

# How Diverse Is the Genus *Wolbachia*? Multiple-Gene Sequencing Reveals a Putatively New *Wolbachia* Supergroup Recovered from Spider Mites (Acari: Tetranychidae)<sup>∇†</sup>

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Received 14 May 2008/Accepted 13 December 2008

**At least 20% of all arthropods and some nematode species are infected with intracellular bacteria of the genus *Wolbachia*. This highly diverse genus has been subdivided into eight “supergroups” (A to H) on the basis of nucleotide sequence data. Here, we report the discovery of a new *Wolbachia* supergroup recovered from the spider mite species *Bryobia* species V (Acari: Tetranychidae), based on the sequences of three protein-coding genes (*ftsZ*, *gltA*, and *groEL*) and the 16S rRNA gene. Other tetranychid mites possess supergroup B *Wolbachia* strains. The discovery of another *Wolbachia* supergroup expands the known diversity of *Wolbachia* and emphasizes the high variability of the genus. Our data also clarify the existing supergroup structure and highlight the use of multiple gene sequences for robust phylogenetic analysis. In addition to previous reports of recombination between the arthropod-infecting supergroups A and B, we provide evidence for recombination between the nematode-infecting supergroups C and D. Robust delineation of supergroups is essential for understanding the origin and spread of this common reproductive parasite and for unraveling mechanisms of host adaptation and manipulation across a wide range of hosts.**

*Wolbachia* is a genus of endosymbiotic alphaproteobacteria infecting a wide range of arthropods and filarial nematodes. In arthropods, *Wolbachia* often manipulates the reproductive mode of its host, causing parthenogenesis, feminization, cytoplasmic incompatibility, or male killing (48). In nematodes, and also in some arthropods, *Wolbachia* is an obligate symbiont required for host fertility (11, 39). It is estimated that more than 20% of arthropod species are infected with *Wolbachia*, including all major insect orders and some crustaceans and chelicerates (16, 31, 33, 60). The genus *Wolbachia* is genetically highly diverse and is divided into eight “supergroups” (A to H) (35). Currently, all of the supergroups are thought to represent one species, *Wolbachia pipientis* (35). Supergroups A and B were described first and are most commonly found among arthropod species (17, 60, 61). Supergroups C and D are restricted to filarial nematodes (9). Since the description of these four supergroups, the number of host taxa investigated has grown and the accuracy of detection has improved (investigating multiple individuals per host species and using a combination of primers). This led to the discovery of new *Wolbachia* strains and new host species. For example, supergroup F was described in 2002 (34), yet the known diversity and geographic range of host species infected with the supergroup is rapidly expanding. It has been detected in both nematodes and several major arthropod orders (in Chelicerata, scorpions

[Scorpiones]; in Hexapoda, termites [Isoptera], weevils [Coleoptera], bush crickets [Orthoptera], cimicids [Hemiptera], lice [Phthiraptera], and louse-flies [Diptera]) (6, 19, 20, 34, 38, 45). Other host taxa for which there is evidence of infection by supergroup F are cockroaches (Blattodea) and ant lions (Neuroptera) (22, 54). Supergroups E and H are less widespread, with E so far found only in springtails (Collembola) (53, 56) and H in one genus of termites (Isoptera) (14). Isolates recovered from Australian spiders (Araneae) have been assigned as supergroup G (43), although it has been argued that these strains are in fact recombinants between supergroups A and B rather than representing a distinct lineage (7). Other diverse *Wolbachia* strains have been detected in fleas (Siphonaptera) (25), the filarial nematode *Dipetalonema gracile* (Spirurida) (19), and the pseudoscorpion *Cordylochernes scorpioides* (Pseudoscorpionida) (63), but these strains have not yet been assigned to a supergroup.

The genotypic diversity of *Wolbachia* mirrors an equally impressive range of phenotypes in terms of host range and manipulative effects. Much is still unknown about the origin and spread of *Wolbachia*, which host taxa were first infected, and how *Wolbachia* was transferred between taxa. Routes of transfer are still unclear; it is, for example, unknown if arthropods or nematodes were first infected (19). Robust phylogenetic analysis and the delineation of supergroups is therefore essential for elucidating the pathways and processes by which these diverse phenotypes have evolved, and recent studies have highlighted the promise of the multiple-gene sequencing approach (4, 6, 14, 19, 44). Such datasets can also reveal evidence concerning the contribution of lateral gene transfer to the evolution and host adaptation of *Wolbachia*.

Here, we describe the characterization of a diverse sample

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† Supplemental material for this article may be found at <http://aem.asm.org/>.

