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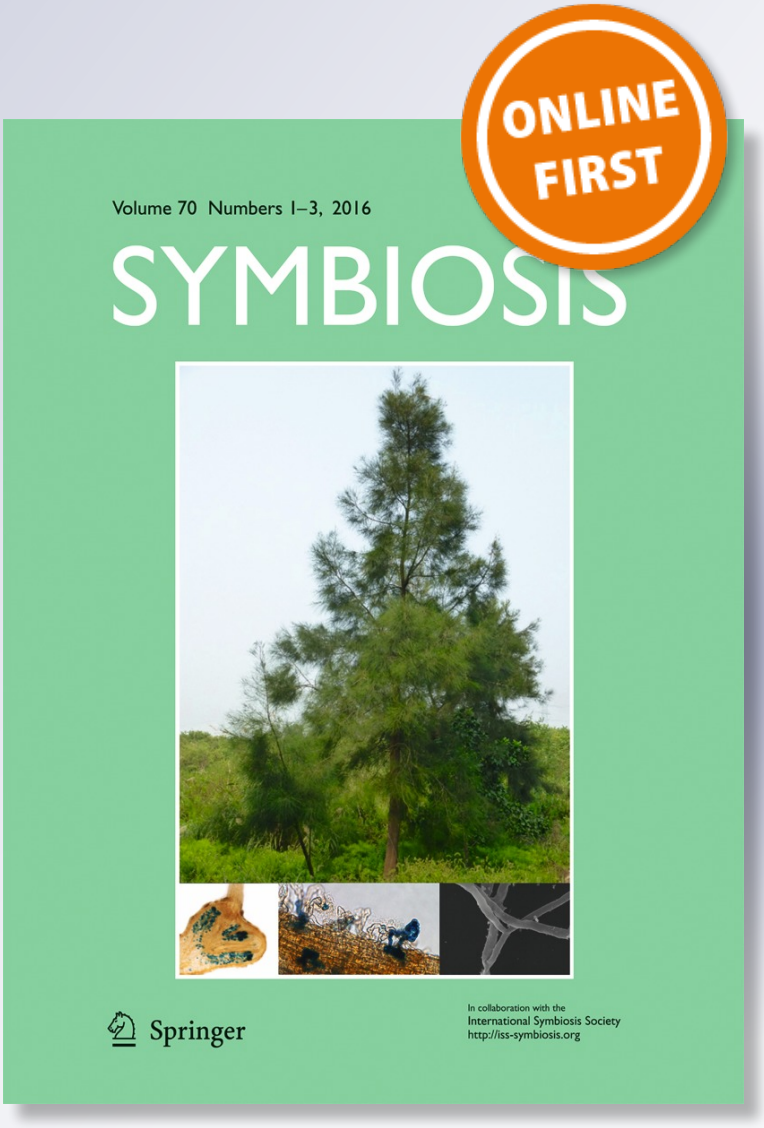

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Genetic characterization of *Wolbachia* from Great Salt Lake brine flies

Amanda Truong[1] · **Mohammad Sondossi**[2] · **Jonathan B. Clark**[1]

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Abstract *Wolbachia* are intracellular prokaryotic endosymbionts associated with a wide distribution of arthropod and nematode hosts. Their association ranges from parasitism to mutualism, and there is growing evidence that *Wolbachia* can have dramatic effects on host reproduction, physiology, and immunity. Although all *Wolbachia* are currently considered as single species, *W. pipientis*, phylogenetic studies reveal about a dozen monophyletic groups, each designated as a supergroup. This study uses 16S rRNA gene sequences to examine the genetic diversity of *Wolbachia* present in three species of Great Salt Lake brine flies, *Cirrula hians*, *Ephydra gracilis*, and *Mosillus bidentatus*. The brine fly *Wolbachia* sequences are highly similar, with an average nucleotide sequence divergence among the three species of 0.00174. The brine fly *Wolbachia* form a monophyletic group that is affiliated with a subset of supergroup B, indicating that this supergroup may be more diverse than previously thought. These findings expand the phylogenetic diversity of *Wolbachia* and extend their host range to taxa adapted to a hypersaline environment.

