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Absence of *Wolbachia* in Nonfilariid Worms Parasitizing Arthropods

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Abstract *Wolbachia* are strictly intracellular maternally inherited α-proteobacteria, largely widespread among arthropods and filariids (i.e., filarial nematodes). *Wolbachia* capacities to infect new host species have been greatly evidenced and the transfer of *Wolbachia* between arthropods and filariids has probably occurred more than once. Interestingly, among nematode species, *Wolbachia* infection was found in filariids but not in closely related lineages. Their occurrence in filariids has been supposed a consequence of the parasitic lifestyle of worms within *Wolbachia*-infected arthropods, implying that nonfilariid worms parasitizing arthropods are also likely to be infected by some *Wolbachia* acquired from their hosts. To further investigate this hypothesis, we have examined seven species of nonfilariid worms of Nematoda and Nematomorpha phyla, all interacting intimately with arthropods. *Wolbachia* infection in nonfilariid parasitic worms was never detected by polymerase chain reaction assays of the 16S rDNA and *wsp* genes. By contrast, some arthropod hosts are well infected by *Wolbachia* of the B supergroup. Then the intimate contact with infected arthropods is not a sufficient condition to explain the *Wolbachia* occurrence in filariids and could underline a physiological singularity or a particular evolutionary event to acquire and maintain *Wolbachia* infection.

Keywords *Wolbachia* · Symbiosis · Nematoda · Nematomorpha

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Introduction

Wolbachia are maternally inherited α-proteobacteria largely widespread among arthropods and filariids (i.e., filarial nematodes), being probably one of the most ubiquitous endosymbionts [1–3]. Their successful spread is attributed to their ability to interfere with host reproduction, which improves their own transmission and persistence among host populations. In arthropods, *Wolbachia* are generally facultative endosymbionts not involved in host survival and act as a key manipulator of host reproduction by inducing feminization, parthenogenesis, male killing, or cytoplasmic incompatibility [1–3]. These manipulations promote the production and the fitness of infected daughters (i.e., the transmitting sex) via negative effects on the fitness of individuals not involved in the transmission (males or uninfected females), allowing *Wolbachia* to invade natural populations. *Wolbachia* reach a high prevalence in numerous arthropod groups using reproductive parasitism, infecting 20–75% of insect species [4, 5]. By contrast, filariid *Wolbachia* have evolved toward a mutualistic pathway and their presence is obligatory required for fertility and survival of many filariid species [6–8]. In this way, *Wolbachia* appear fixed throughout filariids, except in a few species that are supposed to have diverged earlier than lineages leading to infected species or to have secondarily lost the infection [9–11]. The great

Wolbachia variation in reproductive effects reflects their high degree of plasticity, but the evolutionary pattern of *Wolbachia* infection remains poorly understood.

Wolbachia can be separated into eight supergroups (A to H) that have diverged 60–100 million years ago (Mya) based on 16SrRNA, *Wolbachia* surface protein (*wsp*), and *ftsZ* phylogenies [12–16]. Some supergroups are specific of arthropods (A, B, E, G, and H) or filariids (C and D), whereas the F supergroup is found in both host types. Arthropod *Wolbachia* can shift host species in complement to vertical transmission, and recurrent horizontal transfers have occurred especially considering the A and B supergroups [5, 16]. In filariids, the acquisition of *Wolbachia* probably predated the diversification of the group and both have coevolved for 100 Mya [9, 17]. Indeed, *Wolbachia* and their filariid hosts show closely parallel phylogenies, contrary to arthropod *Wolbachia* [17]. However, *Wolbachia* transfers between arthropods and filariids have perhaps occurred more than once, as evidence being that both arthropod and filariid *Wolbachia* are found in the F supergroup [18]. To date, the direction and mechanisms of horizontal transfer remain unclear.

Wolbachia have not been found in nematode groups other than filariids, even in closely related lineages [19]. The occurrence of *Wolbachia* in filariids has been thus supposed a consequence of the parasitic lifestyle of worms [9, 19]. Contrasting to free-living nematodes, many of the filariid species are vectored by arthropods and are intimately in contact with their *Wolbachia*, increasing the potential of interspecific transfer. This implies that nonfilariid worms parasitizing arthropods are also likely to be infected by some *Wolbachia* acquired from their hosts. Except filariids, three major families of Nematoda parasitize arthropods: Mermithidae, Steinernematidae, and Heterorhabditae. Mermithidae include species that kill, sterilize, or alter host development and have been reported from a variety of arthropods, in a variety of environments, and often infecting large percentages of host populations [20]. Steinernematidae and Heterorhabditae species transmit the symbiotic bacteria *Xenorhabdus* sp. and *Photorahbdus* sp., which are lethal to their host, worms feeding on the host cadaver [21]. Outside of Nematoda, the Nematomorpha phylum (horsehair worms) is relatively unknown but largely widespread. Horsehair worms grow in adult large worms whose size exceeds the length of the host by a considerable amount. Indeed, when the development has been completed, the horsehair worm occupies most of the host cavity, with the exception of the head and the legs [22].