<sup>∇</sup> Published ahead of print on 19 December 2008.

TABLE 1. Sampling details of tetranychid species analyzed in this study

Code	Species	Country	Locality	Host plant name		Collection date	Reproductive mode <sup>a</sup>
				Common	Scientific		
NL12	<i>B. praetiosa</i>	Netherlands	Amsterdam	Grasses and herbs		May 2004	A
FR16	<i>B. sarothamni</i>	France	Vireux	Common Broom	<i>Cytisus scoparius</i>	July 2006	S
NL14	<i>Bryobia</i> species I	Netherlands	Nieuweschans	Vetches	<i>Vicia</i> sp.	May 2006	A
ITA11	<i>Bryobia</i> species V	Italy	S. Felice Circeo	Grasses and herbs		May 2005	A
T2	<i>T. urticae</i>	Unknown	Unknown	Cucumber	<i>Cucumis sativus</i>	— <sup>b</sup>	S

<sup>a</sup> A, asexual; S, sexual (for details, see reference 41).

<sup>b</sup> Maintained in the laboratory on bean (*Phaseolus vulgaris*) for over 10 years.

of *Wolbachia* strains by multiple-gene sequencing of the 16S rRNA gene and three protein-coding genes (*gltA*, *groEL*, and *ftsZ*) that had been used earlier for supergroup descriptions (14, 19, 35). We report the discovery of a new *Wolbachia* supergroup (K) in a spider mite species of the genus *Bryobia* (*Bryobia* species V; Acari: Tetranychidae) and show that other tetranychid species (*Bryobia praetiosa*, *Bryobia sarothamni*, *Bryobia* species I, and *Tetranychus urticae*) harbor B-group *Wolbachia*. The cocurrence of two supergroups (B and K) within a single host genus is very unusual and has previously been noted only for supergroups A and B, which often occur in the same arthropod species and are known to recombine (7, 60, 61). We further rationalize the supergroup structure by proposing that the strains recovered from fleas (Siphonaptera) (19, 25) and the filarial nematode *D. gracile* (Spirurida) (19) correspond to the new supergroups I and J, respectively.

#### MATERIALS AND METHODS

**DNA isolation, amplification, and sequencing.** We analyzed *Wolbachia* strains from four *Bryobia* species (*B. praetiosa*, *B. sarothamni*, and *Bryobia* species I and V) and *T. urticae* (Table 1). The *Bryobia* species were sampled within Europe between 2004 and 2006, maintained as isofemale lines in the laboratory, and identified using 28S rRNA and cytochrome *c* oxidase subunit I sequence data (42). An infected strain of *T. urticae* has been maintained in the laboratory for over 10 years. DNA was extracted from single individuals using the cetyltrimethylammonium bromide method as described by Ros and Breeuwer (41).

*FtsZ* was amplified and sequenced using the primer sets *ftsZ1* (5'-CCGTATGCCGATTGCAGAGCTTG-3') and *ftsZ2* (5'-GCCATGAGTATCACTTGGCT-3') (30) and *ftsZf1* (5'-GTTGTCGCAATACCGATGC-3') and *ftsZr1* (5'-CTTAAGTAAGCTGGTATATC-3') (61). *groEL* was amplified using the primers *groEL-F* (5'-CAACRGTGRSRRYAACGTGCDGG-3') and *groEL-R* (5'-GATADCCRCGRTCAAAYTGC-3'), hand designed from available *Wolbachia* and *Rickettsia* genome sequences (2, 37, 62) and tested on isolates representative of supergroups A and B (samples kindly donated by Robert Butcher, University of Bath). *gltA* was amplified and sequenced as described by Casiraghi et al. (19).

PCR amplifications were performed in 25- $\mu$ l reaction mixtures containing 2.5  $\mu$ l 10 $\times$  Super *Taq* buffer (HT BioTechnology, Cambridge, United Kingdom), 1.25  $\mu$ l bovine serum albumin (10 mg/ml), 5  $\mu$ l deoxynucleotide triphosphate mixture (1 mM of each nucleotide), 0.2  $\mu$ l of each primer (20  $\mu$ M each), 0.2  $\mu$ l of super *Taq* (5 U/ $\mu$ l) (HT BioTechnology), 13.15  $\mu$ l water, and 2.5  $\mu$ l of DNA extract. The PCR cycling profile for *ftsZ* was 35 cycles of 30 s at 95°C, 30 s at 51°C, and 1 min at 72°C, and for *groEL* it was 35 cycles of 1 min at 95°C, 1 min at 49°C, and 1.5 min at 72°C. The PCR products were purified using a DNA extraction kit (Fermentas, St. Leon-Rot, Germany). The purified products were directly sequenced using the ABI Prism BigDye Terminator Sequence Kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) according to the manufacturer's instructions but diluted 16 times. Both strands of the products were sequenced using the same primers used in the PCR amplification. Sequences were run on an ABI 3700 automated DNA sequencer.

