



Species in *Wolbachia*? Proposal for the designation of ‘*Candidatus Wolbachia bourtzisii*’, ‘*Candidatus Wolbachia onchocercicola*’, ‘*Candidatus Wolbachia blaxterii*’, ‘*Candidatus Wolbachia brugii*’, ‘*Candidatus Wolbachia taylorii*’, ‘*Candidatus Wolbachia collembolicola*’ and ‘*Candidatus Wolbachia multihospitum*’ for the different species within *Wolbachia* supergroups

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ARTICLE INFO

Article history:

Received 22 October 2014

Received in revised form 21 May 2015

Accepted 27 May 2015

Keywords:

Wolbachia supergroups

Wolbachia Candidatus

Wolbachia taxonomy

ANI

DDH

ABSTRACT

Wolbachia are highly extended bacterial endosymbionts that infect arthropods and filarial nematodes and produce contrasting phenotypes on their hosts. *Wolbachia* taxonomy has been understudied. Currently, *Wolbachia* strains are classified into phylogenetic supergroups. Here we applied phylogenomic analyses to study *Wolbachia* evolutionary relationships and examined metrics derived from their genome sequences such as average nucleotide identity (ANI), *in silico* DNA–DNA hybridization (DDH), G + C content, and synteny to shed light on the taxonomy of these bacteria. Draft genome sequences of strains wDacA and wDacB obtained from the carmine cochineal insect *Dactylopius coccus* were included. Although all analyses indicated that each *Wolbachia* supergroup represents a distinct evolutionary lineage, we found that some of the analyzed supergroups showed enough internal heterogeneity to be considered as assemblages of more than one species. Thus, supergroups would represent supraspecific groupings. Consequently, *Wolbachia pipientis* nomen species would apply only to strains of supergroup B and we propose the designation of ‘*Candidatus Wolbachia bourtzisii*’, ‘*Candidatus Wolbachia onchocercicola*’, ‘*Candidatus Wolbachia blaxterii*’, ‘*Candidatus Wolbachia brugii*’, ‘*Candidatus Wolbachia taylorii*’, ‘*Candidatus Wolbachia collembolicola*’ and ‘*Candidatus Wolbachia multihospitum*’ for other supergroups.

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Introduction

Wolbachia is a genus of endosymbiotic bacteria that are wide spread in nature. *Wolbachia* endosymbionts do not have a free-living phase and are under confinement to particular hosts. It is estimated that *Wolbachia* may be found in 40% of arthropod species [106], while a previous report calculated 60% [44]. *Wolbachia* endosymbionts have been found associated with nematodes from the Onchocercidae family [22,54]. Interactions with their

hosts range from parasitism to mutualism. In arthropods are mostly considered as parasites since they may manipulate host reproduction by mechanisms like parthenogenesis, feminization, male killing, and cytoplasmic incompatibility [12,80,100]. However, *Wolbachia* symbiosis has been implicated in host fitness [15,94], or as being necessary for oogenesis [25]; in nematodes they are regarded as mutualistic and essential for survival [58]. *Wolbachia* symbiosis is outstanding as it may cause host speciation events [11].

Some insects and their endosymbionts have a parallel evolutionary history, and cospeciation events have been described for both host and bacteria, especially primary endosymbionts [1,10,21,79,87]. For endosymbionts that have cospeciated with

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their hosts, endosymbionts in different hosts would be distinct species. It seems that cospeciation is rare in *Wolbachia* and insects as their phylogenies are usually not congruent [1,49,86,88]. Thus, adaptations to different hosts would not necessarily mean bacterial speciation. *Wolbachia* infections in insects may be recent in some cases [41,48], implying a short host–symbiont interaction that would not lead to speciation. Recent *Wolbachia* acquisitions may come from horizontal transfers from close or even distant insects [41]. In filarial nematode–*Wolbachia* associations, congruence between *Wolbachia* phylogenies and those of their host has been documented [9,17,32]. In this case, cospeciation between bacteria and their worm hosts seems to have occurred and a single origin of this symbiosis for supergroups C and D has even been proposed [32]. *Wolbachia* have become essential for nematode development and play an important role in host embryogenesis [58]. Nematodes treated with antibiotics cannot reach adulthood [13,93].

