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Species in *Wolbachia*? Proposal for the designation of '*Candidatus* Wolbachia bourtzisii', '*Candidatus* Wolbachia onchocercicola', '*Candidatus* Wolbachia blaxteri', '*Candidatus* Wolbachia brugii', '*Candidatus* Wolbachia taylori', '*Candidatus* Wolbachia collembolicola' and '*Candidatus* Wolbachia multihospitum' for the different species within *Wolbachia* supergroups

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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 *w*DacA and *w*DacB 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 multihospitis' 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 *w*Di and *w*Ls, which were available at http://nematodes.org/genomes/index_filaria.html [22]. Genomes of *Wolbachia* strains *w*DacA (Bioproject PRJNA274701) and *w*DacB (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 *w*Men was obtained from the *Strepsiptera* Genome Project [68], and genomes of strains *w*Fol, *w*Oc and *w*Cte 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 *w*Men, *w*Fol, *w*Oc, and *w*Cte. 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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Results and discussion

Genome-based relationships

Predicted evolutionary relationships between all 34 complete or almost complete *Wolbachia* genomes currently available and our two *Wolbachia* strains from *D. coccus*, *w*DacA and *w*DacB (Table 1) based on a set of conserved proteins is shown in Fig. 1. The distinctiveness of *Wolbachia* supergroups was evidenced by each forming a different and well-supported clade. The A and B supergroups clustered together in a branch separated from the C, D and F supergroups as previously observed by Nikoh et al. [69], for a set of six genomes using 52 ribosomal proteins. The phylogenetic reconstructions were also in agreement with a previous analysis obtained with a set of 90 orthologous genes from only eleven sequenced *Wolbachia* strains [22]. All the *Wolbachia* strains associated with *Culex* mosquitoes (*W. pipientis* representatives) tightly clustered in a single clade within supergroup B. The *Wolbachia* strain *w*Bol1-b from *Hypolimnas bolina* was also phylogenetically close to the *W. pipientis* strains. *Wolbachia* strains from *D. coccus* clustered into distinct supergroups. *w*DacA was resolved as the most phylogenetically distant strain within supergroup A, whereas *w*DacB was a member of supergroup B having *w*VitB from *Nasonia vitripennis* as its closest sequenced relative. The strain *w*Fol associated with the springtail *Folsomia candida* was found placed as the most distant of all the analyzed *Wolbachia* supergroups as reported previously [36].

G + C content

The G + C content of *Wolbachia* genomes ranged from 32.1% to 38.4% (Table 1) evidencing an enrichment of AT nucleotides as is common in endosymbionts [67]. Mean G + C contents of supergroups A, B, C and D were 35.5%, 34.0%, 32.4% and 33.4%, respectively. The sequenced representative of supergroup F had a G + C content of 36.3%. Although the analyzed genomes may not completely comprise the natural variation present in *Wolbachia* supergroups, it is worth noting that each supergroup seems to have a characteristic G + C content (Table 1).

In silico *DDH and ANI estimates*

DDH is the "gold standard" for species delineation in prokaryotes but it is not applicable for non-cultivable bacteria like *Wolbachia*. ANI represents a suitable surrogate for wet lab DDH as correlation analyses indicate that strains showing ANI higher or equal than 95–96% shared DDH values higher or equal than 70% and are thus considered to be of the same species [38]. Genome sequences also allow the estimation of *in silico* DDH values, and

Table 1
Characteristics of the sequenced *Wolbachia* genomes used in this work.