**Amplification and cloning of the 16S rRNA gene.** To check for multiple infections (by different *Wolbachia* strains or by other bacteria), part of the

16S rRNA gene was amplified and cloned for the four *Bryobia* samples. DNA was extracted from five pooled individuals per isofemale line following the cetyltrimethylammonium bromide method (41). Part of the 16S rRNA gene was amplified using the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1513R (5'-ACGGYTACCTTGTTACGACTT-3') (59) in a 25- $\mu$ l reaction mixture containing 2.5  $\mu$ l 10 $\times$  Super *Taq* buffer, 5  $\mu$ l deoxynucleotide triphosphate mixture (1 mM of each nucleotide), 0.5  $\mu$ l of each primer (10  $\mu$ M each), 0.2  $\mu$ l of super *Taq* (5 U/ $\mu$ l), 13.3  $\mu$ l water, and 3  $\mu$ l of DNA extract. The PCR cycling profile was 35 cycles of 1 min at 94°C, 1 min at 54°C, and 1 min at 72°C. The PCR products were purified using the method of Boom et al. (13). The purified products were ligated into a vector (pGEM-T Easy Vector System) and then used to transform JM109 competent cells, according to the manufacturer's instructions (Promega, Madison WI). Plasmids were recovered for 10 colonies per sample, using miniprep procedures (46). The plasmids were sequenced using the M13 forward primer and the internal 16S rRNA gene primers 704f (5'-GTAGAGGTRAAATTCGTAAA-3') and 765r (5'-CTGTTTGCTCCCCACGCYTTG-3') (modified from the method of Bandi et al. [10]) as described above. For *T. urticae*, the 16S rRNA gene was sequenced directly using the same primers and PCR protocol.

**Data assembly and phylogenetic analyses.** The sequences obtained in this study were combined with sequences obtained from GenBank representing all currently known supergroups of *Wolbachia* (see Table S1 in the supplemental material). We analyzed alignments of 636 bp for *gltA*, 489 bp for *ftsZ*, 491 bp for *groEL*, and 1,255 bp for the 16S rRNA gene. Sequences for which at least two of the three protein-coding genes were available were included. The 16S rRNA gene data set contained a few additional samples (for which data on the mentioned protein-coding genes are unknown) to explore the full range of *Wolbachia* diversity. Sequences were identified by the name of the host species. The sequences were aligned using ClustalX version 1.8.0 with default settings (52). Phylogenetic analyses were performed using neighbor-joining (NJ), maximum likelihood (ML), and Bayesian methods for each locus separately and for a concatenated data set of the three protein-coding genes. PAUP\* version 4.0b10 (50) was used to select the optimal evolution model by critically evaluating the selected parameters (51) using the Akaike Information Criterion (1). For the protein-coding genes, we tested if the likelihood of models could be further improved by incorporating specific rates for each codon position (47). This approach suggested the following models: 16S rRNA gene (HKY + I + G), *gltA* (TIM + G), *ftsZ* (TrN with site-specific rates for each codon position), *groEL* (submodel of GTR with rate class "a b c d e" and site-specific rates), and the concatenated data set (submodel of GTR + I + G with rate class "a b c d e"). Under the selected models, the parameters and tree topology were optimized using the successive-approximations approach (49). NJ analyses (p distances) and ML analyses (heuristic search, random addition of sequences with 10 replicates, and TBR branch swapping) were performed in PAUP. The robustness of nodes was assessed with 100 bootstrap replicates. However, as PAUP does not allow site-specific rates in bootstrap analysis, ML bootstrapping for *ftsZ* and *groEL* was performed with gamma distributed rates with 100 bootstrap replicates. Bootstrap values were then plotted on the phylogeny obtained with the original model with site-specific rates. Bayesian analyses were performed as implemented in MrBayes 3.1.2 (40). The models used were GTR + I + G (16S rRNA gene), GTR + G (*gltA*), and GTR with separate rates for each codon position (*ftsZ* and *groEL*). For the concatenated data set, the same models were used for each gene partition. Analyses were initiated from random starting trees. Two separate Markov chain Monte Carlo (MCMC) runs, each composed of four chains (one cold and three heated), were run for 6,000,000 generations. The cold chain was sampled every 100 generations, and the first 15,000 generations were

