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wsp Gene Sequences from the *Wolbachia* of Filarial Nematodes

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Abstract. *Wolbachia* endosymbiotic bacteria are widespread in arthropods and are also present in filarial nematodes. Almost all filarial species so far examined have been found to harbor these endosymbionts. The sequences of only three genes have been published for nematode *Wolbachia* (i.e., the genes coding for the proteins FtsZ and catalase and for 16S rRNA). Here we present the sequences of the genes coding for the *Wolbachia* surface protein (WSP) from the endosymbionts of eight species of filaria. Complete gene sequences were obtained from the endosymbionts of two different species, *Dirofilaria immitis* and *Brugia malayi*. These sequences allowed us to design general primers for amplification of the *wsp* gene from the *Wolbachia* of all filarial species examined. For these species, partial WSP sequences (about 600 base pairs) were obtained with these primers. Phylogenetic analysis groups these nematode *wsp* sequences into a coherent cluster. Within the nematode cluster, *wsp*-based *Wolbachia* phylogeny matches a previous phylogeny obtained with *ftsZ* gene sequences, with a good consistency of the phylogeny of hosts (nematodes) and symbionts (*Wolbachia*). In addition, different individuals of the same host species (*Dirofilaria immitis* and *Wuchereria bancrofti*) show identical *wsp* gene sequences.

Wolbachia endosymbiotic bacteria are widespread in arthropods, where they typically induce reproductive manipulations such as parthenogenesis, cytoplasmic incompatibility (CI), feminization of genetic males, and death of male embryos [10, 20]. These bacteria belong to the alpha-Proteobacteria and are phylogenetically related to the genera *Anaplasma*, *Cowdria*, and *Ehrlichia* [15]. *Wolbachia* endosymbionts have also been found in filarial nematodes (family Onchocercidae), where they are thought to be obligatory symbionts [2–4, 8, 12, 17]. The following filarial species have been shown to harbor *Wolbachia*: *Dirofilaria immitis* and *D. repens* from the subfamily Dirofilariinae; *Brugia malayi*, *B. pahangi*, *Litomosoides sigmodontis*, *Onchocerca gutturosa*, *O. ochengi*, *O. volvulus*, *O. gibsoni*, and *Wuchereria bancrofti* from the subfamily Onchocercinae [3, 7]. The sequences of only three genes have been published for nematode *Wolbachia*, coding for the proteins FtsZ and catalase and for 16S rRNA [3, 7, 17]. One of the most abundantly expressed proteins in the arthropod endosymbiont is the *Wolbachia* surface protein (WSP) [6]. This protein contains transmembrane domains and a standard signal peptide for secretion and shows homologies to the major outer membrane proteins of *Ehrlichia* spp. and related genera [13, 14]. This indicates that WSP is a membrane protein of the bacterial outer envelope [6]. The *wsp* gene has proved to be very useful for phylogenetic studies of arthropod *Wolbachia* [19, 23]. Bacterial surface proteins are also commonly used both for the development of antibody-based staining and detection systems, as well as for the development of antibody-based purification methods. WSP could thus become useful for the development of methods for the detection and for purification of *Wolbachia* from the host tissues. There is indeed an increasing interest in methods for *Wolbachia* staining [8] and purification, particularly for protein and genome studies [5]. Here we report the sequencing of the *wsp* gene from the *Wolbachia* of eight species of filarial nematodes.

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Materials and Methods

Filarial parasite material. The following filarial species were included in this study: *Dirofilaria immitis*, *D. repens*, *Brugia malayi*, *B. pahangi*, *Litomosoides sigmodontis*, *Onchocerca gibsoni*, *O. ochengi*, and *Wuchereria bancrofti*. The origins of most of the samples examined are reported in Bandi et al. [3]. In addition, the following *D. immitis*, *D. repens*, and *W. bancrofti* specimens were examined: *D. immitis* collected from a cat in Parma (Italy), from two dogs in Kobe (Japan), from two dogs in Athens (USA), and from one dog in Cuba; *D. repens* collected from one dog in Milano and one in Pavia (Italy); and *W. bancrofti* obtained from humans in the North Coast of Papua New Guinea and from Sri Lanka. For *W. bancrofti*, DNAs from pooled samples (microfilariae) were analyzed. For the other species, DNAs from individual specimens were examined.