Wolbachia strain	Host species	GenBank accession number	Genome status	Number of contigs	Genome size (bp)	G + C%	Super group	Reference
*w*Mel	*Drosophila melanogaster*	AE017196	Complete	1	1,267,782	35.2	A	[102]
*w*MelPop	*Drosophila melanogaster*	AQQE00000000	WGS	80	1,239,155	35.1	A	[101]
*w*Ri	*Drosophila simulans* Riverside	CP001391	Complete	1	1,445,873	35.2	A	[52]
*w*Ha	*Drosophila simulans*	CP003884	Complete	1	1,295,804	35.1	A	[30]
*w*Sim	*Drosophila simulans*	AAGC00000000	WGS	629	1,063,100	35.4	A	[84]
*w*Au	*Drosophila simulans*	LK055284	Complete	1	1,268,461	35.2	A	[91]
*w*Rec	*Drosophila recens*	JQAM00000000	WGS	43	1,126,656	35.2	A	[66]
*w*Suzi	*Drosophila suzukii*	CAOU02000000	WGS	110	1,415,350	35.2	A	[89]
*w*Dwi	*Drosophila willistoni*	AAQP00000000	WGS	260	1,145,915	38.4	A	Remington et al. (unpublished)
*w*Ana	*Drosophila ananassae*	AAGB00000000	WGS	464	1,440,750	35.7	A	[84]
*w*Gmm	*Glossina morsitans morsitans*	AWUH00000000	WGS	241	1,019,510	35.2	A	[14]
*w*Uni	*Muscidifurax uniraptor*	ACFP00000000	WGS	256	867,873	35.2	A	[52]
*w*DacA	*Dactylopius coccus*	PRJNA274701	WGS	456	933,576	35.0	A	This study
*w*No	*Drosophila simulans*	CP003883	Complete	1	1,301,823	34.0	B	[30]
*w*Pip_Pel	*Culex quinquefasciatus*	AM999887	Complete	2	1,482,455	34.2	B	[51]
*w*Pip_JHB	*Culex quinquefasciatus*	ABZA00000000	WGS	21	1,543,661	34.2	B	[85]
*w*Pip_Mol	*Culex molestus*	HG428761	Complete	1	1,340,443	33.9	B	[74]
*w*Pip	*Culex pipiens molestus*	CACK00000000	WGS	888	1,479,531	34.3	B	Sinkins et al. (unpublished)
*w*Di	*Diaphorina citri*	AMZJ00000000	WGS	124	1,240,904	34.0	B	[83]
*w*Bol1-b	*Hypolimnas bolina*	CAOH00000000	WGS	144	1,377,933	33.9	B	[28]
*w*AlbB	*Aedes albopictus*	CAGB00000000	WGS	156	1,162,431	33.7	B	[63]
*w*DacB	*Dactylopius coccus*	PRJNA274698	WGS	321	1,282,277	34.2	B	This study
*w*VitB	*Nasonia vitripennis*	AERW00000000	WGS	523	1,107,643	33.9	B	[50]
*w*Cte	*Ctenocephalides felis*	SRR1222150	Raw data	–	–	–	B	[36]
*w*Oo	*Onchocerca ochengi*	HE660029	Complete	1	957,990	32.1	C	[24]
*w*Ov	*Onchocerca volvulus* strain Cameroon	HG810405	Complete	1	960,618	32.1	C	Cotton et al. (unpublished)
*w*Di	*Dirofilaria immitis*	PRJEB4154[a]	WGS	2	921,012	32.7	C	[22]
*w*Bm strain TRS	*Brugia malayi*	AE017321	Complete	1	1,080,084	34.2	D	[35]
*w*Bn	*Wuchereria bancrofti*	ADHD00000000	WGS	763	1,052,327	34.0	D	[26]
*w*Ls	*Litomosoides sigmodontis*	PRJEB4155[a]	WGS	10	1,048,936	32.1	D	[22]
*w*Fol	*Folsomia candida*	SRR1222159	Raw data	–	–	–	E	[36]
*w*Cle	*Cimex lectularius*	AP013028	Complete	1	1,250,060	36.3	F	[69]
*w*Oc	*Osmia caerulescens*	SRR1221705	Raw data	–	–	–	F	[36]
*w*Men	*Mengenilla moldrzyki*	SRX095325	WGS	–	–	–	F	[68]

[a] Accessions numbers correspond to the European Nucleotide Archive database as submitted by the original authors.

