A phylogenetic analysis of filarial nematodes: comparison with the phylogeny of *Wolbachia* endosymbionts

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SUMMARY

Infection with the endosymbiotic bacteria *Wolbachia* is widespread in filarial nematodes. Previous studies have suggested concordance between the phylogeny of *Wolbachia* with that of their nematode hosts. However, there is only one published molecular phylogenetic study of filarial species, based on the 5S rRNA gene spacer. The phylogeny proposed by this study is partially incongruent with previous classifications of filarial nematodes, based on morphological characters. Furthermore, both traditional classifications and molecular phylogenies are, in part, inconsistent with the phylogeny of *Wolbachia*. Here we report mitochondrial cytochrome oxidase I (COI) gene sequences for 11 species of filaria and for another spirurid nematode which was included as an outgroup. In addition, 16S rRNA, *wsp* and *ftsZ* gene sequences were generated for the *Wolbachia* of several filarial species, in order to complete the available data sets and further resolve the phylogeny of *Wolbachia* in nematodes. We used these data to evaluate whether nematode and *Wolbachia* phylogenies are concordant. Some of the possible phylogenetic reconstructions based on COI gene were congruent with the phylogeny of *Wolbachia* and supported the grouping of the rodent filaria *Litomosoides sigmodontis* with the lymphatic filariae (i.e. *Brugia* spp. and *Wuchereria* spp.) and the sister group relationship of *Dirofilaria* spp. and *Onchocerca* spp. However, the placement of the *Wolbachia*-free filaria *Acanthocheilonema viteae* is ambiguous and dependent on the phylogenetic methods used.

Key words: filarial nematodes, phylogeny, *Wolbachia*, COI gene, 16S rDNA, *ftsZ*, *wsp*.

INTRODUCTION

Wolbachia endosymbiotic bacteria are widespread in arthropods (Werren, 1997). These bacteria are phylogenetically related to the genera *Anaplasma*, *Cowdria* and *Ehrlichia* and have been assigned to the alpha 2 subclass of the proteobacteria (O'Neill *et al*. 1992). *Wolbachia* has also been found in filarial nematodes (order Spirurida; family Onchocercidae) (Sironi *et al*. 1995; Bandi *et al*. 1998). The major human and animal filariasis agents have been shown to harbour *Wolbachia*: *Brugia malayi*, *B. pahangi*, *Wuchereria bancrofti*, *Onchocerca volvulus*, *O. ochengi*, *O. gibsoni*, *O. gutturosa*, *Dirofilaria immitis*, *D. repens*, *Litomosoides sigmodontis* (Bandi *et al*. 1998; Taylor *et al*. 1999). A model filarial species of rodents, *Acanthocheilonema viteae*, has been shown not to harbour *Wolbachia* (McLaren *et al*. 1975; Bandi *et al*. 1998; Hoerauf *et al*. 1999; Taylor & Hoerauf, 1999). *Wolbachia* endosymbionts are known to be vertically transmitted from mother to offspring in both arthropods and nematodes (Werren, 1997; Kozek, 1977; Kozek & Figueroa, 1977; Taylor *et al*. 1999). Strict vertical transmission of symbionts is expected to result in matching of the phylogenies of host and symbiont. Comparisons of host and symbiont phylogenies can therefore provide a powerful approach to understand patterns of transmission in host–symbiont associations (Hafner & Nadler, 1988). In particular this approach can be used (1) to assess whether horizontal transmission occurred in addition to vertical transmission and (2) to differentiate between parasite loss from certain lineages and previous horizontal transmission events (Moran & Baumann, 1994; Bandi *et al*. 1995). In arthropods, the phylogeny of *Wolbachia* is not always congruent with that of their hosts, thus indicating that horizontal transmission phenomena have occurred (Werren, 1997).

In previous studies, the phylogeny of filarial nematodes has been compared to the phylogeny of *Wolbachia* endosymbionts, which was based on the protein coding gene *ftsZ* (Bandi *et al*. 1998). Comparison of the phylogeny of filarial nematodes with that of their *Wolbachia* bacteria did not produce definitive answers. There is indeed just one published molecular phylogenetic study including a representative sample of filarial species. This study was based on the analysis of the 5S rRNA gene spacer (Xie, Bain & Williams, 1994). In addition, traditional classifications based on morphological characters do not necessarily represent the phylogeny of filarial nematodes. All the genera included in our study (see Materials and Methods section) are assigned to a single taxon in some classifications (e.g.

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Sonin, 1985; Skryabin, 1991). In the classification reported by Anderson & Bain (1976; see also Anderson, 1992), these genera are assigned to 2 different subfamilies. However, the positioning of the genus *Onchocerca* is not congruent in Anderson & Bain's classification (1976) and in the molecular phylogeny of Xie *et al*. (1994). According to Anderson & Bain (1976), the genus *Onchocerca* is assigned to the subfamily Onchocercinae, which also encompasses the genera *Brugia*, *Wuchereria*, *Litomosoides* and *Acanthocheilonema*. The genus *Dirofilaria* is placed in the subfamily Dirofilarinae. On the other hand, the phylogenetic reconstruction of Xie *et al*. (1994) places the genus *Onchocerca* as the sister group of the genus *Dirofilaria*.

Wolbachia phylogeny has been shown to be in part congruent with Xie *et al*.'s (1994) molecular phylogeny and in part with Anderson & Bain's (1976) classification (Bandi *et al*. 1998). In agreement with Xie *et al*.'s (1994) grouping of the genera *Onchocerca* and *Dirofilaria*, the endosymbionts of *Dirofilaria* spp. cluster with the endosymbionts of *Onchocerca* spp. (this disagrees with Anderson & Bain's (1976) classification). On the other hand, in agreement with Anderson & Bain's grouping of the lymphatic filariae (*Brugia* spp. and *Wuchereria* spp.) with *Litomosoides* spp., the endosymbionts of the lymphatic filariae cluster with those of *L. sigmodontis* (this disagrees with Xie *et al*.'s (1994) phylogeny).

