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Detection and phylogenetic analysis of *Wolbachia* in Collembola

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Summary

Wolbachia **are obligatory, cytoplasmatically inherited α-Proteobacteria which are known for infecting the reproductive tissues of many arthropods. Their prevalence in the large group of Collembola, however, is not known, except for PCR detection in the parthenogenetically reproducing species *Folsomia candida* (Order: Entomobryomorpha; Family: Isotomidae). In this study, fluorescence *in situ* hybridization on microscopic sections of *F. candida* specimens indicated that *Wolbachia*-related bacteria were restricted to tissues of the ovary and brain. PCR with primers designed to detect 16S rRNA genes of *Wolbachia* were positive with specimens from all of five geographically independent *F. candida* breeding stocks and with three parthenogenetic species from another order (Poduromorpha; Family Tullbergiidae), i.e. *Mesaphorura italica*, *M. macrochaeta* and *Paratullbergia callipygos*. In contrast, negative results were obtained with the two sexually reproducing species, *Isotoma viridis* (Isotomidae) and *Protaphorura fimata* (Poduromorpha; Onychiuridae). The *ftsZ* gene of *Wolbachia* could be PCR-amplified from all *Wolbachia*-positive hosts with the exception of *M. macrochaeta*. The phylogenetic distances of the *ftsZ* and 16S rRNA gene sequences reflected the phylogenetic distances of the host organisms but the sequences of *Wolbachia* were relatively closely related, indicating that *Wolbachia* infections took place after the Collembola had diversified. Our study confirms a monophyletic branch (supergroup E) of Collembola colonizing *Wolbachia* and indicates that this group is a sister group of supergroup A, the latter harbouring a high diversity of host organisms within the group of insects.**

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Introduction

Wolbachia are a coherent group of obligatory, intracellular α-Proteobacteria that have been detected in many arthropods and in several filarial nematodes. In arthropods, *Wolbachia* infections can result in manipulations of host reproduction, for example cytoplasmatic incompatibility, parthenogenesis, feminization of genetic males, or male-killing [for reviews see (O'Neill *et al*., 1997; Werren, 1997; Stouthamer *et al*., 1999)]. Within arthropods, host organisms of *Wolbachia* have been found in major groups, such as Insecta, Crustacea and Arachnida. However, despite its high abundance in terrestrial ecosystems and the considerable richness of species, little is known about Collembola (springtails) as potential hosts for *Wolbachia*.

Collembola are typical soil inhabitants and part of the mesofauna (Bardgett and Griffiths, 1997). Adult Collembola have a body length in the range of 0.2–3 mm and they can spend most of their lives either in the soil matrix or on the soil surface, e.g. in the litter layer. Phylogenetically, Collembola are considered to be more primitive and older than insects (Frati *et al*., 1997). Based on fossil evidence, the first Collembola appeared as early as 380 million years ago (Whalley and Jarzembowski, 1981). Together with insects, Collembola form the taxon of Hexapoda (six-legged arthropods) but a recent study indicates that this taxon is not monophyletic (Nardi *et al*., 2003). Interestingly, as for many insects, parthenogenesis is widespread in Collembola (Goto, 1960). This lifestyle allows a species to increase its population's size more quickly than by sexual reproduction (Koivisto and Braig, 2003). For Collembola, parthenogenesis has not yet been connected to the presence of *Wolbachia*.

In soil, Collembola feed on plant residues, fungi or living and dead animals and they play an important role in the ecology of microbial symbionts in the rhizosphere (Hopkin, 1997). Collembola are important within the terrestrial food web (Hunt *et al*., 1987; Rusek, 1998). Their chewing activity results in a breakdown of plant matter and inoculation of the food with gut bacteria. The guts of Collembola harbour a highly active and dense bacterial community that undergoes dramatic changes during each molting cycle (Borkott and Insam, 1990; Thimm *et al*., 1998). In addition to the gut, other body compartments of Collembola may also serve as bacterial habitats. Intracellular bacteria were first detected by transmission electron

microscopy (TEM) in the fat body and ovarial tissue of *Folsomia candida* (Order: Entomobryomorpha, Family: Isotomidae) (Palévody, 1972). In a later study with *F. candida*, *Wolbachia*-related 16S rRNA sequences were detected by means of PCR (Vanderkerckhove *et al*., 1999). Fluorescence *in situ* hybridization (FISH) with a universal bacterial gene probe recently confirmed the occurrence of intracellular bacteria in different tissues of *F. candida* (Thimm and Tebbe, 2003) but the location of *Wolbachia* cells in *F. candida* or other Collembola has not been characterized yet.