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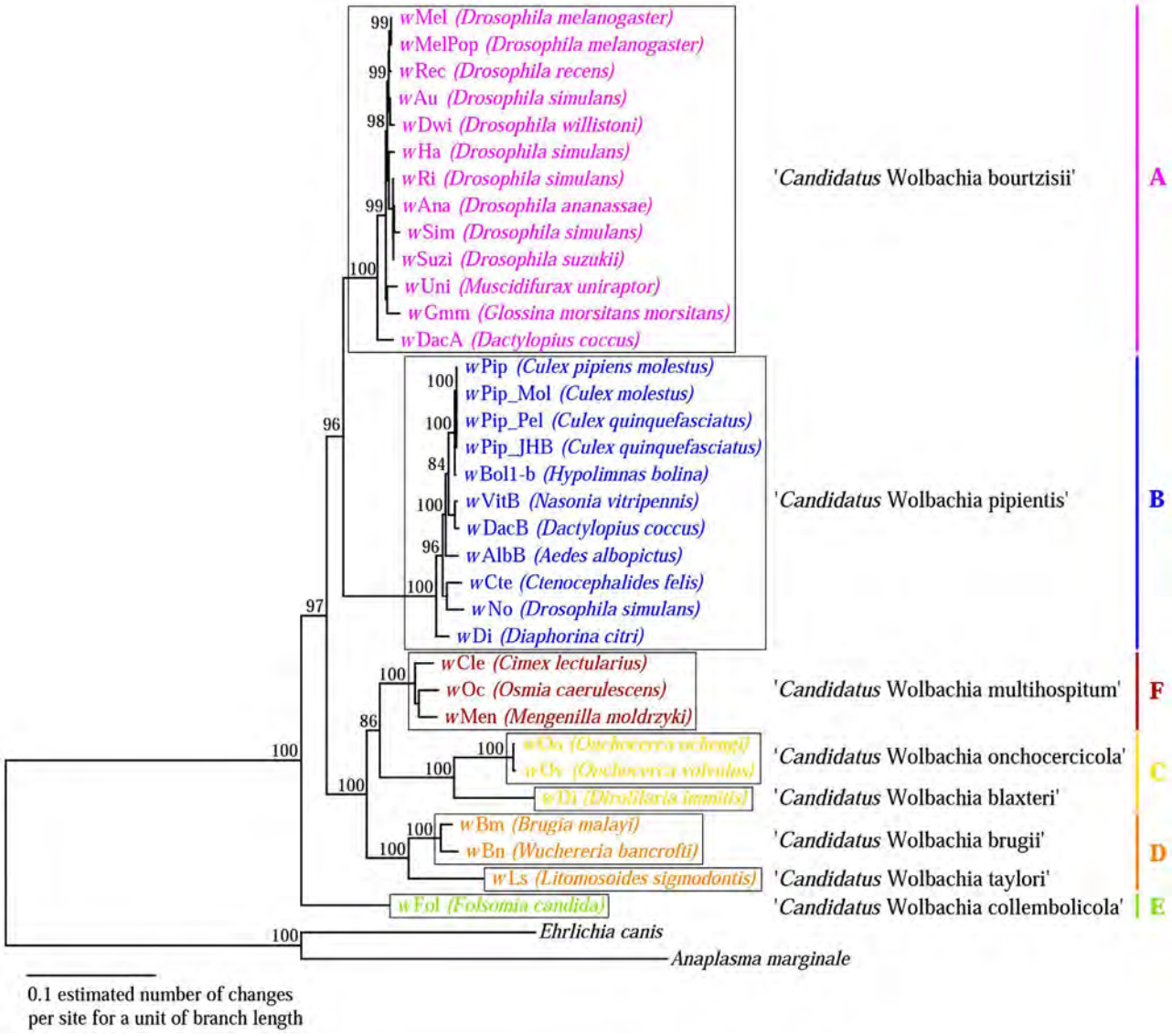

Fig. 1. Phylogenomic tree showing evolutionary relationships between *Wolbachia* strains inferred with PhyML based on a concatenated alignment of 31 marker proteins detected with AMPHORA2 and analyzed with the JTT substitution model. Hosts for each strain are indicated in forceps. Strains included in the new designations for each species name are boxed. Proposed species names are shown next to the boxes. Supergroups are shown to the right. Numbers at the branch points represent bootstrap support values based on 100 replicates. The scale bar represents the estimated number of amino acid changes per site for a unit of branch length.

these correlate very well with wet lab DDH [3]. Based in these criteria other bacteria as *Ensifer* and *Rhizobium* have been reclassified [71].

In silico DDH and ANI values were calculated for all pairs of analyzed *Wolbachia* genomes (Tables 2 and 3, respectively). Strains from different supergroups showed maximum ANI and *in silico* DDH values of 86.8% and 34.6%, respectively indicating that *Wolbachia* is comprised of several different species as previously suggested based on fewer ANI comparisons [75]. Within each supergroup, most members showed ANI values over 96% (Table 3) and *in silico* DDH values over 70% (Table 2), relatedness levels that are consistent with single species. However, in some supergroups there were strains with enough differences to put them below or near the borderline for species delineation. In supergroup A *Dactylopius* strain *w*DacA, and in supergroup B *Drosophila simulans* strain *w*No and *Diaphorina citri* strain *w*Di, showed *in silico* DDH values <62%, below the species circumscription level with all members of their own supergroups and ANI values just above of the species cut-off level (Table 2). In the phylogenomic analyses, strains *w*DacA, *w*No, *w*Di occupied peripheral positions within their supergroups (Fig. 1). *w*AlbB from *Aedes albopictus* and *w*DacB from *D. coccus* in supergroup B, also showed *in silico* DDH values below or very close to 70% with their supergroup neighbors, although these differences were not reflected by low ANI values (Table 2).

Within supergroup B, *in silico* DDH and ANI values were high among representative sequenced strains of *Wolbachia pipientis* (*w*Pip strains). Values were also high between *w*Bol1-b and *W. pipientis*. In contrast, comparisons between *w*Pip strains and other members of supergroup B produced values that were just over or slightly under the cutoff limits recommended for species delineation. The genome from strain *w*VitB had ANI value of around 97.4% when compared with the *w*Pip strain (Table 3) but most of their DDH estimates were below 70% (Table 2).