Further data are clearly needed to resolve the phylogeny of filarial nematodes to evaluate whether the phylogeny of *Wolbachia* matches the phylogeny of their nematode hosts. We therefore decided to generate a new set of DNA sequence data from the mitochondrial genomes of filarial worms. The gene coding for the cytochrome oxidase I (COI) was sequenced for those filariae that are known to harbour *Wolbachia*. We also included in our study *A. viteae*, which does not harbour *Wolbachia* (see above), and *Thelazia lacrimalis* (order Spirurida; family Thelaziidae) which was used as an outgroup. In addition, we generated sequence data from the genes coding for the small-subunit ribosomal RNA (16S rDNA) and for the FTSZ and WSP proteins of the *Wolbachia* of some filarial species in order to complete the available data sets and produce more complete *Wolbachia* phylogenies.

MATERIALS AND METHODS

Parasite material

Eleven species of filarial parasites of the family Onchocercidae were included in the present study: *Brugia malayi*, *B. pahangi*, *Dirofilaria immitis*, *D. repens*, *Litomosoides sigmodontis*, *Onchocerca gibsoni*, *O. gutturosa*, *O. ochengi*, *O. volvulus*, *Wuchereria bancrofti* and *Acanthocheilonema viteae*. In particular, specimens of *B. malayi*, *B. pahangi*, *L. sigmodontis* and *A. viteae* were provided by the TRS Laboratories Inc., Athens, GA, USA; specimens of *D. repens* and *D. immitis* were collected from dogs in Milano (Italy); specimens of *O. gibsoni* from naturally infected cattle in Queensland, Australia; specimens of *O. gutturosa* and *O. ochengi* from naturally infected cattle in Northern Cameroon; specimens of *O. volvulus* from humans in Ghana; specimens of *W. bancrofti* from humans in Sri Lanka. For *W. bancrofti*, DNA from a pooled sample (microfilariae) was analysed. For the other species, DNAs from individual specimens were examined. Three specimens of the outgroup species, *Thelazia lacrimalis*, were collected from a horse in Italy.

DNA extraction, primer design, PCR conditions, DNA sequencing

For most of the worms, crude DNA preparations were obtained through proteinase-K treatment (Bandi *et al*. 1994). In the case of *D. immitis* and *D. repens*, after a proteinase-K treatment, DNA was purified according to standard phenol–chloroform procedures (Sambrook, Fritsch & Maniatis, 1989). A degenerate primer pair (COIfilF: 5′-T(ATG)T-CT(AT)T(AG)(ATG)T(ATG)ATTCGTT-3′ and COIfilR: 5′-AC(ATG)ACATAATAAGTATCAT-G-3′) was designed on the basis of regions of COI gene conserved among the nematodes species *O. volvulus*, *Ascaris suum* and *Caenorhabditis elegans* (accession numbers: NC_001861.1; NC_001327.1; U80438/CELT19B4). Polymerase chain reaction (PCR) was performed in 20 μl volumes under the following final conditions: 1 × buffer including 1·5 mM $MgCl_2$ (Amersham Pharmacia Biotech), 0·2 mM of each dNTP, 1 μM each of forward and reverse primers, and 1 unit of *Taq* DNA Polymerase (Amersham Pharmacia Biotech). The thermal profile we used was: 94 °C 45 sec, 52 °C 45 sec, and 72 °C 90 sec for 40 cycles. Since COIfilF and COIfilR gave good amplifications only from 3 species, *B. pahangi*, *B. malayi* and *D. repens*, PCR products were cloned (using the pGEM-T Vector System II, Promega) and sequenced (using ABI technology; 3 clones for each species were sequenced). On the basis of the regions conserved in the sequences obtained for the three species and in the sequence of *O. volvulus* available in the data bases, we designed the following non-degenerate primers: COIintF (5′-TGATTGG-TGGTTTTGGTAA-3′) and COIintR (5′-ATAA-GTACGAGTATCAATATC-3′). These primers generated products of the expected size from all the species included in this study under the cycling conditions indicated above. The PCR products obtained were gel-purified (using the NucleoSpin® Nucleic Acid Purification Kit, Clontech) and directly sequenced using ABI technology. The sequences obtained have been deposited in the EMBL Data Library (accessions: AJ271610-AJ271619, AJ272117). The positions of COI primers on the

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complete mitochondrial genome of *O. volvulus* are: COIfilF: 2390–2408; COIfilR: 3416–3396; COIintF: 2519–2538; COIintR: 3207–3186.

In addition to the COI data from filarial nematodes, *Wolbachia* gene sequences were generated for *O. gutturosa*, *O. gibsoni* and *D. repens* (16S rDNA; accessions: AJ276498; AJ276499; AJ276500), *O. volvulus* and *O. gutturosa* (*wsp*; accessions: AJ276496; AJ276497) and *O. volvulus* (*ftsZ*; accession: AJ276501). These sequences were generated using methods described by Sironi *et al.* (1995), Bandi *et al.* (1998) and by Bazzocchi *et al.* (2000).

We tested 3 individual specimens of the outgroup species *T. lacrimalis* for the presence of *Wolbachia* using published general *Wolbachia* primers for *ftsZ* (ftsZfl and ftsZrl; Werren, Zhang & Guo, 1995), *wsp* (WSPintF and WSPintR; Bazzocchi *et al.* 2000) and 16S rDNA (99f and 994r; O'Neill, Gooding & Aksoy, 1993). In addition, we tested *T. lacrimalis* for the presence of *Wolbachia* using further general primers for 16S rDNA and *ftsZ*: 16SWolbF (5′-GAAGATAATGACGGTACTCAC-3′) and 16SWolbR3 (5′-GTCACTGATCCCACTTTA-AATAAC-3′); *fts*ZUNIF (5′-GG(CT)AA(AG)-GGTGC(AG)GCAGAAGA-3′) and *fts*ZUNIR (5′-ATC(AG)AT(AG)CCAGTTGCAAG). We designed these primers on the basis of the sequences now available for *Wolbachia* from groups A–D; these primers are thus more conserved among the different wolbachiae than those used previously (which were based on sequences of *Wolbachia* from only groups A and B). The PCR cycling conditions are as described in Bandi *et al.* (1994), but with an extension time of 2 min. DNA isolated from the *Wolbachia*-infected filarial species *D. immitis*, *D. repens*, *B. malayi*, and *O. ochengi* and from a *Wolbachia*-infected strain of *Culex pipiens* were included as positive controls. DNA from *A. viteae* was included as a negative control.

