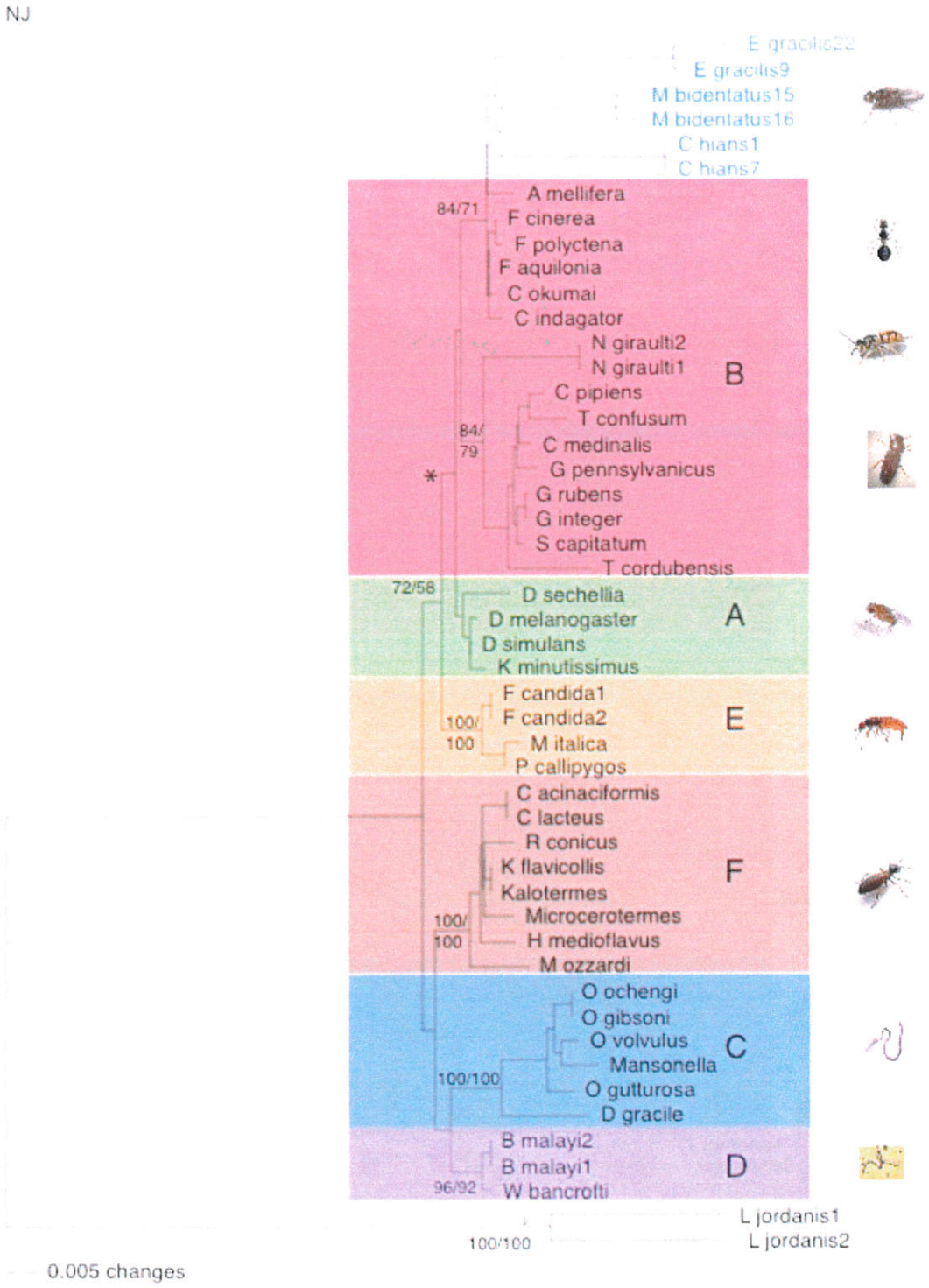

Figure 5. *Wolbachia* phylogeny of the 16s rRNA gene including two sequences from each species of brine fly. Bootstrap values shown represent well-supported clades. Supergroup B (asterisk) is no longer well-supported with the addition of brine fly *Wolbachia* sequences.