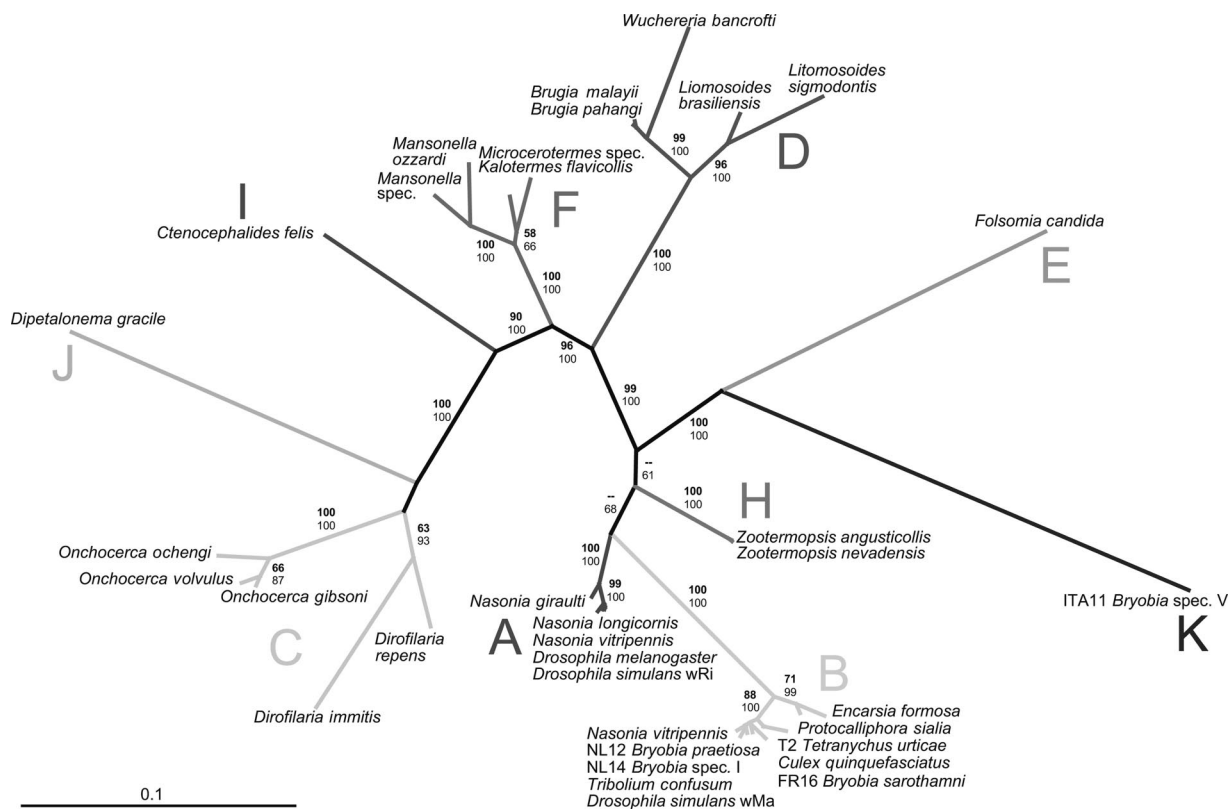


FIG. 1. Concatenated phylogenetic tree (ML, unrooted) based on *Wolbachia* sequences of three protein-coding genes (*gltA*, *ftsZ*, and *groEL*) (1,616 bp). Strains are characterized by the names of their host species. ML bootstrap values (top numbers, in boldface) based on 100 replicates and Bayesian posterior probabilities (bottom numbers) are depicted (only values larger than 50 are indicated). Values for recently diverged taxa within supergroups are sometimes excluded for clarity of presentation. The bar indicates a branch length of 10% likelihood distance.

later discarded (a burn-in of 25%). Posterior probabilities were computed from the remaining trees. We checked whether the MCMC analyses ran long enough by using the program AWTY (<http://ceb.csit.fsu.edu/awty>). Stationarity (stable topologies and posterior probabilities with increasing run time) was assumed when there was convergence between the two MCMC runs and when the cumulative posterior probabilities of splits stabilized; in all analyses, 6,000,000 generations proved sufficient.