Clear examples of the existence of different species within supergroups were observed for nematode strains *w*Di of

Table 2
In silico DNA–DNA hybridization (DDH).

		in silico DNA-DNA[a] hybridization																														
Strain	Supergroup	*w*Mel	*w*MelPop	*w*Rec	*w*Au	*w*Dwi	*w*Uni	*w*Gmm	*w*Ana	*w*Ri	*w*Suzi	*w*Sim	*w*Ha	*w*DacA	*w*Pip	*w*Pip_Mol	*w*Pip_Pel	*w*Pip_JHB	*w*Bol1-b	*w*VitB	*w*DacB	*w*Di	*w*AlbB	*w*No	*w*Oo	*w*Ov	*w*Di	*w*Bm	*w*Bn	*w*Ls	*w*Cle
*w*Mel	A	100.0	77.7	89.3	93.9	70.5	75.8	71.2	72.8	71.9	72.1	75.1	73.3	57.0																		
*w*MelPop	A		100.0	95.3	93.6	92.6	76.2	71.7	72.8	72.1	72.3	74.9	73.2	57.6																		
*w*Rec	A			100.0	90.6	94.0	75.4	72.0	73.0	70.9	71.2	74.3	75.2	57.6																		
*w*Au	A				100.0	64.7	75.6	70.8	72.4	70.8	71.2	74.3	73.4	57.0																		
*w*Dwi	A					100.0	75.4	70.5	73.4	72.0	71.9	74.4	72.6	57.0																		
*w*Uni	A						100.0	74.5	73.5	73.7	73.8	73.3	73.6	58.6																		
*w*Gmm	A							100.0	67.1	65.1	66.1	67.9	69.5	56.4																		
*w*Ana	A								100.0	98.0	97.9	95.6	72.0	57.1																		
*w*Ri	A									100.0	98.6	85.2	69.3	55.9																		
*w*Suzi	A										100.0	90.7	69.5	55.9																		
*w*Sim	A											100.0	72.1	56.9																		
*w*Ha	A												100.0	56.2																		
*w*DacA	A													100.0																		
*w*Pip	B														100.0	100.0	96.7	96.4	83.1	71.5	69.5	58.8	63.7	57.3								
*w*Pip_Mol	B															100.0	84.4	67.6	81.5	70.8	68.5	58.2	63.0	56.6								
*w*Pip_Pel	B																100.0	86.3	78.0	68.6	67.5	56.7	61.9	55.2								
*w*Pip_JHB	B																	100.0	77.2	68.8	67.5	56.3	61.3	55.0								
*w*Bol1-b	B																		100.0	69.8	68.0	58.1	61.9	57.6								
*w*VitB	B																			100.0	83.4	61.5	66.0	61.1								
*w*DacB	B																				100.0	59.6	62.6	58.6								
*w*Di	B																					100.0	59.0	54.5								
*w*AlbB	B																						100.0	58.9								
*w*No	B																							100.0								
*w*Oo	C																								100.0	96.1	29.2					
*w*Ov	C																									100.0	29.1					
*w*Di	C																										100.0					
*w*Bm	D																											100.0	69.8	36.5		
*w*Bn	D																												100.0	38.7		
*w*Ls	D																													100.0		
*w*Cle	F																														100.0	

[a] Values <70% are shaded.

Table 3
Average nucleotide identity (ANI) percentage values between Wolbachia strains.