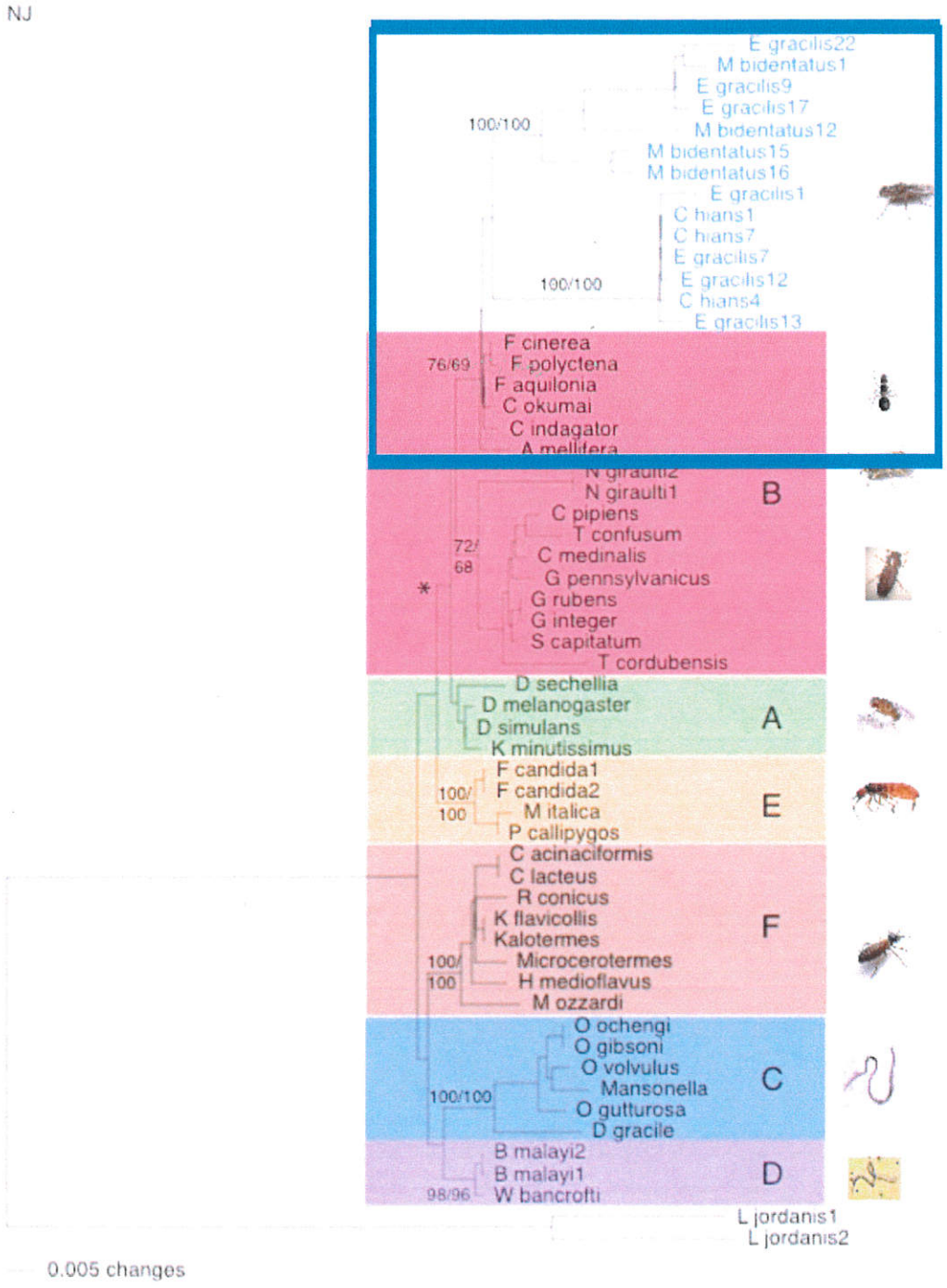

Figure 6. Phylogenetic analysis of *Wolbachia* 16s rRNA sequences from various hosts, including 14 brine fly samples. Blue text identifies all of the brine fly sequences obtained in this study. The blue box highlights a possible new supergroup, consisting of *Wolbachia* from brine flies and some hymenoptera. An asterisk marks the branch that led previously to the supergroup B clade.