Keywords *Wolbachia* · Endosymbionts · Hypersaline environments · Ephydridae · Brine flies

Electronic supplementary material The online version of this article (doi:10.1007/s13199-016-0446-3) contains supplementary material, which is available to authorized users.

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1 Introduction

The genus *Wolbachia* comprises a group of endosymbiotic bacteria found in many species of arthropods and nematodes (Zug and Hammerstein 2012). *Wolbachia* have been found to induce numerous effects on the reproductive mechanisms of their hosts, including sperm-egg cytoplasmic incompatibility; killing of male progeny; feminization of males; and parthenogenesis, in which unfertilized eggs develop by default into female progeny (Stouthamer et al. 1999; Werren et al. 2008). Each of these manipulations has the effect of increasing the number of infected females, thereby enhancing the long-term survival of the endosymbiont. *Wolbachia* have complicated associations with their hosts that include mutualism, commensalism, and parasitism (Hentschel et al. 2000). In addition to their influence on host biological, ecological, and evolutionary processes, these bacteria have generated much excitement because of potential in controlling insect pests and disease vectors, such as those associated with malaria and dengue fever (Bian et al. 2013; Walker et al. 2011).

Molecular phylogenetic analyses reveal as many as 16 monophyletic groups of *Wolbachia*, designated as supergroups (Glowska et al. 2015). Whereas the host distribution for each supergroup is limited, there is little congruence between host phylogeny and endosymbiont phylogeny. This suggests that *Wolbachia* may be transferred fairly frequently between hosts and that *Wolbachia* infection does not always persist following host speciation events (Aikawa et al. 2009; Klasson et al. 2009; Woolfit et al. 2009). Within some supergroups, specific genotypes of *Wolbachia* may be more prevalent than others, owing to their favorable effects on host fecundity (Serga et al. 2014). In addition, genetic transfer between *Wolbachia* and other endosymbionts may also occur (Nikoh and Nakabachi 2009).

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To date, *Wolbachia* have been found in a large number of insect species examined (Hilgenboecker et al. 2008; Zug and Hammerstein 2012), but their presence in many invertebrate taxa is unknown. In particular, there is no identification of *Wolbachia* from host species inhabiting extreme environments. Great Salt Lake (GSL), in northern Utah, is the largest saline lake in the western hemisphere and is characterized by salinity levels that range from 6 % to full saturation. Among the few animals that live and reproduce in the lake's waters are the brine shrimp, *Artemia franciscana*, and shore flies (known as brine flies) of the Diptera family Ephydridae. As a group, shore flies are particularly adaptable and are prominent in a number of marginal habitats, including hot springs, oil ponds, highly saline lakes, and inland alkaline pools and marshes (Foote 1995).

The family Ephydridae (shore flies) is one of the largest in the order Diptera, with nearly 2000 species described (Wirth et al. 1992). Brine flies are among the most conspicuous inhabitants of Great Salt Lake, the center of a valuable wetland ecosystem in the Great Basin of North America. Few invertebrates possess the historical, cultural, and biological legacy achieved by GSL brine flies and their prominence is well-documented in the famous accounts of the western expeditions of John Fremont (1845) and Howard Stansbury (1852). During the summer months, brine flies reach densities estimated at 600 million adults per km shoreline (Felix and Rushforth 1980). Although Great Salt Lake shorelines are dynamic and transitory, using a conservative average of 4800 km yields an estimate of nearly three trillion adults, and, depending on the time of year, an equal number of larvae and pupae.

This study uses partial 16S rRNA gene sequences to examine *Wolbachia* associated with three species of GSL brine flies: *Ephydra gracilis*, *Cirrula hians*, and *Mosillus bidentatus*. To our knowledge, this study represents the first description of *Wolbachia* from hosts that inhabit an extreme environment. The brine fly *Wolbachia* reported here are monophyletic and affiliated with supergroup B, which includes a diverse number of insect hosts. The brine fly *Wolbachia* form a distinct lineage that is affiliated with a subset of supergroup B *Wolbachia*, revealing two well-supported clades. These results augment the diversity of *Wolbachia* and suggest that increased sampling may lead to the identification of additional supergroups of this fascinating endosymbiont.