**Analysis of recombination.** Alignments were checked for signs of intragenic recombination using the software package RDP3 (36) and by visual inspection. The programs used in the RDP3 software package were RDP, Geneconv, Bootscan, MaxChi, Chimaera, and Sister Scanning. Analyses were run with default settings, except for window and step sizes, which were varied during independent analyses. Analyses were performed for total data sets and reduced data sets with one or two strains representing each supergroup. We have included only those cases of recombination that were detected by at least two of the listed programs and supported by visual inspection.

**Nucleotide sequence accession numbers.** All new sequences were deposited in the GenBank database under accession numbers EU499315 to EU499334 (see Table S1 in the supplemental material).

## RESULTS

Phylogenetic analysis of four *Wolbachia* loci (a total of 2,871 bp) showed the existence of two highly divergent *Wolbachia* strains within the family Tetranychidae (Fig. 1 and 2). The species *Bryobia* species V harbors a *Wolbachia* strain that does not cluster with any of the previously described supergroups. It forms a distinct phylogenetic lineage, highly divergent from all known supergroups (2.8 to 5.5% pairwise difference of the 16S rRNA gene). The three other *Bryobia* species and *T. urticae*

harbor (different) *Wolbachia* strains that cluster with *Wolbachia* supergroup B.

Cloning of the 16S rRNA gene showed that each *Bryobia* mite is infected with a single *Wolbachia* lineage, and no double infections with other bacteria were found. The 10 clones sequenced per species showed minimal sequence differences (<1%), and those differences that were detected may reflect cloning artifacts (9). For each sample, a majority-rule consensus sequence was created. Among the 10 clones sequenced for *Bryobia* species I, one clone was identified as belonging to the genus *Streptococcus* ("oral clone"). This clone was considered contamination and discarded.

Our analysis confirms supergroups A to F and H as distinct, coherent phylogenetic clades, supported by high bootstrap and posterior probability values (Fig. 1 and 2). These clades were consistently recovered for each locus separately (data not shown), although support for supergroup A is low in the 16S rRNA gene phylogeny (Fig. 2). Apart from these known supergroups, three further distinct *Wolbachia* lineages were observed: a lineage found in *Ctenocephalides felis* and *Orchopeas leucopus* (both Siphonaptera) (19, 25), a lineage found in *D. gracile* (Spirurida) (19), and a lineage in *Bryobia* species V (Tetranychidae; this study). These lineages are highly distinct from all other supergroups for all analyzed loci. We propose to name these lineages supergroups I, J, and K, respectively (Fig. 1 and 2; see Table S1 in the supplemental material).