		ANI[a] percentages for strain																														
Strain	Supergroup	*w*Mel	*w*MelPop	*w*Rec	*w*Au	*w*Dwi	*w*Uni	*w*Gmm	*w*Ana	*w*Ri	*w*Suzi	*w*Sim	*w*Ha	*w*DacA	*w*Pip	*w*Pip_Mol	*w*Pip_Pel	*w*Pip_JHB	*w*Bol1-b	*w*VitB	*w*DacB	*w*Di	*w*AlbB	*w*No	*w*Oo	*w*Ov	*w*Di	*w*Bm	*w*Bn	*w*Ls	*w*Cle
*w*Mel	A	100.0	100.0	99.6	99.6	99.4	97.9	97.3	97.4	97.8	97.7	97.9	97.7	95.0																		
*w*MelPop	A		100.0	99.6	99.6	99.4	97.9	97.4	97.5	97.8	97.7	97.8	97.6	94.9																		
*w*Rec	A			100.0	99.6	99.5	97.9	97.4	97.5	97.6	97.6	97.6	97.8	94.9																		
*w*Au	A				100.0	99.9	98.0	97.4	97.4	97.6	97.6	97.7	97.6	94.9																		
*w*Dwi	A					100.0	97.8	97.2	97.2	97.5	97.4	97.7	97.5	94.5																		
*w*Uni	A						100.0	97.6	97.7	97.6	97.6	97.6	97.5	95.0																		
*w*Gmm	A							100.0	96.9	96.9	96.9	96.9	97.0	94.6																		
*w*Ana	A								100.0	99.9	99.9	99.8	97.7	94.7																		
*w*Ri	A									100.0	100.0	99.9	97.7	94.7																		
*w*Suzi	A										100.0	99.9	97.7	94.7																		
*w*Sim	A											100.0	97.5	94.8																		
*w*Ha	A												100.0	94.8																		
*w*DacA	A													100.0																		
*w*Pip	B														100.0	100.0	99.9	99.9	99.1	97.4	97.3	95.8	96.3	95.3								
*w*Pip_Mol	B															100.0	99.8	99.8	99.0	97.2	97.2	95.7	96.1	95.2								
*w*Pip_Pel	B																100.0	99.9	98.9	97.2	97.1	95.6	96.1	95.1								
*w*Pip_JHB	B																	100.0	98.9	97.1	97.2	95.6	96.1	95.2								
*w*Bol1-b	B																		100.0	97.2	97.1	95.7	96.2	95.3								
*w*VitB	B																			100.0	98.8	95.7	96.2	95.6								
*w*DacB	B																				100.0	95.5	96.1	95.4								
*w*Di	B																					100.0	95.4	94.8								
*w*AlbB	B																						100.0	95.3								
*w*No	B																							100.0								
*w*Oo	C																								100.0	99.4	86.1					
*w*Ov	C																									100.0	86.1					
*w*Di	C																										100.0					
*w*Bm	D																											100.0	96.3	88.8		
*w*Bn	D																												100.0	88.8		
*w*Ls	D																													100.0		
*w*Cle	F																														100.0	

[a] Values <96% are shaded.

Dirofilaria immitis and *w*Ls in supergroups C and D, respectively (Tables 2 and 3). In these cases, both strains occupied peripheral positions within their supergroups (Fig. 1). Also, *in silico* DDH and ANI values supported the differentiation of these strains from other members of their respective supergroups supporting that they belong to different species.

Cospeciation between nematodes and *Wolbachia* is reflected in the fact that more distantly related nematodes [32], also have more distantly related *Wolbachia*. In supergroup D, *Wolbachia* from related nematode genera *Brugia* and *Wuchereria* are more closely related to each other than to *Wolbachia* from the more distant genus *Litomosoides* (Fig. 1, Tables 2 and 3). It is worth noting that *Wolbachia* infecting *Brugia* and *Wuchereria* are at the borderline for species definition by *in silico* DDH. Similarly, in supergroup C, *Wolbachia* from two species of *Onchocerca* are related but showed very low *in silico* DDH and ANI values to the endosymbiont of *D. immitis* (Fig. 1, Tables 2 and 3). Taken together, these data seem to indicate that each nematode genus harbors a different *Wolbachia* species. Nevertheless, the presence of supergroup F *Wolbachia* in both nematodes and insects, and its loss in some filarial species in

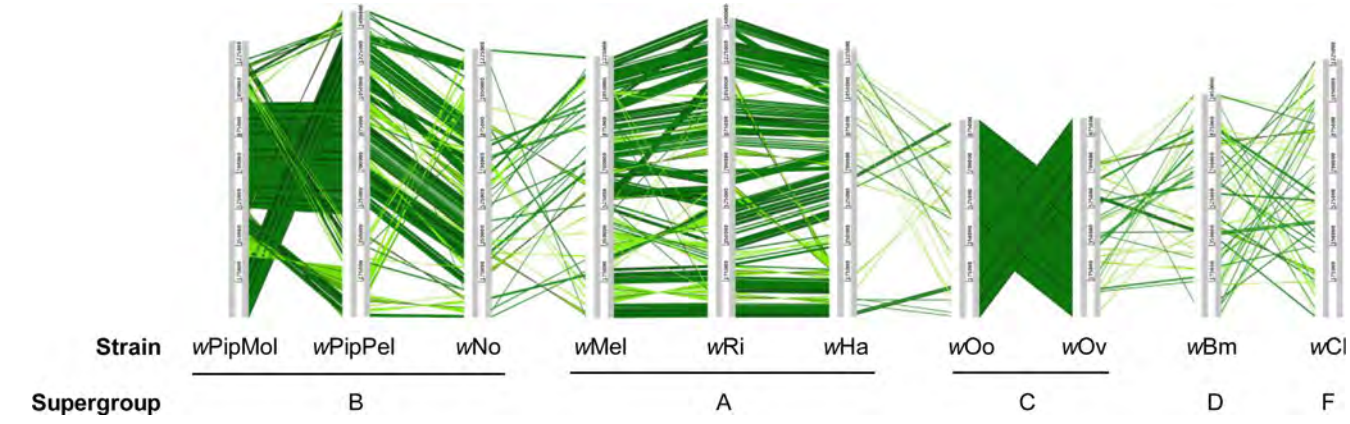

Fig. 2. Synteny between complete genomes of *Wolbachia* strains. Sequence blocks oriented in the same or inverted directions are shown in dark or light green, respectively. (For interpretation of the references to color in this figure caption, the reader is referred to the web version of this article.)