2 Materials and methods

2.1 DNA isolation and PCR amplification

Adult brine flies were collected from the marina of the south shore of Great Salt Lake in 2010 and 2011. *Cirrula hians*, *Ephydra gracilis*, and *Mosillus bidentatus* were identified and separated based on gross morphology, and DNA was isolated from individual flies using the MasterPure™ DNA Purification Kit (Epicentre® Biotechnologies). To provide some measure of the *Wolbachia* diversity within a species, DNA from multiple individuals was pooled prior to the final 16S rRNA gene amplification reactions.

Control primers for the mitochondrial *cytochrome oxidase subunit I* gene (COI) and the nuclear rRNA internal transcribed spacer region (ITS-1) were also used to confirm the identity of each host species. *Wolbachia* DNA was detected for all individuals from each species, using polymerase chain reaction (PCR) with primers specific for *Wolbachia* the 16S rRNA gene. Primer sequences are as follows:

Wolbachia 16S rRNA (forward): 5′-TTGTAGCCTGCTATGGTATAACT.
Wolbachia 16S rRNA (reverse): 5′-GAATAGGTATGATTTTCATGT.
COI (LepR1): 5′-TAAACTTCTGGATGTCCAAAAAATCA.
COI (LepF1): 5′-ATTCAACCAATCATAAAGATATTGG.
ITS-1 (7246): 5′-GCTGCGTTCTTCATCGAC.
ITS-1 (7247): 5′-CGTAACAAGGTTTCCGTAGG.

2.2 DNA sequencing and phylogenetic analysis

Wolbachia PCR products were visualized by electrophoresis and were cloned with the TOPO© Cloning Kit (Invitrogen). Purified plasmids were sequenced from both strands using T7 and SP6 primers. Sequences have been deposited in GenBank (http://www.ncbi.nlm.nih.gov/) under the accession numbers KX139035-KX139040. The brine fly amplicons were examined for chimeric sequences using Mallard version 1.02 (Ashelford et al. 2006), using a 99 % cutoff value and a log-linear curve for identifying outliers. The reference sequence used was the *E. coli* K-12 16S rRNA (NR_102804). The brine fly *Wolbachia* 16S rRNA gene sequences were aligned with those from selected supergroups using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/). Table 1 provides details of the sequences included in the analyses.

The initial phylogenetic analysis included 900 positions within the 16S rRNA gene using parsimony and distance methods as implemented by PAUP 4.0b10. The parsimony analysis used random addition of sequences (ten replicates) and TBR branch-swapping. 684 characters were constant, 61 were parsimony-uninformative, and 155 were parsimony-informative. The distance analysis used Neighbor-joining with the Kimura two-parameter sequence correction. Sequences from two non-*Wolbachia* rickettsias, *Ehrlichia* and *Anaplasma*, were used as outgroups. Branch support for both the parsimony and distance trees was evaluated with bootstrapping, using 1000 replicates for each analysis. The phylogenetic analysis was extended with maximum