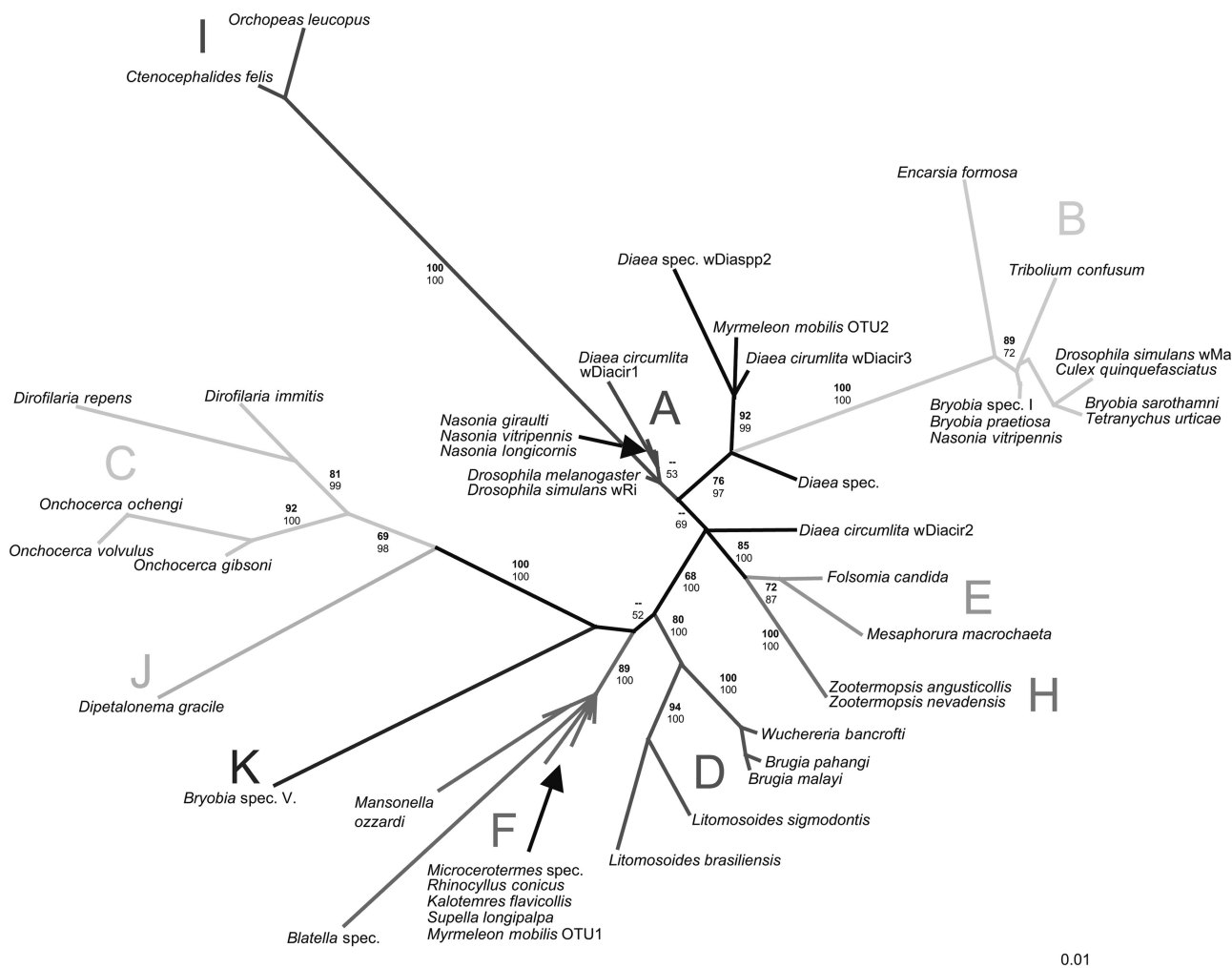


FIG. 2. Unrooted ML phylogenetic tree of *Wolbachia* 16S rRNA gene sequences (1,255 bp). Strains are characterized by the names of their host species. ML bootstrap values (top numbers, in boldface) based on 100 replicates and Bayesian posterior probabilities (bottom numbers) are depicted (only values larger than 50 are indicated). Values for recently diverged taxa within supergroups are sometimes excluded for clarity of presentation. The bar indicates a branch length of 1% likelihood distance.

The 16S rRNA gene phylogenetic tree includes additional samples of interest. These represent newly described supergroups or were recovered from host species only recently discovered to harbor *Wolbachia* (21, 22, 25, 34, 54). Our analysis also includes isolates assigned to supergroup G (43) (Fig. 2; see Table S1 in the supplemental material). The phylogeny of the 16S rRNA gene is less well resolved than the phylogenies of the protein-coding genes. Supergroup A lacks support, and samples from the spider genus *Diaea* (Araneae) and the ant lion *Myrmeleon mobilis* (Neuroptera) fall between or within supergroups A and B. This finding is therefore consistent with a previous suggestion by Baldo and Werren (7) that the *Wolbachia* strains from *Diaea* are supergroup A/B recombinants rather than forming an independent lineage (supergroup G), as proposed by Rowley et al. (43). These observations also challenge the common assumption that the 16S rRNA gene is a reliable phylogenetic marker that is recalcitrant to recombination. Our data reveal at least one clear case of recombination in the 16S

rRNA gene (Fig. 3). Recombination has occurred between *Wolbachia* strains from *M. mobilis* and isolates from supergroups B (*B. praetiosa*) and F (*Kaloterms flavicollis*) ( $P < 0.05$  for MaxChi, Chimaera, Bootscan, and Siscan) (Fig. 3a). Exact breakpoints could not be determined, and it remains unclear which sequence represents the recombinant. A second, less evident, recombination event was revealed between strains from supergroups E (*Folsomia candida*), I (*C. felis*), and B (*B. praetiosa*) ( $P < 0.05$  for MaxChi and Chimaera) (Fig. 3b). In this case, recombination may have occurred at several places along the sequences and between all strains. This could reflect recombination events in the distant past. Clear recombination is also evident in the striking mosaic structure observed within *gltA* between strains from supergroups C and D (Fig. 4) ( $P < 0.001$  in all cases). The sequence of *Wuchereria bancrofti* (supergroup D) is a combination of the sequence of *Brugia malayi* (D) and *Onchocerca gibsoni* (C) (Fig. 4). Strains of these two supergroups infect filarial nematodes but are highly divergent





TABLE 2. Minimum, maximum, and average p distances within and between *Wolbachia* supergroups for each of the analyzed genes