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the onchocercids, contravene the idea of cospeciation in general [33].

Genome synteny

Structural genome comparisons in other bacteria like rhizobia have shown that chromosome synteny is very well conserved within a species and less maintained between different species [39,77]. Synteny was used as a further criterion to distinguish *Wolbachia* species. It has been observed that levels of genome synteny are higher within than between *Wolbachia* supergroups [30], as it is evidenced in Fig. 2 for ten finished genomes. *Wolbachia* genomes have high levels of repetitive DNA and mobile genetic elements that lead to DNA rearrangements that diminish synteny even between related strains [30,35,52,102]. Genome rearrangements in other organisms represent recombination barriers and could lead to genetic isolation [76]. Strain *w*No showing significant divergence by *in silico* DDH and ANI values is less syntenic with its supergroup siblings. It would be worth investigating if a speciation process could start within a supergroup by *Wolbachia* strains developing novel genomic rearrangements, as discussed previously [30].

Conclusions

We showed here that *Wolbachia* supergroups represent distinct evolutionary lineages based on phylogenomics, G + C content, ANI, *in silico* DDH and synteny. Our results support the previous proposal that *Wolbachia* from different supergroups should be considered as genetically distinct clades not only from implications related to host confinement and their biology [72], but on the basis of molecular evidence [30,53,78]. Furthermore, we found heterogeneity within supergroups. The more divergent strains within each supergroup were recovered as outliers in the phylogenomic analyses. Not all strains, however, seem to have accumulated enough nucleotide sequence differences to show ANI values lower than 95–96%, used to delineate different species, with other distant strains in their supergroups (Table 3). Nevertheless, within a supergroup significant genome content differences were evidenced by low *in silico* DDH (less than 70%) and genome synteny among supergroup members was not always high. Thus, our novel analyses indicate that different species may occur inside a supergroup. Consequently, supergroups would have a supraspecific status.

Given the evidence reviewed and presented here, the name *W. pipientis* (Hertig 1936) [43], should be applied only to supergroup B strains. As *Wolbachia* are still uncultivable, the proper designation for supergroup B strains should be '*Candidatus* Wolbachia pipientis'. In order to distinguish the different species within *Wolbachia*, we propose the designation '*Candidatus* Wolbachia bourtzisii' for strains in supergroup A, '*Candidatus* Wolbachia onchocercicola' for endosymbionts of genus *Onchocerca* in supergroup C, '*Candidatus* Wolbachia blaxterii' for endosymbionts of *D. immitis* in supergroup C, '*Candidatus* Wolbachia brugii' for endosymbionts of nematodes from *Brugia* and *Wuchereria* species in supergroup D, '*Candidatus* Wolbachia taylorii' for endosymbionts of nematodes from *Litomosoides* species in supergroup D, '*Candidatus* Wolbachia collembola' for endosymbionts of Collembola arthropods in supergroup E and '*Candidatus* Wolbachia multihospitis' for *Wolbachia* strains hosted by nematodes and arthropods in supergroup F.

Description of '*Candidatus* Wolbachia bourtzisii'

'*Candidatus* Wolbachia bourtzisii' (bourt.zi'si.i. N.L. gen. n. *bourtzisii*, of Bourtzis, in honor of Kostas Bourtzis, as a recognition for his studies on *Wolbachia* and other bacteria associated with arthropods).

The description of the species '*Candidatus* Wolbachia bourtzisii' is based on the studies reported by Louis and Nigro [62], Sacchi et al. [82], Texeira et al. [94], and Zhukova and Kiseleva [105]. Cell size is 0.5 μm in *D. simulans*, and 0.5–1.0 μm in *D. melanogaster*. Cells are roundish and less frequently rod shaped and are surrounded by three enveloping membranes. The first is the plasmatic membrane and the second represents the outer part of the cell wall. The third one, closely related to the cytoplasm of the host cell, forms a vacuole for each single microorganism. Ribosomes and nucleic acid fibrils are observed in the cytoplasm. In *D. melanogaster* individual bacterial cells are distributed throughout the host cell cytoplasm, occasionally occurring as small groups. Bacteria occur in the ovarioles in high numbers and in germline cells like cytocytes, oogonia, oocytes and nurse cells.