Table 1 Sources of *Wolbachia* 16S rRNA Sequences

Host species	Class / Order	Accession no.
Supergoup A		
Koreoculio minutissmus	Insecta / Coleoptera	AB604664
Drosophila melanogaster	Insecta / Diptera	AE017196
Drosophila simulans	Insecta / Diptera	DQ412085
Supergroup B		
Oreina cacaliae-1	Insecta / Coleoptera	HG326132
Oreina cacaliae-1	Insecta / Coleoptera	HG326135
Strophosoma capitatum	Insecta / Coleoptra	GQ167625
Trilobium confusum	Insecta / Coleoptera	X62247
Cacoxenus indagator	Insecta / Diptera	EU390865
Culex pipiens	Insecta / Diptera	X61768
Culex quinquefasciatus	Insecta / Diptera	AF397409
Drosophila simulans-1	Insecta / Diptera	DQ235288
Drosophila simulans-2	Insecta / Diptera	AF390864
Formica aquilonia	Insecta / Hyemnoptera	GU592785
Formica cinerea	Insecta / Hyemnoptera	GU592780
Formica polyctena	Insecta / Hyemnoptera	GU592787
Nansonia vitripennis	Insecta / Hymenotpera	M84686
Trichogramma cordubensis	Insecta / Hymenoptera	L02883
Cubitermes sp	Insecta / Blattodea (Isoptera)	EF417899
Odontotermes horni	Insecta / Blattodea (Isoptera)	GQ422896
Cnaphalocrocis medinalis	Insecta / Lepidoptera	HQ336509
Gryllus integer	Insecta / Orthoptera	U83096
Gryllus pennsylvanicus	Insecta / Orthoptera	U83090
Gryllus rubens	Insecta / Orthoptera	U83092
Bryobia praetiosa	Arachnida / Trombidiformes	EU499317
Bryobia sarothamni	Arachnida / Trombidiformes	EU499315
Tetranychus urticae	Arachnida / Trombidiformes	EU499319
Supergoup C		
Mansonella ozzardi	Nematoda: Secernentea / Spirurida	AJ279034
Onchocera gibsoni	Nematoda: Secernentea / Spirurida	AJ276499
Onchocera guttarosa	Nematoda: Secernentea / Spirurida	AJ276498
Onchocera ochengi	Nematoda: Secernentea / Spirurida	AF172401
Onchocera volvulus	Nematoda: Secernentea / Spirurida	AF069069
Supergoup D		
Brugia malayi-1	Nematoda: Secernentea / Spirurida	NR074571
Brugia malayi-2	Nematoda: Secernentea / Spirurida	AF051145
Wuchereira bancrofti	Nematoda: Secernentea / Spirurida	AF093510
Supergoup E		
Folsomia candida-1	Thysanura (Entognatha) / Collembola	EU831094
Folsomia candida-2	Thysanura (Entognatha) / Collembola	AF179630
Mesaphorura italica	Thysanura (Entognatha) / Collembola	AJ575104
Paratullbergia callipygos	Thysanura (Entognatha) / Collembola	AJ509026
Supergoup E		
Coptotermes acinaciformis	Insecta / Blattodea (Isoptera)	DQ837197
Coptotermes lacteus	Insecta / Blattodea (Isoptera)	DQ837199
Kalotermes flavicollis	Insecta / Blattodea (Isoptera)	Y11377
Microcerotermes sp	Insecta / Blattodea (Isoptera)	AJ292347
Rhinocyllus conicus	Insecta / Coleoptera	M85267

likelihood, using MEGA 7.0.16. The following parameters were used: a gamma distribution (five discrete categories) with invariant sites as a model of nucleotide substitution; nearest-neighbor interchange; 1000 bootstrap replicates. The phylogeny of an expanded dataset of that included 58 taxa was examined using the same parameters described above. For the parsimony analysis, 659 characters were constant, 82 were parsimony-uninformative, and 159 were parsimony-informative.

3 Results

To examine the genetic evidence for the *Wolbachia* endosymbiont in GSL brine flies, total DNA was isolated from individuals each of three species: *Cirrula hians* (four individuals), *Ephydra gracilis* (six individuals), and *Mosillus bidentatus* (six individuals). Each individual was examined with *Wolbachia*-specific 16S rRNA primers and all 16 single-fly samples showed successful amplification. Genomic DNA from each individual of that species was pooled and the 16S rRNA gene was amplified in new reactions, yielding products that were then cloned and sequenced. BLAST searches against a bacterial 16S rRNA dataset revealed a close affiliation of the brine fly sequences to *Wolbachia* sequences from supergroup B, which includes endosymbionts from a number of insect hosts.

Definitive supergroup affiliation of *Wolbachia* is usually assessed through multilocus sequence typing (MLST), which examines five protein-coding genes (Baldo et al. 2006). However, individual genes, including the 16S rRNA gene, have proven useful in examining phylogenies of *Wolbachia* (Augustinos et al. 2011; Ros et al. 2009; Salunke et al. 2010) and were used here for a preliminary placement of the brine fly *Wolbachia*. To examine the phylogenetic signal of this alignment, 33 *Wolbachia* 16S rRNA gene sequences from various hosts representing the six main supergroups were included in an initial phylogenetic analysis, along with the six brine fly sequences (Fig. 1). The sequences included from each supergroup were chosen by first identifying the taxa that were the most similar and the least similar to those from the