Distance parameter	Value <sup>b</sup>				
	<i>gltA</i>	<i>ftsZ</i>	<i>groEL</i>	Concatenated	16S rRNA gene <sup>c</sup>
Within					
Minimum <sup>a</sup>	0.0032 (H)	0.0021 (H)	0 (H)	0.0019 (H)	0 (H)
Maximum	0.0907 (C)	0.0732 (C)	0.0536 (C)	0.0675 (C)	0.0173 (C)
Avg	0.0425	0.0273	0.0256	0.0331	0.0084
Between					
Minimum	0.0613 (AB)	0.0556 (AH)	0.0672 (IF)	0.0716 (AH)	0.0135 (AE)
Maximum	0.2484 (IK)	0.1396 (CK)	0.1711 (JK)	0.2248 (EJ)	0.0658 (IJ)
Avg	0.1764	0.1120	0.1276	0.1477	0.0361

<sup>a</sup> Excluding supergroups with only one strain.

<sup>b</sup> The supergroup(s) between or within which the minimum or maximum distance was found is indicated in parentheses.

<sup>c</sup> Excluding strains that were not assigned to a supergroup.

typing, in which the alleles at a set of housekeeping genes define strains and clonal groups within a named species, and multilocus sequence analysis, in which the same procedure provides evidence concerning the boundaries between closely related “species” (23). The level of diversity within each supergroup varies (e.g., supergroup C is very diverse) (Fig. 1 and 2), but all currently recognized supergroups are well supported (A to F and H), and the levels of divergence between the supergroups are generally more akin to inter- rather than intraspecies differences (Table 2). Similarly, the newly defined supergroups I, J, and K are highly divergent from all other supergroups and are well supported by all analyzed loci. More strikingly, for all four analyzed genes, the distances between these new supergroups and any current supergroups are equal to (in one case, between I and F for *groEL*) or larger than (in all other cases) the minimum distance between current supergroups. Moreover, the maximum observed distance between all supergroups for all genes involves either one or both of the new supergroups, indicating the high diversity of these groups. Therefore the putative new supergroups more than match the criteria for sequence “distinctness” on which current supergroups are based.

In our opinion, this validates the designation of these supergroups and the possible promotion of *Wolbachia* supergroups to species status. The occurrence of occasional gene transfer between supergroups does not preclude such a reevaluation, as by this token, many closely related named bacterial species should be lumped together. Although there is currently no universal conceptual basis for species (or supergroup) assignment, the equivalence of supergroups and species would help to bring *Wolbachia* systematics into line with other bacterial taxa and would encourage debate as to the evolutionary and ecological significance of these major *Wolbachia* clades.

The 16S rRNA gene data set was supplemented with recently identified strains of interest. Within the ant lion *M. mobilis* (Neuroptera), two distinct strains were detected (22), one of which clusters with supergroup F. The other strain clusters with samples previously identified as supergroup G (see below). Thus, two divergent strains infect this ant lion species, although this should be further investigated using other genes as our data and previous studies point to the possibility of recombination at the 16S rRNA gene. Furthermore, *Wolbachia* strains from two cockroach species, *Blattella*

sp. and *Supella longipalpa* (Blattodea), and a weevil, *Rhinocylus conicus* (Coleoptera), cluster with supergroup F based on 16S rRNA gene data (34, 54). This shows that, like supergroups A and B, supergroup F is also widespread among different arthropod orders (Table 3). Supergroup G was described for *Wolbachia* strains from two species of the spider genus *Diaea* (Araneae) (43). We included these samples in our 16S rRNA gene analysis, and Fig. 2 shows that these samples fall in between supergroups A and B. Based on analyses of *wsp* sequences, Baldo and Werren (7) showed that *Wolbachia* strains defined as supergroup G are recombinants between strains of supergroups A and B. Recombination is apparently also present at the 16S rRNA gene (Fig. 3). This is illustrated for the sequence from *M. mobilis*, which clusters with samples from the two *Diaea* species in the 16S rRNA gene phylogenetic tree (Fig. 2). This sequence shows signs of recombination events with strains from supergroups B (*B. praetiosa*) and F (*K. flavicollis*) (Fig. 3a). Only part of the 16S rRNA gene is sequenced for the *Diaea* samples, making exact inference of relationships impossible. A more detailed investigation including other genes should be performed to infer the exact relationship of these samples.