DNA analysis. DNA was extracted according to standard phenol–chloroform procedures [16] or through proteinase-K treatment [2, 17]. The *Wolbachia wsp* gene was PCR amplified using primers WSPestF (5′-TTAGACTGCTAAAGTGGAATT) and WSPestR (5′-AAACCACTGGGATAACAAGA). These primers were designed on the basis of conserved portions of the non-coding regions of the arthropod *wsp* sequences available in Genbank (accessions: AF020070, AF020066, AF020067, AF020071). These primers were used to obtain the complete *wsp* coding sequence from two model filarial species: *D. immitis* and *B. malayi*. Polymerase chain reaction (PCR) was performed in 20-μl volumes under the following final conditions: 1× buffer (Bioline), 2 mM $MgCl_2$, 0.2 mM of each dNTP, 1 μM each of forward and reverse primers, and 1 unit of BIO-X-ACT™ DNA polymerase (Bioline). The thermal profile we used was: 94°C 45 s, 52°C 45 s, and 72°C 90 s for 35 cycles. The high-fidelity DNA polymerase (BIO-X-ACT™) was employed to minimize incorporation errors during PCR synthesis. PCR amplification products were cloned by using the pGEM-T Vector System II (Promega). Three clones for each species were extracted, purified, and sequenced with ABI technology. The sequences obtained from *D. immitis* and *B. malayi* clones were aligned. A pair of primers, WSPintF: (5′-TAG(CT)TACTACATTCGCTTGCA) and WSPintR (5′-CCAA(CT)AGTGC(CT)ATAAAGAAC), were designed on the basis of regions conserved between the two sequences. These primers were used on all filarial samples listed above. PCR conditions were as previously described, but with annealing temperature at 50°C. PCR products were gel purified and sequenced directly with ABI technology. The sequences obtained have been deposited in the EMBL Data Library (accessions: AJ252061, AJ252062, AJ252175–AJ252180).

Data analysis. The sequences obtained were aligned to the prealigned *wsp* sequences available for arthropod *Wolbachia* [23]. Phylogenetic analysis was done on these sequences according to both character state and distance matrix-based procedures by using PAUP 4.0 (Sinauer Associates), MacClade (Sinauer Associates), and Treecon [18]. Both nucleotide and amino acid alignments were analyzed. On nucleotide alignments, phylogenetic analysis was done on the first, the second, the third, or the first plus second codon positions. Sequences of the surface proteins of *Ehrlichia* and *Cowdria* were used as outgroups in some analysis. Insertions and deletions (indels) were not considered in most of the analyses. When included, indels were treated as single substitution events regardless of their length. Search for transmembrane domains and secretion signals on the amino acid sequences was done using the Tmpred algorithm [9]. Similarity search against the databases was effected using the basic local alignment search tool [1] in the BLAST network service (National Center for Biotechnology Information, Bethesda, MD).

Results and Discussion

The primers we designed in the non-coding regions, on the basis of available arthropod *wsp* sequences, gave amplification from only four of the eight nematode species examined (*D. immits*, *D. repens*, *B. malayi*, and *B. pahangi*). However, PCR amplification bands at standard conditions (annealing at 55°C and 1.5 mM $MgCl_2$) were quite faint in most cases. To obtain better PCR amplifications, we used lower annealing temperature and higher $MgCl_2$ concentration (see Materials and Methods). We then focused the work on two model filarial species: *D. immitis* and *B. malayi*. These are thought to represent two different branches of filaria evolution [22]. In addition, the endosymbionts of *D. immitis* and *B. malayi* represent the two evolutionary branches (C and D) of filarial *Wolbachia* [3]. PCR products were cloned for these two filarial species, and the three clones we sequenced for each species showed identical sequences. Database searching with these sequences gave the highest similarity scores against *Wolbachia wsp* genes. Alignment between *D. immitis*, *B. malayi*, and *Drosophila simulans wsp* genes confirmed the similarity of the three sequences (EMBL alignment accession: ds41508). For both *D. immitis* and *B. malayi*, we obtained a full-length gene sequence (711 bp for *D. immitis*, EMBL accession AJ252062, and 723 bp for *B. malayi*, EMBL accession AJ252061). These sequences contained one open reading frame that codes for a protein of 237 (*D. immitis*) or 241 (*B. malayi*) amino acids. The 24 N-terminal amino acids appear to be almost identical to the signal secretion sequence observed in arthropod *Wolbachia* [6]. In addition, analysis of the nematode-derived protein sequences predicts a transmembrane domain (*D. immitis*: amino acids 118–135 of the mature protein; *B. malayi*: 106–127), which overlaps the second transmembrane domain of arthropod *wsp* sequences (*D. simulans*: 111–128). The newly obtained sequences are thus very likely to code for a surface protein homologous to the *wsp* of arthropod *Wolbachia*.