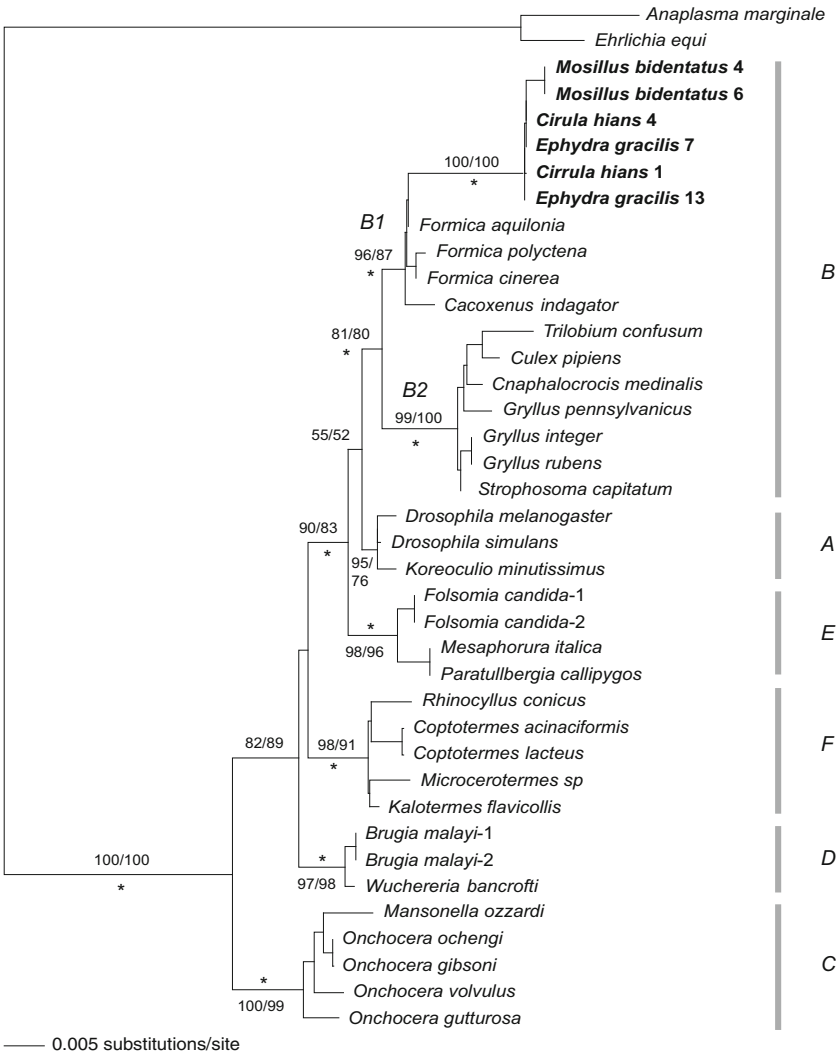

Fig. 1 Preliminary *Wolbachia* phylogeny based on partial 16S rRNA sequence comparisons. Host names are provided and include three species of Great Salt Lake brine flies (bold typeface; clone designation shown after species name). Distance tree is presented with bootstrap values for both Neighbor-joining (*before slash*) and parsimony (*after slash*) shown. Asterisks (*) identify nodes with more than 70 % bootstrap from a separate maximum likelihood analysis (see Supplemental Fig. S1). Supergroups are identified as indicated. B1 and B2 are new designations used to identify the major split within supergroup B

brine flies. So that each supergroup had at least three representatives, a third taxon was included with an intermediate divergence. In cases where multiple taxa met these criteria, additional sequences were included as well.

The *Wolbachia* sequences from brine flies are affiliated with a subset of supergroup B sequences from the ants *Formica cinerea, Formica polyctena,* and *Formica aquilonia*, and a fly (*Cacoxenus indagator*). This clade is referred to as B1 in the tree in Fig. 1. The other supergroup B sequences, referred to as B2, (*Culex pipiens, Tribolium confusum, Gryllus rubens, Gryllus pennsylvanicus, Strophosoma capitatum, Cnaphalocrocis medinalis* and *Gryllus integer*) form a distinct clade that is also well-supported. The overall supergroup topology is consistent with trees obtained from other studies, including MLST (Doudoumis et al. 2012) and phylogenomics (Ramírez-Puebla et al. 2015), indicating that the 16S rRNA gene sequences contain useful phylogenetic information. Consistent with other explorations of the 16S rRNA gene (Augustinos et al. 2011; Ros et al. 2009; Salunke et al. 2010), supergroup A, which includes *Wolbachia* from some insects, including *Drosophila,* is not as well supported as other supergroups. It is worth noting that the bootstrap support for the both the expanded supergroup B clade is strong, as is the support for B1 and B2. The *Wolbachia* phylogeny was also examined using maximum likelihood (Supplemental Fig. S1). With the exception of reduced support for supergroup A, the overall phylogeny is identical to that shown in Fig. 1. It is clear that the brine fly *Wolbachia* are affiliated with a subset of supergoup B sequences, designated here as B1.