The phylogenetic analysis of *Wolbachia* is currently mainly based on three different genes, i.e. the 16S rRNA gene, with a rather small sequence divergence between different *Wolbachia* (Vanderkerckhove *et al*., 1999), the *ftsZ* gene, a gene that is involved in the cell division process (Lo *et al*., 2002), and the *wsp* gene, encoding for a surface protein (van Meer *et al*., 1999). Based on the phylogenetic analysis of the *ftsZ* gene, four major groups, called supergroups A, B, C and D, have been differentiated among *Wolbachia*. Supergroups C and D exclusively comprise *Wolbachia* isolated from filarial nematodes with a high correlation of host and *Wolbachia* phylogeny (Casiraghi *et al*., 2001). In contrast, supergroups A and B include hosts of a large number of different insects and there seems to be no strict overall-correlation between the phylogeny of the hosts and *Wolbachia*. Interestingly, the only *ftsZ* gene that was analysed from *Wolbachia* from a member of the Collembola group, which was again *F. candida*, could not be assigned to any of the four supergroups, and it was concluded that *F. candida* represents a new supergroup (Lo *et al*., 2002). This analysis confirmed an earlier phylogenetic analysis of the 16S rRNA gene in which a supergroup E for *F. candida* was already suggested (Vanderkerckhove *et al*., 1999).

We conducted this study (i) to elucidate which compartments of the body of *F. candida* were colonized with *Wolbachia* and (ii) to investigate whether Wolbachia from different Collembola were of monophyletic origin in comparison to Wolbachia from other host organisms. For the latter purpose, we included Collembolan species from two different orders (Poduromorpha, Entomobryomorpha) and three different families (Isotomidae, Onychiuridae, Tullbergiidae) and *F. candida* specimens of several different breeding stocks obtained from the US, France and Germany. The selection of Collembolan species included those known to have a parthenogenesis-lifestyle and those with sexual reproduction (Table 1). Furthermore, by combining the phylogenetic analyses of both the 16S rRNA genes and the *ftsZ* genes we wanted to stabilize a potential Collembola-specific phylogenetic group and identify its relation to the other supergroups A to D.

Results

Colonization of F. candida *with* Wolbachia

FISH was conducted to confirm the occurrence of *Wolbachia*-like bacteria in the body cavity and to analyse whether specific tissues were preferentially colonized. The specificity of the chosen probe for *Wolbachia* 16S rRNA, W2, was tested with *Sinorhizobium meliloti* (*Proteobacteria*, alpha subgroup) as a negative control and FISH indicated no hybridization (data not shown). We chose EUB388 as a probe to detect all eubacterial cells. In microscopic sections of *F. candida* we found bacteria in the gut and in different tissues (Fig. 1A and C). Relatively high numbers of bacterial cells could be detected in the tissues of the ovaries (Fig. 1C) and frequently, but not always, bacterial cells were also detected in the fat body (Fig. 1A). The density of the bacterial cells that were detected in the fat body varied between specimens. In contrast to results with EUB388, we found that W2-hybridizing bacteria were restricted to the tissues of the brain (Fig. 1B) and the ovaries (Fig. 1D). The *Rickettsia*-like bacteria observed in the fat bodies did not hybridize with the *Wolbachia*-specific gene probe.

Occurrence of Wolbachia *in different* Collembola

With a *Wolbachia*-specific PCR (Werren *et al*., 1995) we obtained amplified products of the predicted size with all *F. candida* (Isotomidae) breeding stocks tested and also with the three species undergoing parthenogenesis, i.e. *Paratullbergia callipygos*, *Mesaphorura italica* and *M. macrochaeta* (all Tullbergiidae). In contrast, no *Wolbachia*-

Table 1. Characterization of Collembola used in this study.