Wolbachia pipientis Hertig 1936 [43], was first observed in cells of the *Culex pipiens* mosquito [42]. Heterogeneity within *Wolbachia* has been revealed by sequence analysis of 16S rRNA genes and protein-coding genes, resulting in its distribution into sixteen phylogenetic supergroups, ten of which are found in arthropods (A, B, E, H, I, K, M, N, P, Q), five in nematodes (C, D, J, L, O) and one comprising both arthropod and nematode endosymbionts (F) [4]. The strains of *Wolbachia* detected in Australian spiders [81], were designed as Supergroup G but it was later revealed that it has a *wsp* gene that is a recombinant between those of A and B supergroups rather than being a distinct new supergroup [8]. A phylogenetic tree based on a multilocus analysis has been recently published giving insight about the relationships between *Wolbachia* supergroups [37]. A consensus of whether supergroups represent lineages of *W. pipientis* or distinct species has not been reached. Sequence divergence between supergroups seemed to indicate that each represented a species [78], however, other studies have indicated that they do not represent isolated genetic entities [7,99], as would be expected from *bona fide* species [61]. *Wolbachia* have been described as highly recombinogenic bacteria [6,7,99]. Multiple infections with different *Wolbachia* are frequent in the same insect individual [98,104], affording the opportunity for recombination between different strains, including not closely related ones [6,104]. Nevertheless, a recent study found that recombination is far higher within supergroups than between them [30]. Recombination events between supergroups are limited to small DNA fragments.

Endosymbiont confinement in a host leads to an inevitable dependence on the host. This is evident upon inspection of endosymbiont genomes, which generally lack many functions required for independent living. Classic taxonomy relying on phenotypic characterization of pure cultures as well as establishing genomic relatedness by DNA–DNA hybridization (DDH) experiments could not be applied to non-cultivable endosymbionts like *Wolbachia*. In the genomic era, however, metrics based on genome sequences like ANI (average nucleotide identity) and *in silico* DDH can be used as replacements for wet lab DDH [3,38], thus allowing the use of similar taxonomic criteria for both cultivable and non-cultivable prokaryotes. Furthermore, it is increasingly acknowledged that phenotypes should not be given as much importance for species delineation as they currently are [20,70,96].

Here, we evaluated the diversity of *Wolbachia* by performing phylogenomic analyses and by analyzing genome-derived metrics like ANI, *in silico* DDH, G+C content and synteny in order to shed light on the taxonomy of these endosymbionts. Additionally, we increased the genomic database of *Wolbachia* by reporting sequences from two strains recently obtained from the carmine cochineal insect *Dactylopius coccus*.

Materials and methods

Genome sequences

Sequences of all reported *Wolbachia* genomes were retrieved from GenBank database, except those of strains wDi and wLs, which were available at <http://nematodes.org/genomes/index-filaria.html> [22]. Genomes of *Wolbachia* strains wDacA (Bioproject PRJNA274701) and wDacB (Bioproject PRJNA274698) were sequenced by a metagenomic approach from dissected cochineal insects of *Dactylopius coccus*. Detailed functional analyses of those genomic sequences will be reported elsewhere (Ramírez-Puebla et al., in preparation). For G+C content determination, contigs of each genome were concatenated, the number of G plus C nucleotides counted and the sum divided by the genome length. Genome of strain wMen was obtained from the *Strepsiptera* Genome Project [68], and genomes of strains wFol, wOc and wCte were only deposited like Sequence Read Archive (SRA) so they were not included in G+C determination because they were not completely sequenced [36].

Phylogenomic analyses

Predicted proteomes were obtained from annotated genomes deposited at GenBank if available. The RAST server was also used for annotating and comparing whole genome sequences [5]. The AMPHORA2 pipeline [103], was used to identify a set of 31 conserved bacterial proteins from complete or draft genomes. Sequencing reads were obtained from the Sequence Read Archive (SRA) database to obtain phylogenetic markers for strains wMen, wFol, wOc, and wCte. Reads were mapped against individual marker genes obtained from fully sequenced *Wolbachia* genomes using the runMapping option from Newbler (Roche). The obtained mapped reads were processed to obtain the markers for these strains by performing tblastn searches against reference protein sequences corresponding to the markers from other sequenced strains. Protein sequences were concatenated using the EMBOSS union web tool (<http://emboss.bioinformatics.nl/cgi-bin/emboss/union>). The concatenated sequences were then aligned using MUSCLE v.3.8.31 [29], and the resulting alignment was processed with Gblocks [18], to obtain conserved protein blocks and eliminate poorly aligned positions and divergent regions. The edited alignment contained 9151 amino acid positions. A maximum-likelihood analysis was then performed using the JTT substitution model under PhyML 3.0 [40]. Branch support values are based on 100 bootstrap replicates. The genomes from *Ehrlichia canis* (GenBank CP000107) and *Anaplasma marginale* (GenBank CP001079) were used as outgroups.

In silico DDH and ANI calculations

DDH estimates were computed using the Genome-to-Genome Distance Calculator version 2.0 [65], as recommended by Auch et al. [2,3], and Meier-Kolthoff et al. [65]. BLAST+ was used for alignment and formula 2 for genome distance estimation. ANI values were calculated as previously proposed [38], using the ANI calculator from the Kostas lab (<http://enve-omics.ce.gatech.edu/ani/>) with default parameters.

Synteny

Syntenic blocks between ten finished *Wolbachia* genomes were identified by BLASTN. Only blocks at least 3000 bp in length and with 80% identity or higher were used to construct the graphs using the Artemis comparison tool [16].

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