There is little sequence divergence among the *Wolbachia* 16S rRNA gene sequences among the *Wolbachia* from different brine fly species. The average nucleotide sequence divergence among the *Wolbachia* from the three host species is 0.00174, a value considerably lower than that for the divergence of *Wolbachia* sequences from the drosophilids *D. melanogaster* and *D. simulans*, of supergroup A (0.00374). The average uncorrected distance of brine fly *Wolbachia* from the four other taxa in B1 is 0.0189, compared to an average distance of 0.0326 from B2. Thus, there is a clear demarcation between these two supergroup B lineages, consistent with the topology of the 16S rRNA tree. Table 2 summarizes additional nucleotide sequence comparisons, which provide further support for a proposed split within supergroup B. into two new supergroups. The maximum nucleotide sequence divergence for the 16S rRNA gene within this supergroup is 0.0385, a value that exceeds the 2 % threshold that is generally used to specify a new supergroup (Augustinos et al. 2011). Nucleotide sequence divergence comparisons were performed with reassigned groups B1 (brine flies + *Formica cinerea, Formica polyctena, Formica aquilonia* and *Cacoxenus indagator*), and B2 (*Nasonia giraulti, Culex pipiens, Tribolium confusum, Gryllus rubens, Gryllus pennsylvanicus, Trichogramma cordubensis, Strophosoma capitatum, Cnaphalocrocis medinalis* and *Gryllus integer*). As seen in Table 2, the 16S rRNA sequence divergences within B1 and B2 are now similar to the divergences observed within the other supergoups. Moreover, the average uncorrected sequence divergence between clade B1 *Wolbachia* and those from clade B2 is 0.0206, consistent with a prominent demarcation between these two clades (value not shown in Table 2).

Table 2 Summary of 16S rRNA nucleotide sequence divergences within supergroup B

Group	Maximum within group uncorrected sequence divergence
Supergroup B (original designation)	0.0385
Brine Flies	0.00341
Group B1 (new designation)	0.0238
Group B2 (new designation)	0.0177

Because of their considerable nucleotide sequence divergence, there is some uncertainty regarding the use of *Ehrlichia* and *Anaplasma* as appropriate outgroups for studies of *Wolabchia* phylogeny that use protein-coding genes (Lo et al. 2002). However, the use of these two taxa as outgroups in this study is unlikely to influence the overall conclusions presented here: (i) 16S rRNA sequence divergence between *Wolbachia* and the outgroups is moderate compared to that of protein-coding genes, with values for the latter, in a study of the *ftsZ* gene, from 0.3 to 1.7 uncorrected nucleotide substitutions per site (Lo et al. 2002); (ii) replacing these two outgroups with midpoint rooting does not change the overall topology of the major clades nor their respective compositions (see Supplemental Fig. S2).

To further examine the diversity of supergroup B, 20 additional 16S rRNA sequences were included in a new phylogenetic analysis, including sequences from the drosophilds, *D. melanogaster* and *D. simulans*. As seen in Fig. 2, the overall topology of the tree is identical to that of Fig. 1, and is congruent with the 16S rRNA trees presented in the comprehensive studies of Augustinos et al. (2011) (74 total sequences) and Salunke et al. (2010) (44 total sequences). The majority of supergroup B sequences (including those from the two drosophilids) fall into clade B2, with a smaller subset, including those from brine flies, forming clade B1. The bootstrap support for these two clades remains high and completely consistent with the tree presented in Fig. 1. It is clear that the subdivision of supergroup B is a persistent and robust feature that becomes apparent with the inclusion of the brine fly sequences. An independent phylogenetic analysis using maximum likelihood provides additional support for this conclusion (see Supplemental Fig. S3).