While horizontal transfer and recombination between arthropod-infecting supergroups A and B are common, they are considered entirely absent in or between nematode-infecting supergroups (3, 4, 5, 32). So far, phylogenies of filarial nematodes and their *Wolbachia* parasites were found congruent, suggesting coevolution between nematodes and *Wolbachia* for nearly 100 million years (9, 18). We illustrate a clear case of recombination between nematode supergroups C and D. Although *Wolbachia*-nematode associations are found to be highly specific, this apparently does not exclude occasional horizontal transfer and recombination. Possibly, there are ecological opportunities (e.g., sharing of the same nematode host or arthropod vector species) allowing strains of different supergroups to recombine.

Some supergroups have taxonomically widespread host ranges, while others are restricted to a single taxon (Table 3). Supergroups A and B are found in the three major arthropod subphyla (Chelicerata, Crustacea, and Hexapoda). Supergroup F is found in the subphyla Chelicerata and Hexapoda and also in the phylum Nematoda, indicating interhost transmission of *Wolbachia* between arthropods and nematodes (19, 38). Trans-

TABLE 3. Distribution of *Wolbachia* supergroups across nematode and arthropod orders

Taxon	Name	Supergroup(s)
Phylum	Arthropoda	
Subphylum	Chelicerata	
Class	Arachnida	
Order <sup>a</sup>	Acarina <sup>b</sup>	B,K
	Araneae	A,B,(G) <sup>d</sup>
	Pseudoscorpionida	? <sup>c</sup>
	Scorpiones	F
Subphylum	Crustacea	
Class	Ostracoda	
Order	Podocopida	A,B
Class	Malacostraca	
Order	Amphipoda	B
	Isopoda	B
Subphylum	Hexapoda	
Class	Entognatha	
Order	Collembola	B,E
Class	Insecta	
Order	Blattodea	F
	Coleoptera	A,B,F
	Dermaptera	?
	Diptera	A,B,F
	Hemiptera	A,B,F
	Hymenoptera	A,B
	Isoptera	A,B,F,H
	Lepidoptera	A,B
	Mecoptera	?
	Neuroptera	F,?
	Onodonta	B
	Orthoptera	B,F
	Phthiraptera	F
	Psocoptera	A,B
	Siphonaptera	I
	Thysanoptera	B,F
	Thysanura	B
Phylum	Nematoda	
Class	Secernentea	
Order	Spirurida	C,D,F,J
	Strongylida	?

<sup>a</sup> Only orders in which *Wolbachia* was found are listed.

<sup>b</sup> Oribatida, Prostigmata, Mesostigmata.

<sup>c</sup> ?, *Wolbachia* was detected, but the supergroup was undetermined.

<sup>d</sup> The existence of supergroup G is disputed; see text.

mission of supergroups A, B, and F between the subphyla is also thought to be common. In contrast, supergroups E, H, I, J, and K are each currently restricted to a single order or even a single species, although there is very little evidence concerning the true distribution of these supergroups. When all subphyla are considered, the Hexapoda contain the highest diversity of *Wolbachia* strains: all supergroups are found except C, D, J (restricted to the phylum Nematoda), and K (found only in the order Prostigmata [Acarina]). Within the subphylum Crustacea, only supergroups A and B are found. The diversity within the subphylum Chelicerata is much greater: so far, supergroups B (spiders [Araneae], mites, and ticks [Acarina]), A (spiders), F (scorpions [Scorpiones]), and K (mites) and possible new strains (pseudoscorpions [Pseudoscorpionida] and spiders) have been reported (6, 16, 29, 43, 63). This suggests multiple independent infections of the Chelicerata with *Wolbachia*. Most of these findings were only recently reported, and

we expect that future investigations within these and other taxa will further enlarge the known diversity of *Wolbachia*.

In conclusion, the combined analysis of the three protein-coding genes *gltA*, *ftsZ*, and *groEL* give a congruent and well-supported representation of the current supergroup designation. This agrees with the findings of Casiraghi et al. (19) and Bordenstein and Rosengaus (14). The phylogeny obtained from 16S rRNA gene sequences is less well resolved, and recombination is observed within this region, rendering it less suitable for supergroup assignment (34). Although a single intragenic recombination event was found for *gltA*, this does not affect the supergroup classification. Sets of primers are available that work well across all known supergroups, and these should help in refining the structure further and for uncovering more diversity in the future.

#### ACKNOWLEDGMENTS

We thank Betsie Voetdijk and Sangeeta Jessurun for assistance with cloning and sequencing and Jan van Arkel for help with the figures. Thanks are due to Robert Butcher and Tim Karr for useful discussions and to Steph Menken for useful discussions and valuable comments on the manuscript.

This research was funded by a grant from The Netherlands Organization for Scientific Research (NWO; ALW4PJ/03-25).

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