Data analysis

The COI gene sequences obtained were aligned with the *O. volvulus* COI sequence available in the databases (Keddie & Unnasch, 1998). An alignment of 649 positions was obtained using the sequence of *T. lacrimalis* as an outgroup. There were no insertions–deletions (indels), so sequence alignment was unambiguous.

The new 16S rDNA, *ftsZ* and *wsp* sequences obtained for the wolbachiae of some filarial nematodes (see above) were aligned with the homologous *Wolbachia* sequences deposited in the data bases. In the case of 16S rDNA and *ftsZ*, *Anaplasma marginale* was used as an outgroup. Alignment of 16S rDNA sequences was obtained with the aid of pre-aligned sequences available in the Ribosomal Data Base Project (Maidak *et al.* 1999). Alignment of *ftsZ* followed the schemes reported by Werren *et al.* (1995) and by Bandi *et al.* (1998). Alignment of *wsp* was identical to the one reported by Bazzocchi *et al.* (2000) (alignment available in the EMBL data library under accession: ds41508).

Phylogenetic analysis was performed using both distance matrix and character state methods. The distance matrix approach used was neighbour-joining (NJ) and distance matrices were constructed using Kimura 2 parameter or Jukes & Cantor corrections. The character state procedures used were maximum likelihood (ML), unweighted maximum parsimony (uMP), weighted maximum parsimony (wMP), and maximum parsimony after successive approximation character weighting (SACW) (for descriptions and references on the different methods see Results and Discussion section; see also Swofford *et al.* 1996).

Phylogenetic analyses were performed using TREECON 1.3B (Van de Peer & De Wachter, 1993, 1994, 1997), PHYLIP v.3.5c (Felsenstein, 1993), PAUP 4.0 (Swofford, 1998) and Puzzle 4.0.2 (Strimmer & von Haeseler, 1996, 1997). For maximum likelihood analysis, the model of sequence substitution in Puzzle was 'HKY' with the '1 variable+1 invariable' model of among-site rate heterogeneity. Puzzle 4.0.2 was also used to estimate the likelihood of the trees shown in Figs 2–4 and to check whether the likelihood values of these trees were significantly different using the Kishino-Hasegawa (1989) test. For these comparisons, analyses were effected on the alignment including all codon positions. The COI data set was analysed including all codon positions, or only the first, the second or the third codon positions. A transversion analysis was also performed.

RESULTS AND DISCUSSION

We have divided the discussion into 3 different sections: (1) we describe the phylogenetic analysis of the expanded *Wolbachia* sequence data sets, (2) we analyse the COI data and evaluate competing phylogenetic hypotheses for filarial nematodes and (3) we conclude by comparing phylogenetic information from both hosts and symbionts.

Phylogenetic analysis of Wolbachia *from filarial nematodes*

The phylogenetic relationships among *Wolbachia* from filarial nematodes have been resolved from previous work, although some important problems remain with rooting of the trees. The phylogenies so far generated, which are based on *ftsZ* and *wsp* genes (Bandi *et al.* 1998; Bazzocchi *et al.* 2000), are congruent. These phylogenies are also congruent with partial phylogenies based on 16S rDNA (Sironi *et al.* 1995; Bandi *et al.* 1998, 1999; Taylor *et al.* 1999). We expanded the available data sets by

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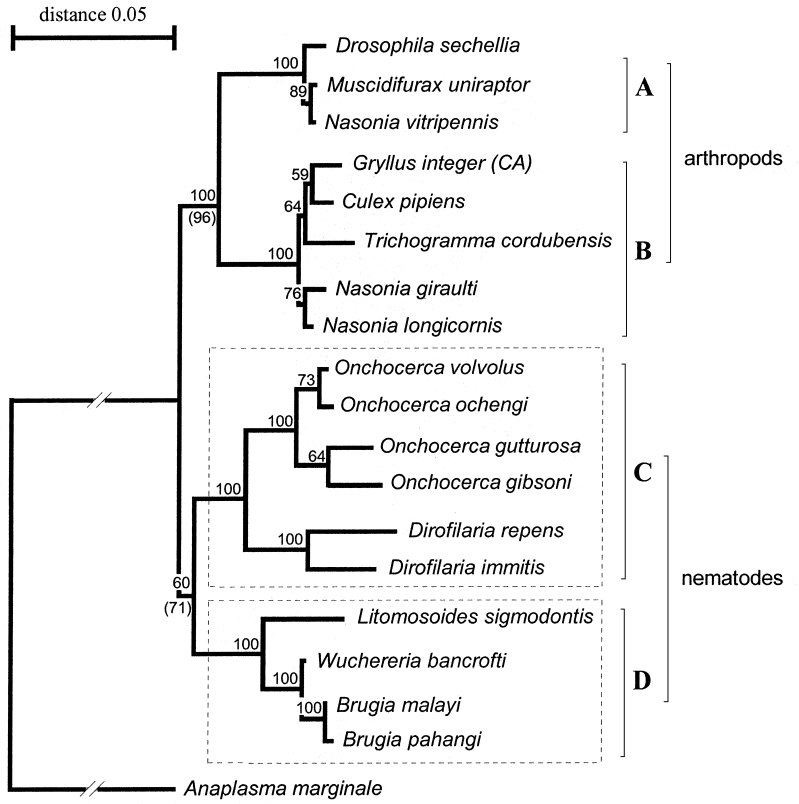

Fig. 1. Tree obtained from the combined data set of 16S rDNA and *ftsZ* sequences of arthropod (A and B) and nematode (C and D) wolbachiae. *Anaplasma marginale* was used as an outgroup. The tree was obtained using the neighbour-joining method after Kimura correction on a sequence alignment in which insertion/deletions were excluded. With the exception of the outgroup, names at the terminal nodes are those of the host species. Bootstrap confidence values after 100 replicates are shown at the nodes. Values in parentheses at the nodes leading to arthropod and nematode wolbachiae were obtained in separate analysis using an alignment which excluded the shortest sequences (the *ftsZ* sequence of the *Wolbachia* of *Wuchereria bancrofti* and the 16S rDNA sequence the *Wolbachia* of *Onchocerca volvulus*; see text for further explanations). The 2 dashed boxes highlight the 2 groups of nematode wolbachiae (C and D). The topology of the subtrees in these boxes was also obtained after analysing the 2 data sets (16S rDNA and *ftsZ*) independently as well as after analysing the *wsp* data set. In addition, the subtrees in the 2 boxes were generated by all analytical procedures, with only minor differences in the positioning of the endosymbionts of *O. gutturosa* and *O. ochengi* relative to the endosymbionts of the other members of the genus *Onchocerca*. The overall topology of the tree in this figure corresponds to the 2 most parsimonious trees obtained using unweighted maximum parsimony, with minor differences in the relative position of the wolbachiae of *Gryllus integer*, *Culex pipiens* and *Trichogramma cordubensis*. The length of the tree is 875 steps (CI: 0·64; RI: 0·79; RC: 0·50). The scale bar indicates the distances in substitutions per nucleotide.