4 Discussion

This study represents the first identification of *Wolbachia* associated with hosts that are adapted to an extreme saline environment. The phylogenetic analysis of *Wolbachia* presented here indicates that the *Wolbachia* found in GSL brine flies are affiliated with a distinct lineage within supergroup B. This result is not particularly surprising since supergroup B comprises a diverse group of insect and acarine hosts, including ants, crickets, flies, wasps, beetles and mites. While supergroups A and B include most arthropod *Wolbachia*, there is little congruence between host and parasite phylogenies. The complex pattern of *Wolbachia* phylogeny is indicative of the ancient association of these endosymbionts with invertebrates and the complications introduced by loss of *Wolbachia* from a host lineage followed by reintroduction from another lineage by horizontal transfer (Lo and Evans 2007).

There are obvious limitations to using a single gene to assess *Wolbachia* supergroups (Baldo and Werren 2007; Bordenstein et al. 2009). A number of recent studies have examined the utility of the 16S rRNA sequence for initial characterization of *Wolbachia* diversity. Some studies show broad agreement between 16S rRNA phylogenies and those constructed with the *ftsZ* gene (Lo et al. 2002) or with expanded multigene datasets (e.g., Doudoumis et al. 2012). The extensive sequence sampling employed here, the congruence with results using more extensive dataset comparisons, including phylogenomics, and the relatively robust bootstrap values indicate that this preliminary analysis has identified a *Wolbachia* lineage that warrants further investigation with