On the basis of the sequences obtained from *B. malayi* and *D. immitis*, a pair of "general" primers was designed (WSPintF and WSPintR) with the aim of amplifying *wsp* from C and D *Wolbachia*. These primers gave amplifications of about 590 bp from all tested nematode species. It was impossible to find sequence stretches conserved among all *Wolbachia* groups (A–D) that were suitable to design more general primers. Indeed, the primers used to amplify the *wsp* gene from arthropod A and B *Wolbachia* (81F and 691 R) [23] also show mismatches in the annealing regions of *wsp* sequences of C and D *Wolbachia*. When we tested these primers on nematodes, we obtained amplifications from

only a few species (*B. malayi*, *B. pahangi*, *L. sigmodontis*; not shown).

The PCR products obtained with primers WSPintF and WSPintR from nematode *Wolbachia* were sequenced directly, and the sequences were aligned to the *wsp* gene available for arthropod *Wolbachia*. We also tried to align *wsp* to the gene sequences available for the major outer membrane proteins of *Anaplasma*, *Ehrlichia*, and *Cowdria* species. While alignment among *wsp* sequences was unambiguous along most of the gene, alignment with the surface-protein genes of *Anaplasma*, *Ehrlichia*, and *Cowdria* appeared unreliable (not shown). As expected from the unreliability of the alignment, phylogenetic analysis showed arthropod and nematode *wsp* sequences closely related relative to the surface-protein genes of *Anaplasma*, *Ehrlichia*, and *Cowdria*. Because of the ambiguity of the alignment, for further phylogenetic analyses we did not include these bacteria.

In agreement with previous studies based on *ftsZ* gene sequencing [3], phylogenetic analysis on *wsp* sequences grouped nematode-derived sequences into two clusters (C and D; Fig. 1). Some analytical procedures also grouped *wsp* sequences from arthropod *Wolbachia* into the expected A and B clusters (Fig. 1a) [21, 23]. Nematode *wsp* sequences appear well differentiated from arthropod *wsp* sequences. However, in the absence of reliably aligned outgroups (see discussion above), the phylogenetic relationships between the four groups of arthropod and nematode *Wolbachia* cannot be determined on the basis of *wsp* analysis. Figure 1 shows two examples of unrooted trees based on an alignment including *wsp* nematode sequences and some *wsp* arthropod sequences that represent groups A and B (for details on the methods used to generate these trees, see Fig. 1 legend). Alignments including all the *wsp* sequences reported in Zhou et al. [23] and in van Meer et al. [19] were examined and produced trees showing similar relationships among nematode-derived sequences. Bootstrap support for the group encompassing nematode sequences (C and D) is very high (100%) in both trees. However, in Fig. 1 a bootstrap support for the cluster encompassing arthropod sequences (A and B) is quite low (58%). Indeed, different analytical procedures produced unrooted trees with different topologies. Figure 1b shows one of the shortest trees found by unweighted maximum parsimony on all codon positions (for details and tree statistics, see Fig. 1 legend). The lengths of trees 1a and 1b are very similar (1a: 558; 1b: 556). In addition, in the absence of suitable outgroups, we cannot determine the rooting of the trees and the relationships between the groups. We can thus limit ourselves to emphasize that comparison between *wsp* sequences allowed us to recognize the grouping of nematode-derived sequences into two clusters (C and D) that correspond to the clusters described on the basis of *ftsZ* sequence comparison.