including sequence data from the 16S rDNA of *O. gutturosa*, *O. gibsoni* and *D. repens*, from the *wsp* gene of *O. volvulus* and *O. gutturosa* and from the *ftsZ* gene of *O. volvulus*. Trees generated for each of the 3 data sets using both distance matrix (NJ after Kimura or Jukes & Cantor corrections) and parsimony methods showed similar overall topologies. These trees were also similar in topology to the trees previously obtained using smaller data sets (Bandi *et al*. 1998, 1999; Bazzocchi *et al*. 2000). All these reconstructions recognized 4 major groups of *Wolbachia*: A and B, which include arthropod *Wolbachia* (Werren *et al*. 1995; Zhou, Rosset & O'Neill, 1998), C and D, which encompass nematode *Wolbachia* (Bandi *et al*. 1998). In the 2 branches of nematode *Wolbachia*, the relationships among the endosymbionts of the different host genera appear stable, with all genes examined supporting the same groupings (not shown, but see dashed boxes in Fig. 1).

A major limit of these phylogenetic reconstructions, and those generated previously (Bandi *et al*. 1998, 1999; Bazzocchi *et al*. 2000) is that root placement is not robust. This likely reflects the large genetic distance between ingroup taxa and available outgroups (Bandi *et al*. 1998). In order to enhance the stability of the rooting of the *Wolbachia* tree we combined the *ftsZ* and 16S rDNA alignments in a single data set. This approach is expected to produce a better phylogenetic estimation (Huelsenbeck, Bull & Cunningham, 1996). Since suitable outgroups are unavailable for *wsp* sequences (Bazzocchi *et al*. 2000), these data were not included in the combined alignment. A tree generated by NJ after Kimura

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correction from the combined 16S rDNA-*ftsZ* data set is shown in Fig. 1. C and D *Wolbachia* cluster as a monophyletic group. The bootstrap value for this grouping is quite low (60%). However, we must consider that we generated this tree using a prudent approach: all indels were excluded from the computation. Inclusion of indels lead to a tree with a higher bootstrap support for the (C plus D) clade (not shown). However, in view of the ambiguity of the alignment with the outgroup (*A. marginale*) in the gap regions of *ftsZ*, it would be more prudent not to consider this result. Unfortunately, most of the 16S rDNA sequences of *Wolbachia* available in the data bases are only partial. It was therefore not possible to generate a combined *ftsZ*-16S rDNA data set including more sequences from arthropod *Wolbachia*. Some of the 16S rDNA and *ftsZ* sequences of nematode *Wolbachia* are also partial (i.e. the *ftsZ* of *W. bancrofti* and the 16S rDNA of *O. volvulus*) and the combined alignment used to generate the tree in Fig. 1 was thus quite short (1080 bp after excluding all undetermined positions and indels). We generated a longer alignment (1890 bp) by excluding *W. bancrofti* and *O. volvulus*. Using this alignment, bootstrap support is stronger (71%) for the group encompassing C and D *Wolbachia* (see Fig. 1 legend).

In conclusion, even though we do not have a definitive rooting for the phylogenetic tree of *Wolbachia*, the combined data set provides some support to the monophyly of nematode *Wolbachia*. This monophyly supports a number of observations from previous studies. Based on the distribution of the indels in the *ftsZ* gene, C and D wolbachiae are identical and are different from both A and B wolbachiae (Bandi *et al*. 1998). In addition, the *wsp* genes of C and D wolbachiae are more similar among each other than each is to those of A and B wolbachiae (Bazzocchi *et al*. 2000). However, since the outgroups are either phylogenetically distant (rDNA and *ftsZ*) or unavailable (*wsp*), the similarities between C and D wolbachiae are best regarded as phenetic (i.e. due to the distance or unavailability of outgroup sequences, we cannot decide whether shared character states in C and D wolbachiae are synapomorphic states, or plesiomorphic states; see discussion by Bandi *et al*. 1998). In the case of WSP protein sequences, inclusion as outgroups of the surface proteins of *A. marginale* and *Cowdria ruminantium* MSP 4 and MAP 1 according to the alignment reported by Braig *et al*. (1998) generated a tree with high bootstrap support (100%) for the clustering of C and D wolbachiae (not shown). However, alignment with these outgroup sequences does not appear fully reliable, and we cannot exclude the possibility that *wsp* genes in arthropod and nematode wolbachiae are paralogous (i.e. homologous sequences that arose by gene duplication and evolved in parallel within a single line of descent; see also discussion by Bazzocchi *et al*. 2000). Rooting problems have also been encountered using 16S rDNA sequences (see for example the 16S rDNA trees reported by Bandi *et al*. 1998 and by Bandi *et al*. 1999). However, despite the above problems, there is an overall consistency among results that suggests the monophyly of nematode *Wolbachia*. We thus tentatively assume that C and D wolbachiae form a monophyletic cluster.

We tested 3 specimens of *T. lacrimalis* for the presence of *Wolbachia* using both published and unpublished primers (see Materials and Methods section). In all cases, *T. lacrimalis* was PCR negative, while all the positive controls gave amplifications of the expected sizes. We tentatively assume that this species, which was included in our study as an outgroup to root the phylogeny of filarial nematodes (see below), does not harbour *Wolbachia*.