The percentage of apoptotic cells in germaria are increased in *D. melanogaster* infected with *w*MelPop. Tetracycline treatments accelerated the time to death in *D. melanogaster* infected with *Drosophila* C virus (DCV) as the bacteria confer resistance to DCV by interfering with virus proliferation. The DNA G + C content is between 35.0 and 38.4 mol% as calculated from genomic sequences. Most strains exhibit a DNA G + C content of 35.2 mol%.

Description of '*Candidatus* Wolbachia onchocercicola'

'*Candidatus* Wolbachia onchocercicola' [on.cho.cer.ci'co.la. N.L. fem. n. Onchocerca a filarial nematode genus; L. suffix – *cola* (from L. masc. or fem. n. *incola*), a dweller, an inhabitant; N.L. fem. n. *onchocercicola*, a dweller of *Onchocerca*].

The description of the species '*Candidatus* Wolbachia onchocercicola' is based on the studies reported by Determan et al. [27], Egyed et al. [31], Horeauf et al. [46], Kozek and Marroquin [55], and Langworthy et al. [59]. Cell size is 0.3 up to 0.8 μm in diameter and 1.5 up to 1.8 μm in length. Cells are generally round or spherical shaped. Bacteria are located in the cytoplasm surrounded by a membrane-bound vacuole. In *Onchocerca lupi* each vacuole contains only one bacterium surrounded by a double membrane. In contrast, in *Onchocerca volvulus* they often form clusters and in *Onchocerca ochengi* some of them contain up to seven bacteria. Wolbachia live in the subcutaneous and connective tissues of their hosts, usually enclosed in fibrous cysts or nodules. In adults and larvae bacterial cells occur in the lateral cords, and in germinal tissues in females. Depletion of the endosymbiont by oxytetracycline in *O. ochengi* results in the death of adults and microfilaria. Also, there is a decline in the quantity of embryos and an increase in the proportion of embryos showing abnormal morphology. In *O. volvulus* doxycycline treatment blocks embryogenesis. The DNA G + C content is 32.1 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia blaxteri'

'*Candidatus* Wolbachia blaxteri' (blax'ter.i. N.L. gen. n. *blaxteri*, of Blaxter, in honor of Mark Blaxter, in recognition of his molecular studies on nematodes and their associated *Wolbachia* symbionts).

The description of the species '*Candidatus* Wolbachia baxteri' is based on the studies reported by Kozek [55,56], McLaren et al., [64], and Sironi et al. [90]. Cell size is 0.3–1.0 μm in diameter and 4.5 μm in length. Cells are spherical or ovoid shaped. Bacteria are contained in an individual membrane-bounded host vacuole. Some bacterial cells have condensations of dense material within their cytoplasm. In *D. immitis* bacteria occur in the reproductive tract mainly in the ovary and the proximal region of the uterus, and are also found in oocytes and in all embryonic stages of microfilariae developing in the uterus. In lateral cords of adults, they occur as clusters that can

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fill most of the hypodermal tissue. Often they appear to surround the hypodermal nuclei. In embryos, five to ten bacteria per host cell are found. Also, bacteria are abundant in oogonia, eggs and early dividing embryos. Treatment with tetracycline blocks embryo development. The DNA G + C content is 32.7 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia brugii'

'*Candidatus* Wolbachia brugii' (bru'gi.i. N.L. gen. n. *brugii*, of Brug, named after S. L. Brug, a Dutch parasitologist who first described the filarial nematode *Brugia malayi*, a model for the study of *Wolbachia*-nematode relationships).

The description of the species '*Candidatus* Wolbachia brugii' is based on the studies reported by Fischer et al. [34], and Landmann et al. [57,58], and Taylor et al. [92]. Cell size is 0.5 μm up to 1 μm. Cells are spherical or have an elongated shape and are surrounded with a double membrane. Bacteria are contained within membrane-bound vacuoles. In *Brugia malayi* clusters of bacteria are detected in microfilaria. In larvae L2, bacterial cells are detected in the hypodermis and in L3 and L4 larvae in the cells of lateral chord, in high numbers. In adult female worms, bacteria are commonly found in the lateral hypodermal cords, in hypodermis, and close to or inside the ovaries. Bacteria are also seen in the cells surrounding the basal lamina of the oviduct. In adult male worms, microfilariae, and third-stage larvae bacteria are detected in the lateral cord, but in lower numbers compared with females and dispersed in focal groups or as individual bacteria. They are also detected in testis and the border of vas deferens. In *Wuchereria bancrofti* bacteria show a similar distribution as in *B. malayi*, in small clusters or as a single bacterium.