Within the nematode clusters, the *wsp* gene phylogeny matches the *Wolbachia* phylogeny based on *ftsZ* [3]. Thus, in agreement with previous observations based on *ftsZ* sequences, all the phylogenetic relationships which are unquestioned for the host nematodes are matched by the *Wolbachia* phylogeny based on *wsp*. In addition, different individuals of the same host species collected from distant locations (*D. immitis* from Italy, Cuba, Japan, and USA; *W. bancrofti* from New Guinea and Sri Lanka) showed identical *wsp* gene sequences. This also agrees with previous *ftsZ* results and confirms that the *Wolbachia*–filaria association is stable and species specific.

Are the trees in Fig. 1 to be regarded as organism trees or as gene trees? Surface proteins of all the bacteria so far examined for the *Ehrlichia-Cowdria* group are coded by multicopy genes [13, 14]. For example, *Ehrlichia chaffensis* has at least six copies (tandemly arranged with intergenic spacers) of the gene coding for the major outer membrane proteins [13]. The proteins coded by these genes show up to approximately 20% amino acid difference. Trees based on these sequences in the *Ehrlichia-Cowdria* group are thus regarded as gene (or protein) trees [14]. In arthropod *Wolbachia*, there is no evidence for the presence of multiple copies of the genes coding for surface proteins. The gene sequences we obtained from filarial nematodes appear closely related among each other and appear also related to arthropod *wsp*. This provides evidence for homology, but does not allow us to decide whether nematode *wsp* is paralogous or orthologous [11] relative to arthropod *wsp*. Indeed, given that we cannot exclude that the ancestor of arthropod and nematode *Wolbachia* had a family of genes coding for surface proteins, we cannot conclude that the genes now present in arthropod and nematode *Wolbachia* derived from the same ancestral repeat (i.e., we cannot conclude that the arthropod *wsp* and nematode *wsp* are orthologous). On the other hand, we could assume that nematode *Wolbachia* originated from arthropod *Wolbachia* (or vice versa). This would make the idea that arthropod and nematode *wsp* are orthologous more parsimonious than the independent reduction of a multigene family down to a single copy.

Despite doubts outlined above about the usefulness of using *wsp* sequences for investigating the relationships among filarial and arthropod *Wolbachia* and, in particular, the absence of suitable outgroups, these sequences appear to be useful for investigating recent phylogenetic history within the nematode *Wolbachia*, in agreement with the results reported for arthropods. How-