To our knowledge, very few parasitic worms other than filariids have been checked for *Wolbachia* infection (but see [19, 30]). Because they represent potential vectors or hosts for *Wolbachia*, we examined five Nematoda (outside the filariid group) and two Nematomorpha species, all interacting intimately with arthropods, for *Wolbachia* infection.

Materials and Methods

Worm Collection

Nematomorphs and Mermithids were isolated directly from living insects from natural populations in France. Mature nematomorphs manipulate the behavior of their terrestrial insect host, making them seek water and jumping into it [37]. In this way, nematomorphs and their hosts (*Nemobius sylvestris* and *Pholidoptera griseoaptera*) were caught in private swimming pools in southern France. Steinernematidae and Heterorhabditae worms come from laboratory rearing, undergoing a simulated natural life cycle that involves the parasitic stage, in which susceptible insect prey (last instar of the Lepidoptera *Galleria mellonella*) are killed by the combined action of the nematode and the symbiotic bacteria (i.e., *Xenorhabdus* sp. and *Photorahbdus* sp.). Worms and their arthropod hosts were stored in 96% ethanol (number and origin reported in Table 1).

Wolbachia Detection

DNA was extracted using a CTAB protocol [23]. *Wolbachia* infection was addressed by two different types of polymerase chain reaction (PCR) assays specific to the 16S rDNA and *wsp* protein coding gene using generalist primer pairs (fD1/rD1 and 16SWolbF/16SWolbR3 for 16S rDNA and 81F/691R, WSPestF/WSPestR or WSPintF/WSPintR for *wsp*) and their PCR cycling conditions described by Weisburg et al. [33], Casiraghi et al. [17], Zhou et al. [16], and Bazzocchi et al. [24]. We have used positive controls from arthropod samples of infected *Culex pipiens* mosquitoes and *Drosophila simulans* Riverside drosophila and negative controls from uninfected *C. pipiens* and *D. simulans* samples. Controls were included in each group of PCR, and DNA quality was controlled by a PCR assay on 18S rDNA using specific primers (1F/4R) along PCR conditions described by Giribet et al. [25]. PCR products were sequenced directly, using the Big Dye Terminator kit (Applied Biosystems) and analyzed on an ABI Prism 310 sequencer. Sequences were first aligned and modified visually using MEGA version 3.1 [38].

Results and Discussion

Wolbachia have undergone frequent shift of host species on an evolutionary timescale while maintaining themselves principally by vertical transmission [5, 16]. Potential

Table 1 List of the parasitic worm species screened for *Wolbachia* infection

Phylum	Family	Species	*n(w)*	18S rDNA	16S rDNA	*wsp*	Athropod host	*n(a)*	*wsp*
Nematomorpha	Paragordiidae	*Paragordius tricuspidatus*	10	+	–	–	Orthoptera	5	+ (B group)
	Spinochordodidae	*Spinochordodes tellinii*	2	+	–	–	Orthoptera	2	+ (B group)
Nematoda	Mermithidae	*Mermis nigrescens*	2	+	–	–	Orthoptera	Not tested	
	Mermithidae	*Romanomermis culii*	1	+	–	–	Diptera	Not tested	
	Steinernematidae	*Steinernema carpocapsae*	>500	+	+ *	–	Lepidoptera	5	–
	Steinernematidae	*Steinernema kraussei*	>500	+	+ *	–	Lepidoptera	5	–
	Heterorhabditae	*Heterorhabditis heliothidis*	>500	+	+ *	–	Lepidoptera	5	–

Note: The list of arthropods refers to host taxa in which worms have been found and is not exhaustive. Quality of DNA was controled by 18S rDNA PCR assays. *Wolbachia* infection was addressed by PCR assays specific to the 16S rDNA and *wsp* genes. +, positive PCR amplification; –, no PCR amplification; *, 16S rDNA PCR are positive for *Xenorhabdus* and *Photorhabdus* bacteria but negative for *Wolbachia* infection; *n*(*w*), number of individual worms tested; *n*(*a*), number of individual arthropods tested.