Order	Family	Species	Origin[a]	Partheno-genesis
Entomobryomorpha	Isotomidae	*Folsomia candida*	Aa, Br, Bs, El, Gö	yes
		Isotoma viridis	Bs	no
Poduromorpha	Onychiuridae	*Protaphorura fimata*	Aa	no
	Tullbergiidae	*Paratullbergia callipygos*	Br	yes
		Mesaphorura italica	Br	yes
		M. macrochaeta	Br	yes

a. Aa: Aachen; Br: Brunoy; Bs: Braunschweig; El: East Lansing, Gö: Görlitz.

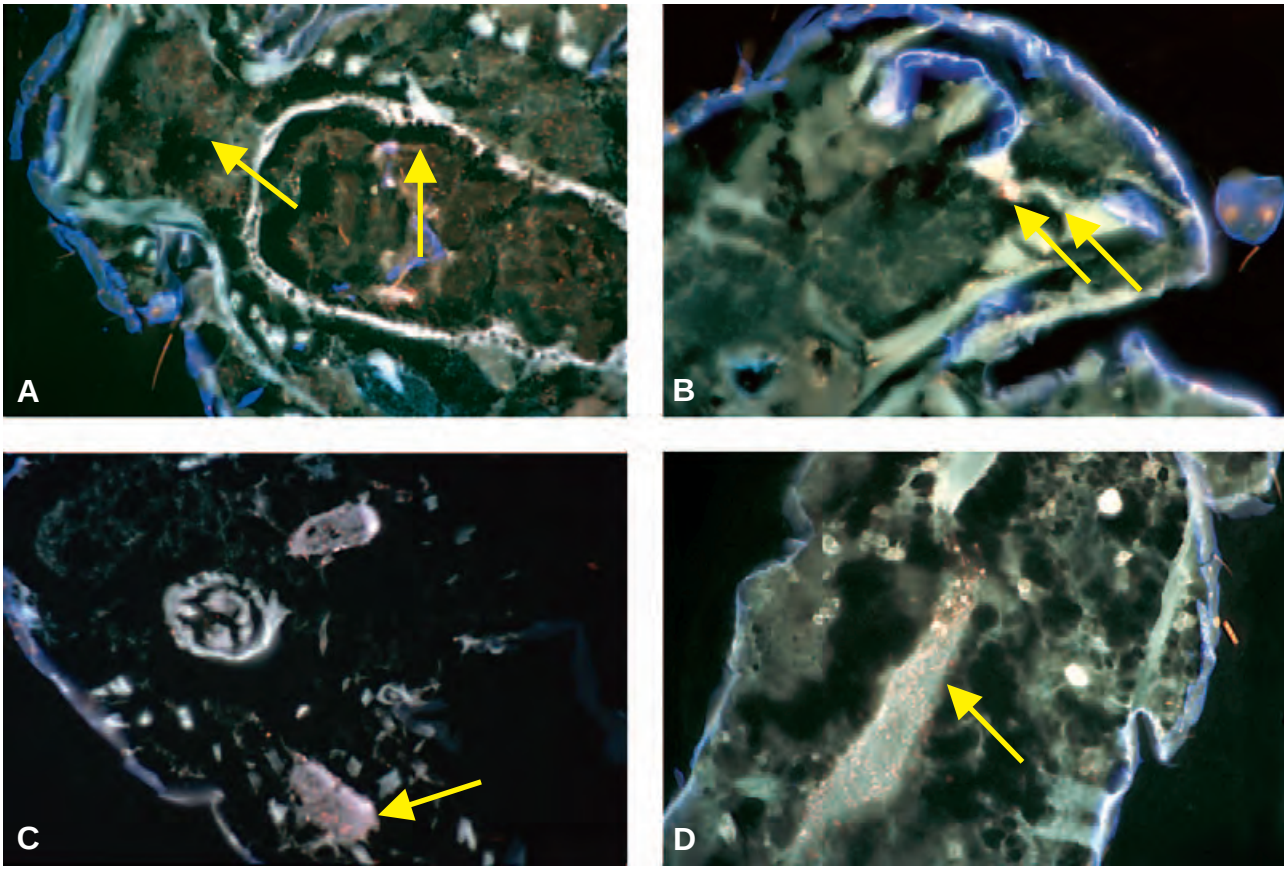

Fig. 1. Fluorescence *in situ* hybridization (FISH) on wax sections of *Folsomia candida*.
A. Proximal region, hybridized with the eubacterial probe EUB338, with bacteria (red) in the gut and fat body (arrow)
B. Head region of the same specimen as in (A), hybridized with the *Wolbachia*-specific probe (W2) showing bright red signals in the brain tissue (arrows)
C. Ovary, cross-section, hybridized with EUB338 (red cells, arrow).
D. Ovary hybridized with the W2-probe, showing tissue cells filled with *Wolbachia* (arrow).

specific PCR products were found with the sexually reproducing specimens of *Protaphorura fimata* (Onychiuridae) and *Isotoma viridis* (Isotomidae) respectively. SSCP analysis indicated that two different PCR products were amplified, one specific for *F. candida* and the other for both Tullbergiidae tested (Fig. 2).

Comparison of 16S rRNA gene sequences

The almost complete 16S rRNA gene sequence (1514 bp) of *F. candida* that we determined in our study showed 100% similarity to a recently published *Wolbachia* sequence from the same host species (Vanderkerckhove *et al*., 1999). In contrast, the 16S rRNA genes of the Tullbergiidae *M. italica*, *M. macrochaeta* and *P. callipygos* were different from *F. candida*, but except for an ambiguous base, identical to each other (AJ422184; AJ575104; AJ509026). Interestingly, the highest similarity to a known sequence in the databases was 99.2% to the corresponding gene of *F. candida*.

The differences between the *F. candida* and the Tullbergiidae sequences were located on both ends (5′ and 3′) of the 16S rRNA gene but not in the region that corresponds to the *Escherichia coli* positions 260–840. In this