Tetracycline treatments dramatically reduce the endosymbiont population in female adults of *B. malayi*. Pyknotic nuclei are observed throughout the ovaries and uteri in the female germline. Microfilaria resulting from a completed embryogenesis after antibiotic treatments, showed defects as abnormal muscle quadrants. Apoptotic nuclei are detected in the ovaries of treated females and become more numerous as the uteri is filled with embryos. The DNA G + C content is between 34.0 and 34.2 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia taylori'

'*Candidatus* Wolbachia taylori' (tay'lo.ri. N.L. gen. n. *taylori*, of Taylor, in honor of Mark J. Taylor, in recognition of his studies on the role of *Wolbachia*–nematode symbiosis in human diseases and his search for treatments).

The description of the species '*Candidatus* Wolbachia taylori' is based on the studies reported by Chagas-Moutinho et al. [19], and Horeauf et al. [45]. Cell size is approximately1 μm and round shaped. Cells present a reduced cell wall and not a typical septum during cell division. Cells are surrounded by a host-derived vacuolar membrane. In *Litomosoides chagasfilhoi*, bacterial cells occur in regions of the hypoderm, in the oocytes, early-stage embryos and complete developed intrauterine microfilariae close to the cell host nucleus. In other filarial tissues, bacteria are found in intracellular vacuoles associated to the nuclear envelope. They are also observed in proximity to the endoplasmic reticulum. TEM suggested a single bacterium per vacuole.

Depletion by tetracycline results in infertility by blocking female worm development and early embryogenesis in *Litomosoides sigmodontis*. The DNA G + C content is 32.1 mol% as calculated from genomic sequences.

Description of '*Candidatus* Wolbachia collembolicola'

'*Candidatus* Wolbachia collembolicola' [col.lem.bo.li'co.la. N.L. n. pl. *Collembola* a lineage of hexapods; L. suffix – *cola* (from L. masc. or fem. n. *incola*), a dweller, an inhabitant; N.L. fem. n. *collembolicola*, a dweller of *Collembola*].

The description of the species '*Candidatus* Wolbachia collembolicola' is based on the studies reported by Czarnetzki and Tebbe [23], Pike and Kingcombe [73], Timmermans and Ellers [95], and Vandekerckhove et al. [97]. Cells detected in hexapod species of the order Collembola. Cell size is 0.2 μm up to 1.4 μm. Cells are pleomorphic from curved to almost hairpin-shaped. Cell wall lacks detectable peptidoglycan layer. Periplasmic space is of around 5–15 nm. Cells are surrounded by a host-derived vacuolar membrane. DNA filaments are visible in a rather diffuse network dispersed throughout the cell and interspersed with ribosomes. Cells occur in aggregations and are found mostly in close association with the rough endoplasmic reticulum in the ovaries. Fat bodies and interstitial cells as detected by TEM techniques or restricted to the ovary and brain as detected by FISH techniques.

Infection is obligatory for host offspring survival. The endosymbiont is sensitive to high-dose of rifampicin and heat treatments. High-dose tetracycline treatment is inefficient for removing cell infections. Bacteria obligate role early in the parthenogenetic developmental process includes egg hatching.

Description of '*Candidatus* Wolbachia multihospitum'

'*Candidatus* Wolbachia multihospitis' (mul.ti.hos'pi.tum. L. adj. *multus* many, numerous; L. n. *hospes -itis*, he who entertains a stranger, a host; N.L. gen. pl. n. multihospitum of numerous hosts, referring to the occurrence of the bacterium on various species of arthropods and nematodes).

The description of '*Candidatus* Wolbachia multihospitis' is based on the studies reported by Ferri et al. [33], Hosokawa et al. [47], and Lefoulon et al. [60]. In *Cimex lectularius* cell size is 0.5 up to 1.2 μm. Cells are rod-shaped. In males, bacterial cells are located in the testis-associated bacteriome, whereas in females they are located in bacteriomes and ovaries. Cells are also detected in the nutritive cord and developing oocytes. In the nematode *Madathamugadia hiepei*, they are detected in young and late embryos. In adult females they are observed in the ovaries and the intestinal wall. In contrast with other nematodes they are absent in the hypodermal lateral chord. In *Cercopithifilaria japonica* and *Mansonella perforata* bacteria are located in the epithelial somatic gonad and in the intestinal wall.

Elimination of the endosymbiont by rifampicin treatments in *C. lectularius* resulted in deformed developing eggs, reduction in the adult emergence rate and prolonged nymphal period. The DNA G + C content is 36.3 mol% as calculated from genomic sequences.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.05.005

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