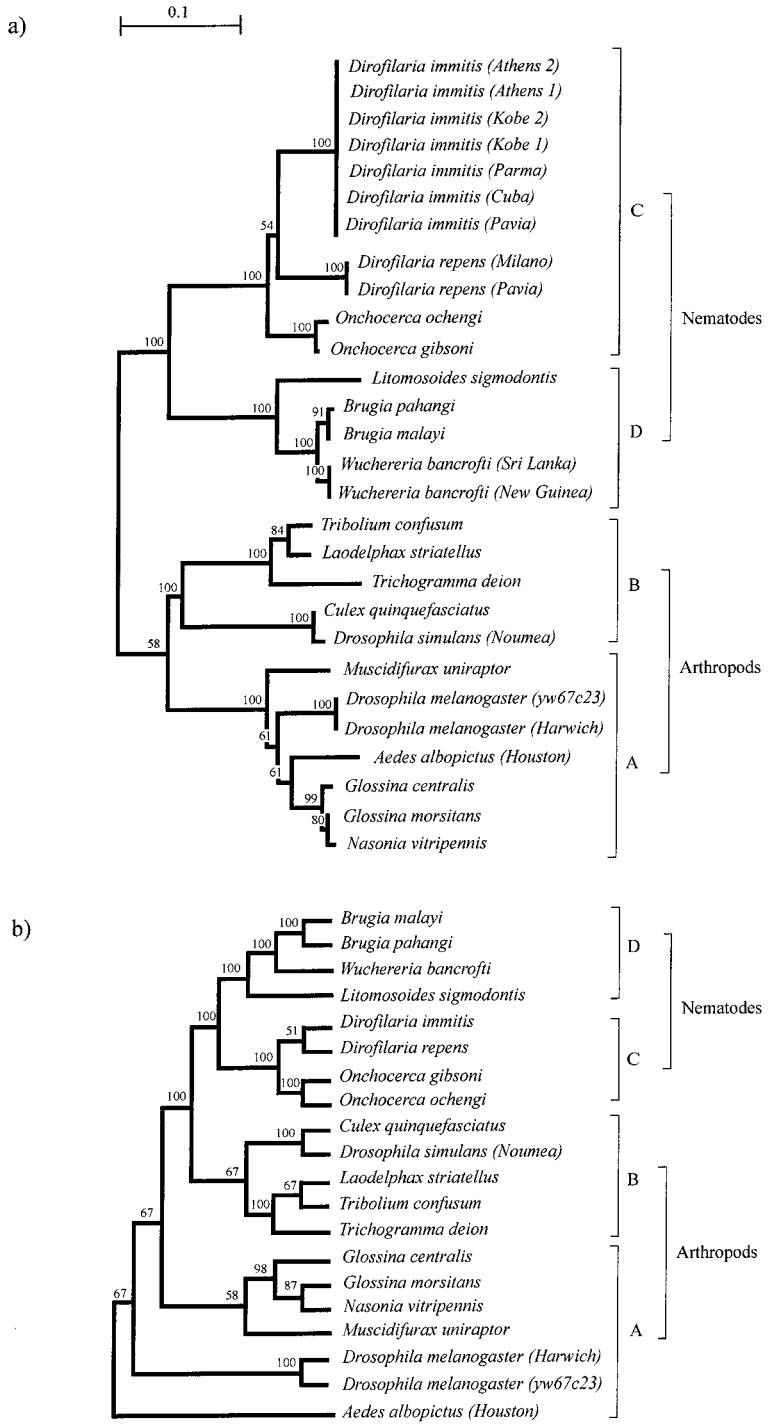

Fig. 1. Examples of trees showing the relationships among *wsp* gene sequences from nematode and arthropod *Wolbachia*. The four main groups of *Wolbachia* (A–D) are indicated. Species names at the terminal nodes are those of the arthropod or nematode hosts. Both trees are to be regarded as unrooted. Figure 1a is a tree obtained using a distance-matrix method (Kimura correction; Neighbor-Joining method; all codon positions included in the alignment; insertion/deletions not taken into account); numbers at the nodes are the bootstrap confidence values obtained after 100 replicates; the scale bar indicates the substitutions per nucleotide; tree length: 558; consistency index after excluding uninformative characters: 0.65; retention index: 0.84. Figure 1b is one of the two shortest trees found by unweighted maximum parsimony on the same alignment with the branch and bound search option in PAUP; tree length: 556; consistency index after excluding uninformative characters: 0.66; retention index: 0.85. Figure 1b is a simplified version of the tree obtained including all the representatives examined for each species. The alignment used to generate these trees has been deposited in the EMBL alignment data base (accession: ds41508).

ever, the level of variation shown by *wsp* in filarial nematodes appears low when compared with the variation observed in arthropod *wsp*. Indeed, while *wsp* sequences in arthropod *Wolbachia* show higher variation than *ftsZ* sequences, in filarial nematodes we observe more variation in *ftsZ* than in *wsp*. For example, there is only one nucleotide substitution out of 600 bp between the *wsp* genes of the *Wolbachia* of *B. malayi* and *B. pahangi*, while their *ftsZ* genes show five nucleotide substitutions over 930 bp. In addition, within the same

nematode species we observed no *wsp* variation, while two synonymous substitutions have been observed in the *ftsZ*s obtained from individuals of the same species [3]. Selective pressures for conserving a given protein sequence in nematode *Wolbachia* cannot explain the patterns observed: we would expect to observe some synonymous substitutions (e.g., between the sequences derived from *B. malayi* and *B. pahangi*). Furthermore, we cannot explain the higher variation in arthropods by invoking selective pressure for amino acid variation: Zhou et al. [23] did not observe any bias towards non-synonymous changes in *wsp*. Similarly, in the *ftsZ* of nematodes [3] no bias towards non-synonymous changes is observed (unpublished observation).

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