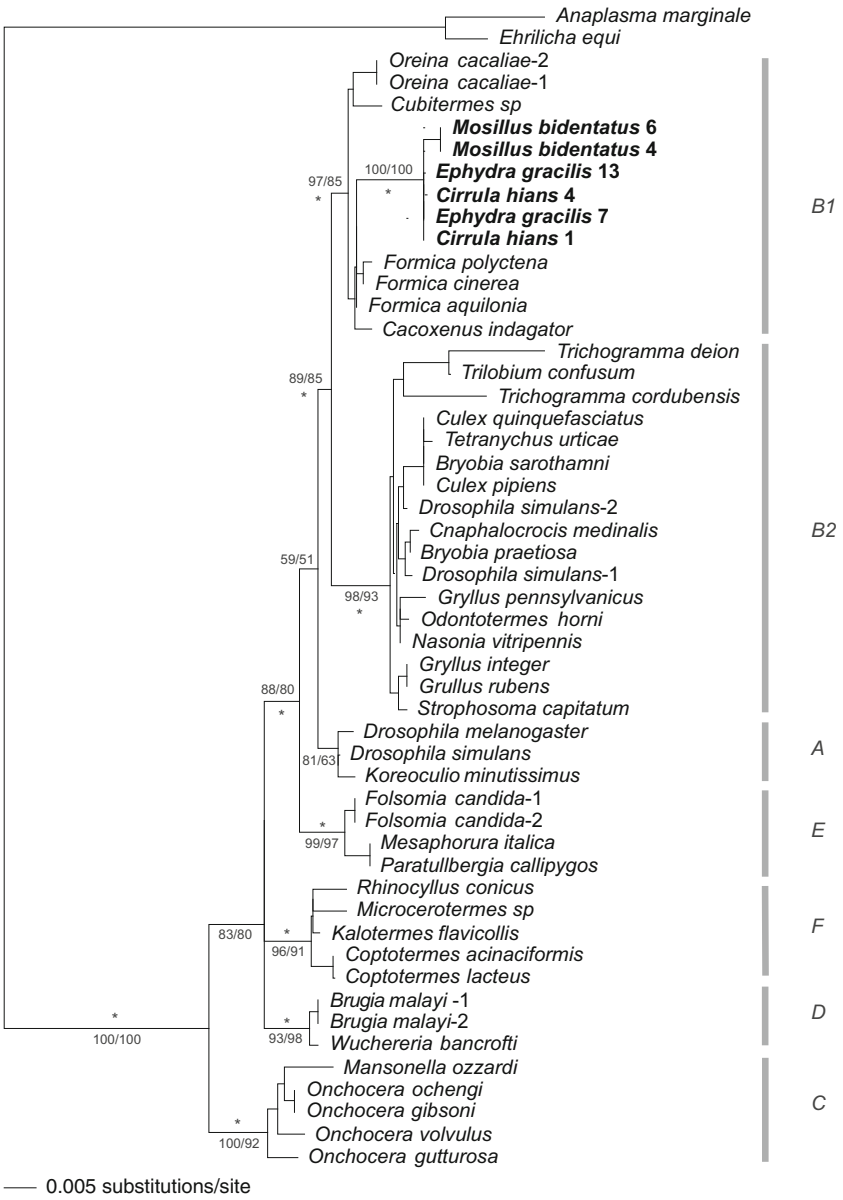

Fig. 2 *Wolbachia* phylogeny based on partial 16S rRNA sequences including enhanced sampling from supergroup B taxa. Brine fly sequences are shown in bold typeface with clone designation shown after the pecies names. Distance tree is presented with bootstrap values for both Neighbor-joining (*before slash*) and parsimony (*after slash*) shown. Asterisks (*) identify nodes with more than 70 % bootstrap support from a separate maximum likelihood analysis (see Supplemental Fig. S2). Supergroups are identified as indicated. B1 and B2 are new designations used to identify the major split within supergroup B

other loci. Perhaps the most important rationale for using the 16S rRNA gene is that it not only provides a suitable phylogenetic signal, but that 16S rRNA primers are sometimes able to identify *Wolbachia* that are otherwise undetectable with other primers, including those used for MSLT. For example, in a *Wolbachia* screen in 37 aphids, Augustinos et al. (2011) reported successful 16S rRNA amplification in 35 samples, whereas amplification of the *ftsZ* gene was successful in only two samples.

Based solely on the 16S rRNA data presented here, it is premature to consider clades B1 and B2 as new supergroups; however, the phylogeny revealed in this study has several practical implications. First, it may help in the design new primers that can be used to amplify *Wolbachia* from host taxa in which the bacteria have not yet been identified. Augustinos et al. (2011) employed this strategy to increase detection of *Wolbachia i*n aphids that were initially refractory to amplification using standard primer sets. Second, with the addition of MLST data from brine flies, the nature of supergroups in defining *Wolbachia*, which is the subject of debate, may be clarified. Third, on a related matter, all *Wolbachia*, regardless of host association, are currently classified as a single species, *Wolbachia pipientis*. As the diversity of these bacteria continues to expand, consideration should be given to proposals (e.g., Ramírez-Puebla et al. 2015) to divide this single taxon into additional species on the basis of supergroup affiliation. Fourth, this study expands the host diversity of *Wolbachia* to include shore flies, a widespread group of adaptable insects, some of which inhabit extreme environments.

Greater resolution of the *Wolbachia* phylogeny presented here can come from MLST analysis, 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 A, E and F, would provide greater confidence for the overall phylogeny of *Wolbachia* and the potential identification of a new supergroup containing brine fly *Wolbachia*. Nevertheless, the congruence of the trees presented here across three separate phylogenetic analyses and including a variety of taxa indicate that the 16S rRNA gene does indeed provide a useful phylogenetic signal, consistent with other studies (Augustinos et al. 2011; Lo et al. 2002; Ros et al. 2009; Salunke et al. 2010).

In addition to their well-documented effects on host reproduction, *Wolbachia* may play important roles in modulating host physiology and immunity (reviewed in Sicard et al. 2014). This may be particularly important in GSL brine flies, which must constantly negotiate the extreme osmotic conditions of the lake. In mosquito cell lines, *Wolbachia* have been shown to induce the production of host antioxidant enzymes (Brennan et al. 2008), and have also been shown in other insects to influence iron homeostasis, which may also be associated with the regulation of oxidative stress (Kremer et al. 2009). Analysis of gene expression using microarrays reveals that *Wolbachia* affect the expression of a number of host genes in *Drosophila*, including those related to heat stress response (Xi et al. 2008). Although not addressed in this study, the identification of *Wolbachia* in brine flies is a first step in dissecting the host effects, if any, imparted by these endosymbionts in the hypersaline environment represented by Great Salt Lake. Our findings further support claims for *Wolbachia* as the most common and diverse endosymbiont found in a broad ecdyzozoan host range.

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