Phylogenetic analysis of COI gene from filarial nematodes

The gene coding for the COI was sequenced from all filarial nematodes included in this study. The sequence obtained from *O. volvulus* was identical to that available in the data bases (Keddie & Unnasch, 1998). In addition, in the case of *D. immitis* and *D. repens* the sequences obtained from worms recovered from different dogs were identical (2 dogs for *D. immitis* and other 2 dogs for *D. repens*). The most simple phylogenetic method we applied to the COI data set of filarial nematodes was NJ on distance matrices recording the percentage nucleotide differences among the sequences, or the nucleotide distances obtained using the corrections of Jukes & Cantor or Kimura (Table 1). On all types of matrices, this method produced identical trees which were similar in some respects to those obtained by Xie *et al*. (1994): *Onchocerca* spp. cluster with *Dirofilaria* spp. and *L. sigmodontis* is placed as a deep branch (Fig. 2A). However, while in the tree of Fig. 2A *A. viteae* is placed as the sister group of (*Onchocerca* spp. plus *Dirofilaria* spp.), Xie *et al*.'s (1994) trees showed *A. viteae* as a deep branch of filaria evolution or as the sister group of *L. sigmodontis*. In the tree of Fig. 2A, *D. repens* does not cluster with *D. immitis*, but is placed as the sister group of *Onchocerca* spp. However, bootstrap support for the positions of *Dirofilaria* spp. and of *A. viteae* were in any case low in the COI NJ trees.

Another basic procedure for phylogenetic analysis is uMP. Using this approach, we obtained the tree shown in Fig. 2B: *A. viteae* appears as the sister group of the lymphatic filariae, *L. sigmodontis* as the deepest branch, and *Dirofilaria* spp. as separate deep branches. This tree thus shows some inconsistencies both with the COI NJ tree (Fig. 2A) and with Xie *et al*.'s (1994) trees. However, the consistency index (CI) of the uMP tree is quite low (0·51 after excluding

Table 1. Distance matrix showing the nucleotide difference among COI gene sequences

(Values below the diagonal are the uncorrected percentage differences. The differences after Kimura correction are above the diagonal. The last row and the last column report the differences relative to the outgroup, *Thelazia lacrimalis*.)

	B. m.	*B. p.*	*W. b.*	*D. i.*	*D. r.*	*L. s.*	*O. gib.*	*O. gut.*	*O. o.*	*O. v.*	*A. v.*	*T. l.*
B. malayi	—	10·687	10·722	17·801	13·863	19·929	15·048	13·854	16·658	15·831	18·347	24·329
B. pahangi	9·826	—	10·862	18·128	14·183	19·813	15·177	13·440	17·361	17·216	18·389	23·359
W. bancrofti	9·937	10·000	—	15·705	12·050	19·519	11·998	12·509	13·748	13·896	16·767	22·031
D. immitis	15·748	15·956	14·038	—	10·465	19·439	13·060	12·486	13·354	12·055	15·778	20·125
D. repens	12·598	12·796	11·041	9·722	—	17·964	9·211	7·725	9·692	9·211	14·445	17·731
L. sigmodontis	17·350	17·220	17·062	17·002	15·920	—	21·067	19·254	20·260	19·587	21·726	23·896
O. gibsoni	13·543	13·586	11·041	11·883	8·642	18·238	—	6·718	7·972	8·063	14·804	20·602
O. gutturosa	12·580	12·179	11·465	11·465	7·325	16·906	6·369	—	7·997	6·898	16·733	18·426
O. ochengi	14·850	15·324	12·500	12·207	9·077	17·684	7·512	7·508	—	2·884	14·937	16·275
O. volvulus	14·173	15·166	12·618	11·111	8·642	17·156	7·562	6·529	2·817	—	16·385	18·474
A. viteae	16·190	16·139	14·944	14·174	13·084	18·692	13·396	12·681	14·937	14·642	—	21·980
T. lacrimalis	20·630	19·905	18·927	17·593	15·741	20·402	17·901	17·516	16·275	16·333	19·003	—

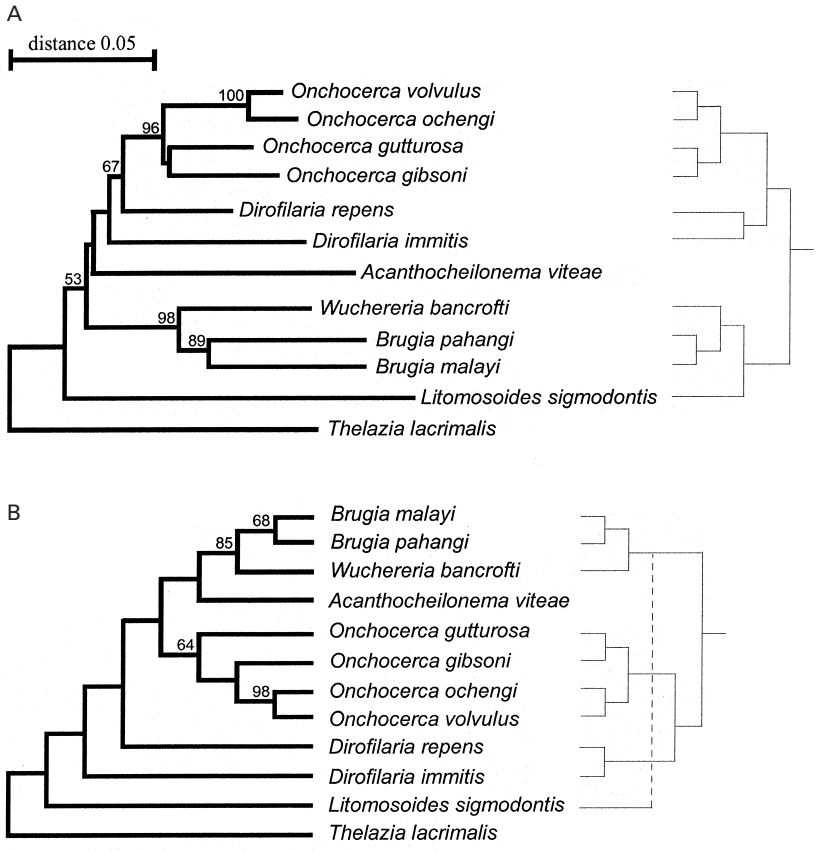

Fig. 2. Phylogeny of filarial nematodes based on COI gene sequences (on the left). *Thelazia lacrimalis* was used as an outgroup. Bootstrap confidence values after 100 replicates are shown at the nodes only for values higher than 50%. For each phylogenetic reconstruction presented, the phylogeny of *Wolbachia* endosymbionts, derived from the tree in Fig. 1, is shown on the right for comparison. (A) Neighbour-joining tree obtained using the Jukes & Cantor correction; the scale bar indicates the distances in substitutions per nucleotide; analysis performed by Treecon 1·3b; tree length: 418 (CI: 0·51; RI: 0·44; RC: 0·22); log likelihood of the tree: −3105·11. (B) Single most parsimonious tree obtained using the branch-and-bound option in Paup 4.0; characters received an equal weight; tree length: 412 (CI: 0·51; RI: 0·46; RC: 0·23); log likelihood of the tree: −3100·00.

uninformative characters) as are bootstrap values for most of the nodes in this tree (Fig. 2B). The CI describes the fit of a set of characters on a tree, and it is an indicator of homoplasy. There is homoplasy when shared character states in different lineages arose independently due to parallel or convergent evolution or to the reversal of character states back to their plesiomorphous (i.e. ancestral) conditions. A CI value of 1 implies no homoplasy, while homoplasy levels increase in the data as the CI decreases.