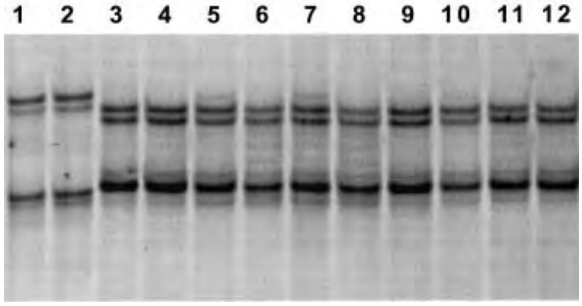

Fig. 2. Non-denaturing polyacrlyamide gel electrophoresis to differentiate partial PCR-amplified 16S rRNA genes of different Collembolan species by single-strand conformation polymorphism: *Mesaphorura macrochaeta* (lane 1), *Paratullbergia callipygos* (2), *F. candida* (lanes 3–12) from different breeding stocks, each in two independent replicates, i.e. Aachen (3, 4), Braunschweig (5, 6), East Lansing (7, 8), Brunoy (9, 10) and Görlitz (11, 12).

region, we found three out of four sites that were similar in Collembola (proposed supergroup E) but do not appear in other groups (Table 2). Furthermore, we found mutations that were restricted to the Collembola group at *E. coli* positions 422, 562, which showed a C in the Collembola but a T in the other groups. In addition, *E. coli* position 1305 had an A in the Collembola group and a G in all other groups.

The phylogenetic analysis with the 16S rRNA genes led to following results: independent of the method for tree reconstruction, all Collembola sequences fell into one single group that was different from sequences of the other supergroups. However, depending on the method, we found small differences within the supergroups A and B and for the position of the branch with the Collembola sequences. In the distance matrix tree, the Collembola group shared a basal internal node with supergroup A (Fig. 3). For the maximum likelihood (ML) tree, 22 852 trees were examined and a stable tree was estimated with a log likelihood of ln L = –3457. In the resulting tree, the Collembola sequences were most closely related to a group of three sequences from supergroup A (Accession Numbers: M84688, M84687, LO2882). The significance of the Collembola branch was $P < 0.001$. In the maximum parsimony (MP) tree, the Collembola branch had a common node with the A/B-clade (bootstrap value 75%). The bootstrap value for the Collembola branch separating from the A/B supergroup was 95%.