mechanistic hypotheses for *Wolbachia* transfer between nonrelated hosts include parasitism, cofeeding on the same host plant, or predation events [26–29]. Here we have investigated the presence of *Wolbachia* in seven phylogenetically distant nonfilariid parasitic worms: two Nematomorpha species (i.e., *Paragordius tricuspidatus* and *Spinochordodes tellinii*), two Mermithidae (Nematoda) species (i.e., *Mermis nigrescens* and *Romanomermis culii*), two Steinernematidae (Nematoda) species (i.e., *Steinernema carpocapsae* and *S. kraussei*), and one Heterorhabditae (Nematoda) species (i.e., *Heterorhabditis heliothidis*). Our sampling is phylogenetically broad—most authors agree that Nematoda is the sister taxon of Nematomorpha [35]—even considering that some of these species have a similar parasitic biology that actually results from evolutionary convergence. These parasitic species have a long-term association with arthropods, as suggested by the Mermithidae and Nematomorpha fossils found in Early Cretaceous Burmese amber dated at 100–110 Mya [34]. Thus, the majority of Mermithidae fossils have been reported from ants, mosquitoes, chironomids, planthoppers, and spiders conserved in amber [34]. Interestingly, *Wolbachia* infection has been reported in some current species belonging to these arthropod groups [4, 5].

Despite their long-term coexistence of parasitic nonfilariid worms with arthropods, *Wolbachia* infection was never detected by our PCR survey using experimental conditions that are able to detect *Wolbachia* in arthropods and in filariids (Table 1). Only bacteria of the *Xenorhabdus* and *Photorhabdus* genus have been actually detected in *Steinernema* and *Heterorhabditis* hosts, respectively (identified by 16S rDNA sequence; data not shown). The possibility of a false negative was reduced by lowering the annealing temperature in reaction with all primer pairs, but this does not change the results. All suggest that *Wolbachia* do not infect nonfilariid worms despite sharing a common arthropod-parasitic lifestyle with filariids.

In contrast to nonfiilarid worms, some of their arthropod hosts are infected by *Wolbachia*. The Wood Cricket (*Nemobius sylvestris* Gryllidae) and the Dark Bush-cricket (*Pholidoptera griseoaptera*) are the natural host of nematomorphs and all crickets tested ($n = 7$) were infected by *Wolbachia* belonging to the B supergroup *(*identified by *wsp* sequence; *data not shown)*. Strengthening these observations, the absence of *Wolbachia* was also reported in *Agamermis unka* (Mermithidae) that parasitizes *Wolbachia*-infected rice planthoppers [30]. The intimate contact with infected arthropods is not a sufficient condition to explain the occurrence of *Wolbachia* in filariids.

The nucleotide divergence between the arthropod and filariid *Wolbachia* argues for an evolutionary separation of about 100 Mya [9]. Similarly, estimates of the divergence of the filariids from other nematodes are of the same order of magnitude [36], suggesting that the *Wolbachia* infection could have occurred quickly after the emergence of the ancestral lineage of extant filariids. Why *Wolbachia* are absent in nonfilariid worms parasitizing infected arthropods remains intriguing. In the Steinernematidae and Heterorhabditae worms, the symbiotic bacteria *Xenorhabdus* and *Photorahbdus* infecting are well known to produce antibiotic [21] and could be an explanation of the absence of *Wolbachia* in these species. However, such an explanation fails in Mermithidae and Nematomorpha because no symbiotic bacterium (i.e., no competitor) was detected. An obvious reason could be the inability of *Wolbachia* to tolerate nonfilariid as nonarthropod cellular environments, underlining in this way a physiological singularity shared by filariids and arthropods to support infection by *Wolbachia*. Indeed, *Wolbachia* genomes encode limited metabolic capacity, resulting from the loss of genetic material following adaptation to the intracellular environment (for review, see [31]), and strong host specificity to the *Wolbachia* endosymbiosis in fialriids (as indicated by strict coevolution) is thus probably the rule. Interestingly, transfer experiments into the naturally *Wolbachia*-free

filariid *Acanthocheilonema vitae* suggest the successful establishment of infection, demonstrating then the capacity of *Wolbachia*-free filariids to be infected [32]. Artificial transfer experiments in nonfilariid worms would help to definitively clarify their possible ability to carry *Wolbachia* infection, but it seems probable that *Wolbachia* could infect only the filariid group among Nematodes and Nematomorphs.

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