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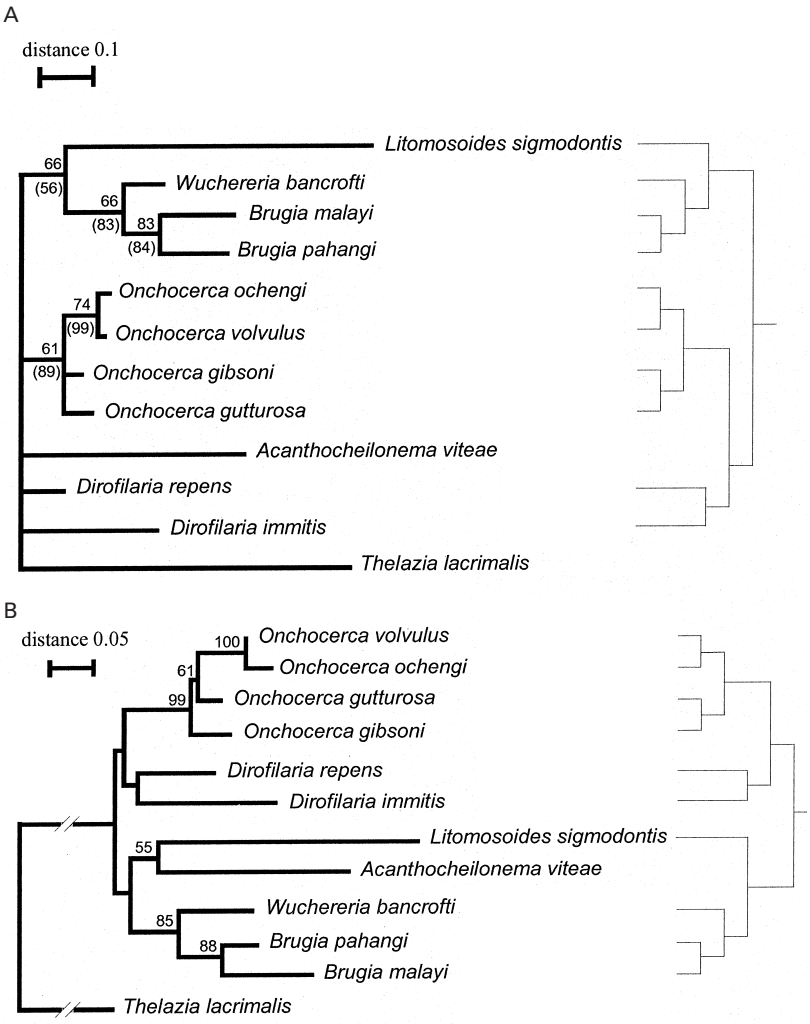

Fig. 3. Phylogeny of filarial nematodes based on COI gene sequences (on the left). *Thelazia lacrimalis* was used as an outgroup. For each phylogenetic reconstruction presented, the phylogeny of *Wolbachia* endosymbionts, derived from the tree in Fig. 1, is shown on the right for comparison. (A) Maximum likelihood tree; values above the branches are the quartet puzzling support values obtained using Puzzle 4.0.2; values in parentheses below the branches are the bootstrap values obtained using Phylip 3.5c (program dnaml; default options); tree length: 419 (CI: 0·51; RI: 0·44; RC: 0·22); log likelihood of the tree: −3104·85. (B) Neighbour-joining tree generated after transversion analysis using TreeconW; bootstrap confidence values after 100 replicates are shown at the nodes only for values higher than 50%; the scale bar indicates the distances in substitutions per nucleotide. Transversion maximum parsimony and weighted maximum parsimony generated trees that showed the same overall topology of the tree in Fig. 3B, with minor differences in the relative positions of the different *Onchocerca* species. Weighted and transversion parsimony were effected using PAUP 4.0 using the branch and bound option. In the case of weighted maximum parsimony, analysis after excluding the third codon positions generated 3 equally parsimonious trees, 1 of which was identical to the tree shown in Fig. 3B (the other 2 trees were slightly different in the positions of *O. gutturosa* and *O. gibsoni*). This tree topology had a length of 55 steps (CI: 0·62; RC: 0·62; RI 0·38) after excluding the third codon positions and uninformative characters; log likelihood of the tree: −3094·64.

Neither of the NJ and uMP trees appear to be robust (low CI and bootstrap values), and this indicates that the COI data set is noisy and that the phylogenetic signal is possibly hidden. Several factors may contribute to this. First, heterogeneity in evolutionary rate across lineages could make phylogenetics misleading. In addition, phylogenetic reconstruction is problematic where short internal branches are associated with long branches leading to the terminal nodes (the so-called 'Felsenstein zone'; Huelsenbeck & Hillis, 1993). In these circumstances, long branches may cluster together, or be placed in deep positions close to the outgroup (the 'long-branch attraction' phenomenon; Felsenstein, 1978, 1988). Relative-rate comparisons on the COI distance matrices (Table 1) show that the rates of molecular evolution of the different lineages of filaria evolution are heterogeneous: the different ingroup filaria species show different distances relative to the outgroup, *T. lacrimalis*. This pattern is also highlighted by the lack of alignment of the terminal nodes of the NJ tree (Fig. 2A). In addition, the