Detection and sequencing of ftsZ *genes*

PCR with primers specifically designed to amplify *ftsZ* genes from *Wolbachia* yielded PCR products of the expected size with all specimens of the five *F. candida* breeding stocks and with *M. italica*, but not with *P. callipygos* and *M. macrochaeta*. Based on the alignment of the four known sequences of the *Wolbachia ftsZ* gene, of which three were determined in this study and one recently published elsewhere (Lo *et al*., 2002), we selected the alternative reverse primer ftsZcol-r. With this primer we were able to obtain PCR products from *P. callipygos* (wCal) but not from *M. macrochaeta*.

In the course of our cloning and sequencing procedure for PCR products, we routinely sequence several clones of each transformation event. When the PCR product that was obtained from eight specimens of *P. callipygos* was analysed, we surprisingly found two slightly different sequences (wCalI, AJ575101; wCalII, AJ575102), even though the specimens were offspring from the same female. Comparing the sequences, there was a remarkable deletion at base 263 in wCalII. Two different *ftsZ* sequences were also recovered from the five *F. candida* breeding stocks (wCanA, AJ509028; wCanB, AJ509027); however, each stock was characterized by only one sequence. The sequence wCanA was found in breeding stocks from Aachen and Brunoy, and wCanB in those from Braunschweig, Görlitz and East Lansing. The *ftsZ* sequence of *M. italica* (wIta, AJ575103) was different from those of *F. candida* and *P. callipygos* respectively. An alignment of the *ftsZ* genes indicated that most of the differences were located in the third triplet position (Wobble base). A higher frequency of differences was found towards the 3′ end of the gene. The complete alignment has been deposited in the FASTA database (Accession Number ALIGN_000586).

Phylogenetic trees with the *ftsZ* genes, independent of the tree construction method, led to similar tree topologies as found with the distance matrix for the 16S rRNA genes (some clades slightly different within supergroup B) (Fig. 4). The ML tree was generated by examination of 6721 trees to a final tree with a log likelihood ln L = –4278. In all trees, the Collembolan *Wolbachia* sequence fell into

Table 2. Variability of nucleotides within the 16S rRNA gene between different phylogenetic lineages (supergroups) of *Wolbachia*.

	Supergroup E		Other supergroups				
E. coli position	*F. candida*	*M. macrochaeta* *M. italica* *P. callipygos*	A	B	C	D	F
76	A	G	W	W	R	A	A
92	G	A	G	G	G	R	G
94	A	G	A	A	R	A	A
156	C	T	T	T	C	T	T
165	G	A	G	G	R	G	G
190	–	G	–, G	–, G	–, G	–, G	–, G
194	G	–	G, –	G, –	G, –	G, –	G, –
841	T	C	T	T	A	T	T
845	A	G	A	A	A	A	A
989	T	C	C	Y	C	C	C
1216	A	G	G	G	G	G	G
1252	T	C	T	T	T	T	T
1254	G	A	G	G	G	G	G
1283	C	T	C	C	C	C	C