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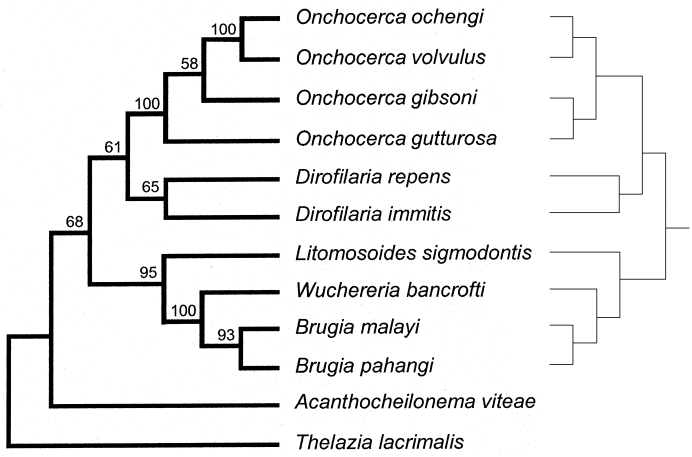

Fig. 4. Phylogeny of filarial nematodes based on COI gene sequences (on the left). *Thelazia lacrimalis* was used as an outgroup. The phylogeny of *Wolbachia* endosymbionts, derived from the tree in Fig. 1, is shown on the right for comparison. Fig. 4 shows the single most parsimonious tree generated by the SACW method using PAUP 4.0 under the branch and bound option and the heuristic search option for bootstrap analysis (for details on the successive weighting approach used, see Results and Discussion section). Bootstrap confidence values after 100 replicates are shown at the nodes. Tree length: 1144 (CI: 0·62; RI: 0·64; RC: 0·40); log likelihood of the tree: −3095·90.

internal deep branches are short compared to the branches leading to the main lineages. Second, the nucleotide distances between some lineages are quite high (e.g. 21·1 % after Kimura correction between *L. sigmodontis* and *O. gibsoni*). This implies the possibility of multiple hits and of convergent evolution (i.e. homoplasy). Indeed, the consistency index of the uMP tree is low (0·51).

A number of phylogenetic methods are available to reduce bias due to evolutionary rate differences and homoplasy. One approach that should produce more reliable trees in the 'Felsenstein zone' is ML (Huelsenbeck *et al*. 1996). An example of a tree generated by ML under the 'non-molecular-clock' assumption is shown in Fig. 3A. This tree shows a multifurcation leading to 5 different branches. The only groupings recognized by ML are (*Onchocerca* spp.) and (lymphatic filariae plus *L. sigmodontis*). Where evolutionary branches are long, transitions can be ignored (or assigned a lower weight) in order to reduce the saturation and 'noise' of the data set. This approach is known as transversion analysis (Swofford *et al*. 1996). Transversion analysis using MP or NJ approaches lead to similar results. Fig. 3B is an example of a tree generated by NJ. This tree corresponds to those generated by transversion MP or wMP (see below), showing the grouping of (*Dirofilaria* spp. plus *Onchocerca* spp.) and of (*L. sigmodontis* plus *A. viteae*). This last group is placed as the sister group of the lymphatic filariae.

Another approach to reduce the level of homoplasy among distantly related lineages is to exclude the most variable characters in the examined data set. In other words, no weight is assigned to characters showing a fast evolutionary rate (*a priori* weighting of characters). This could also compensate for evolutionary rate differences among lineages. In comparisons among nematode species using mitochondrial DNA, the saturation observed at the third base positions suggest that these positions are too variable and should be excluded (Blouin *et al*. 1998). wMP excluding the third codon positions generated 3 equally parsimonious trees (CI after excluding the uninformative characters: 0·62). The relationships among the main lineages in these trees were identical to those depicted by the tree generated by transversion analysis (Fig. 3B). Minor differences among the 3 different wMP trees were observed in the relationships among *Onchocerca* spp. The 0·62 CI of these trees indicates that while homoplasy is still present, it is lower than that of uMP (0·51 CI). Bootstrap values for some nodes of the wMP trees were still low. NJ after Kimura or Jukes & Cantor corrections on distance matrices obtained after excluding the third codon positions generated trees similar to those generated by wMP and transversion analysis (i.e. with *L. sigmodontis* and *A. viteae* clustering with the lymphatic filariae). Minor differences were observed also in this case in the relationships among *Onchocerca* spp.

The next step in our analyses was MP using the SACW method, which is an *a posteriori* weighting approach (Farris, 1969; Carpenter, 1988). Based on the tree generated by uMP (Fig. 2B), the homoplasy of each character in the data set was estimated using the rescaled CI (RC) (Swofford *et al*. 1996). Each character was then weighted on the basis of its RC value. This approach was repeated until the trees found in 2 successive iterations were identical. The single most parsimonious tree obtained using the SACW approach is shown in Fig. 4: *Dirofilaria* spp. cluster together and are placed as the sister group of *Onchocerca* spp., *L. sigmodontis* appears as the sister group of the lymphatic filariae and *A. viteae* is shown as the deepest branch of filaria evolution. CI of this tree is still 0·62 (after exclusion of uninformative

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characters), but the bootstrap values are higher than those of the *a priori* wMP tree. In particular, the bootstrap support for the grouping (*L. sigmodontis* plus lymphatic filariae) is 95 % (Fig. 4). It should be noted that simulation studies showed that weighted parsimony is one of the methods with the highest probability of finding the correct tree in the 'Felsenstein zone' (Huelsenbeck *et al*. 1996). The likelihood of the SACW tree was compared to those of the 4 trees in Figs 2 and 3 using the Kishino-Hasegawa test (1989). However, the likelihood of these 5 trees was not significantly different.

CONCLUSION

It was not the purpose of this paper to produce consensus trees from the dozens of different trees that can be generated using dozens of different approaches. The basic question we addressed was whether the phylogeny of *Wolbachia* matches the phylogeny of its filarial hosts. On the one hand, if we exclude the rooting problems, the phylogeny of *Wolbachia* in filarial nematodes appears robust (Fig. 1). On the other hand, the phylogeny of filarial nematodes appears unstable (Figs 2–4). We emphasize that some of the phylogenetic reconstructions obtained for filarial nematodes (Figs 3B and 4) are perfectly congruent with the phylogeny of *Wolbachia*. Furthermore, these reconstructions were obtained using approaches which are thought to be suitable for situations showing among-lineages evolutionary rate variation, short internal branches associated with long terminal branches, and high levels of divergence among taxa. In particular, wMP, SACW MP, and transversion analyses showed sister group relationships between *L. sigmodontis* and the lymphatic filariae and between *Dirofilaria* spp. and *Onchocerca* spp. It is interesting that the existence of a phylogenetic relationship between the genera *Onchocerca* and *Dirofilaria* has recently been suggested on the basis of the time of the third moulting (Bain *et al*. 1998) and is also supported by the previous molecular phylogeny based on 5S rRNA gene spacer (Xie *et al*. 1994).

The positioning of *A. viteae* appears more ambiguous: different approaches lead to different phylogenetic placements. The trees generated by tansversion analysis and wMP placed *A. viteae* as the sister group of *L. sigmodontis* (Fig. 3B). This would imply that *Wolbachia* has been lost along the lineage leading to *A. viteae* (Fig. 5A) (as stated before, *Wolbachia* has not yet been found in this species). The tree produced by SACW MP (Fig. 4), placing *A. viteae* as the sister group of the *Wolbachia*-harbouring filariae, might imply that the symbiosis with *Wolbachia* has been acquired after the separation of the lineage leading to *A. viteae* (Fig. 5B). Alternatively, the common ancestor of *A. viteae* and

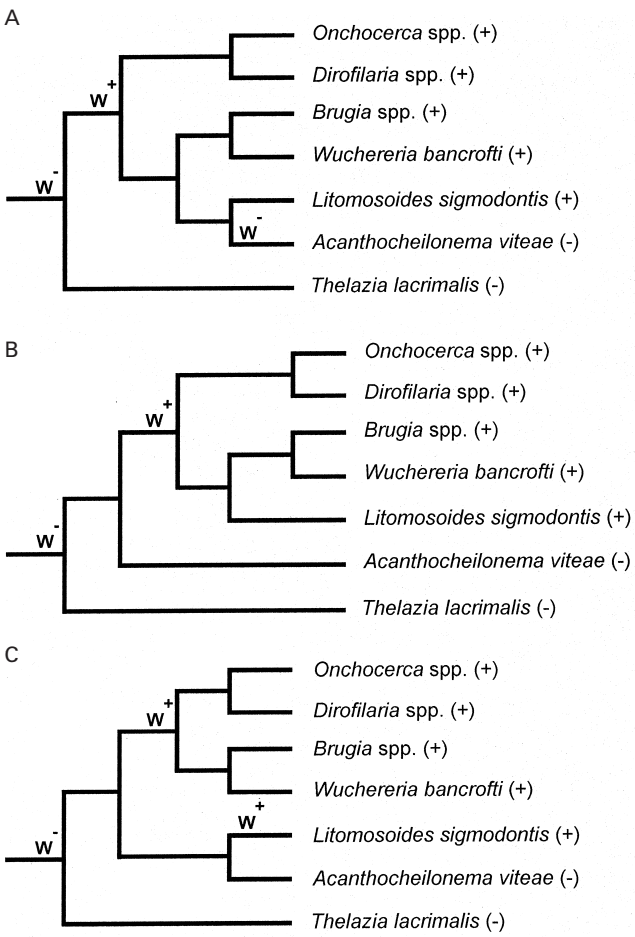

Fig. 5. Possible patterns of *Wolbachia* infection during the evolution of filarial nematodes. (A) Phylogeny of filarial nematodes corresponding to that shown in Fig. 3B: primitive infection of the common ancestor of filarial nematodes and successive loss of *Wolbachia* along the lineage leading to *Acanthocheilonema viteae*. (B) Phylogeny of *Wolbachia* corresponding to that shown in Fig. 4: infection after the separation of the lineage leading *A. viteae* from the other filarial nematodes. (C) Phylogeny of filarial nematodes suggested by Xie *et al*. (1994): infection after the separation of the lineage leading to *A. viteae* and *Litomosoides sigmodontis* and secondary infection in the lineage leading to *L. sigmodontis* (possibly by horizontal transmission of *Wolbachia* from lymphatic filariae). (+) indicates the species harbouring *Wolbachia*; (−) indicates the species not harbouring *Wolbachia*. W^+ indicates the acquisition of *Wolbachia* during evolution. W^- indicates the primitive absence or the loss of *Wolbachia*.

of the other filariae could have been infected, and the symbiosis could have been lost after the separation of the *A. viteae* lineage. For a discussion of the evolution of the genus *Acanthocheilonema* (partial synonym of *Dipetalonema*), see Chabaud & Bain (1976).

In Fig. 5 the presence/absence of *Wolbachia* is also mapped on the phylogenetic tree proposed by Xie *et al*. (1994) (Fig. 5C). This tree would imply that *Wolbachia* has been lost along the lineage leading to

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A. viteae, and/or that some horizontal transmission of *Wolbachia* has occurred. However, there are some problems with this analysis. A distance matrix derived from Xie *et al.*'s (1994) data set highlights differences in the rate of molecular evolution. Furthermore, transversion analysis using this data set placed *L. sigmodontis* as the sister group of the lymphatic filariae (not shown). Another major problem in Xie *et al.*'s (1994) data set was the absence of a suitable outgroup: the species used as outgroup was *Ascaris lumbricoides*, which does not belong to the branch of the spirurid nematodes and is thus quite distant from filarial nematodes.

Based on our analysis of COI gene sequences, we cannot propose a definitive phylogeny for filarial nematodes. In particular, we cannot define the position of the *Wolbachia*-free filarial *A. viteae*. However, we believe that our analyses provide a phylogenetic framework for those filariae that harbour *Wolbachia*, supporting the clustering of *L. sigmodontis* with the lymphatic filariae and the clustering of *Dirofilaria* spp. and *Onchocerca* spp. The phylogeny of these filariae thus appears congruent with that of their *Wolbachia* endosymbionts. In addition, we can perhaps look at the different phylogenies of filarial nematodes from a different perspective, using the character 'presence of *Wolbachia*'. From this perspective, the most parsimonious tree is the one placing *A. viteae* as the sister group of the *Wolbachia*-harbouring filariae (tree in Fig. 5B): this tree implies only 1 evolutionary step (i.e. an acquisition of *Wolbachia* after the separation of the *A. viteae* lineage). This phylogenetic hypothesis, which is supported by SACW analysis on COI gene sequences and provides the most parsimonious explanation to the distribution pattern of *Wolbachia*, can be tested using data sets derived from other molecules.

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