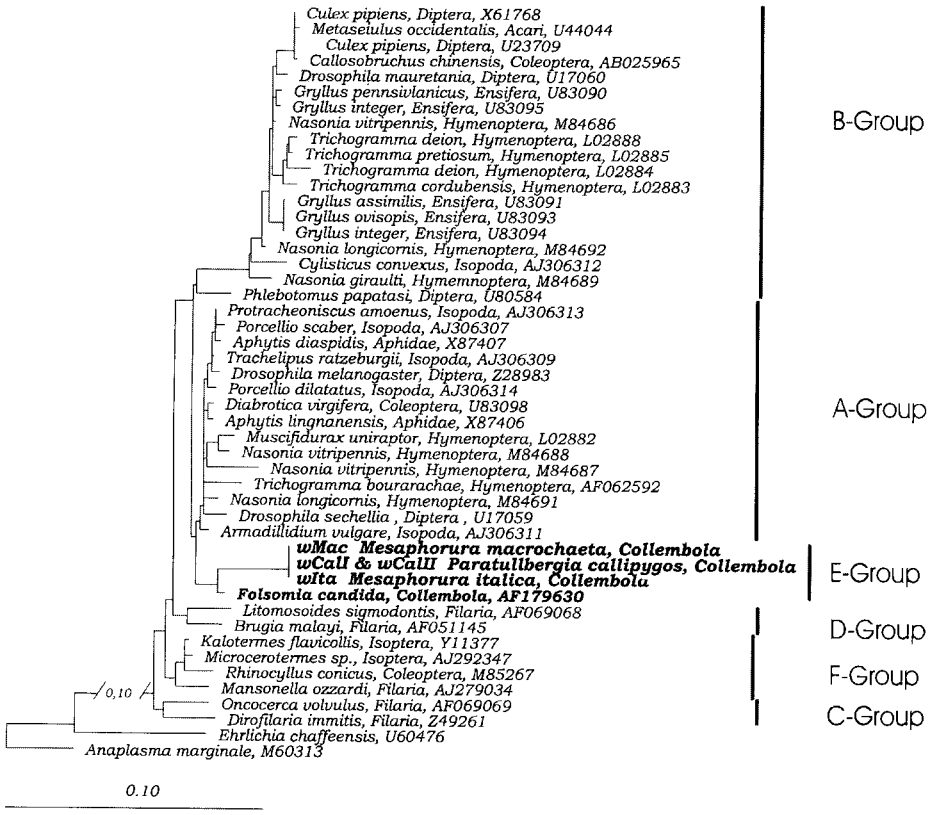

Fig. 3. Phylogenetic tree of *Wolbachia*, for the 16S-rRNA gene. The tree was calculated with the Phylip Distance Method. The 16S rRNA gene of *Anaplasma marginale* was used as an outgroup. The species names in the tree indicate arthropod or nematode hosts from which the sequence were obtained.

the proposed supergroup E, which had a common node with the A Group. In the ML tree, the Collembola branch had a significance of $P < 0.001$. In the MP tree, the branch with Collembola-derived *Wolbachia* sequences had a bootstrap value of 94%; the common branch with the supergroup A had a value of 56%. In all trees, the *F. candida* sequences were found in one clade and the Tullbergiidae sequences in the other. The *P. callipygos* sequences (wCalI, wCalII) were more related to each other then to the *M. italica* sequence (wIta), as shown in the distance matrix tree (Fig. 4).

Discussion

Wolbachia live in close relationship to their host organisms and they are not viable outside of them. Their cytoplasmatic transfer from one generation to the next implies that they infect the host germ line by means of maternal inheritance. Therefore, it is typical to find *Wolbachia* cells colonizing the eggs and the ovarial tissues of infected adult arthropods or nematodes. Our analyses with *Wolbachia*-specific gene probes, FISH and microscopy of micro sections of *F. candida* confirmed the lifestyle of a reproduction parasite in Collembola by locating the cells in tissue of the ovaries. In addition, we found *Wolbachia* hybridizing in the brain region of *F. candida*. The brain tissue of *Drosophila melanogaster* (Insecta, Diptera) can also be colonized by *Wolbachia* resulting in a degeneration of the tissue and early death of the host (Min and Benzer, 1997). In *D. melanogaster* these virulent rapidly proliferating forms (*popcorn*) of *Wolbachia* co-exist with non-virulent strains. In *F. candida* we are not sure about the effect that brain tissue-located *Wolbachia* cause because we did not have any *F. candida* free of *Wolbachia* as a negative control. However, the specimens of *F. candida* we worked with did not indicate that there were symptoms of a disease.

Rickettsia-like organisms (RLO) had already been detected in ovary tissues of *F. candida* by transmission electron microscopy (TEM) as early as 1972 (Palévody, 1972) and our findings suggested that these bacteria were in fact *Wolbachia*. In addition, similar bacterial cells were detected by TEM recently in fat bodies and intestinal cells and it was concluded that they also were *Wolbachia* (Vanderkerckhove *et al*., 1999). We also detected, infrequently and at different densities, bacterial cells in the fat body of *F. candida* using FISH. However, the bacteria in fat body and intestinal cells hybridized with the universal

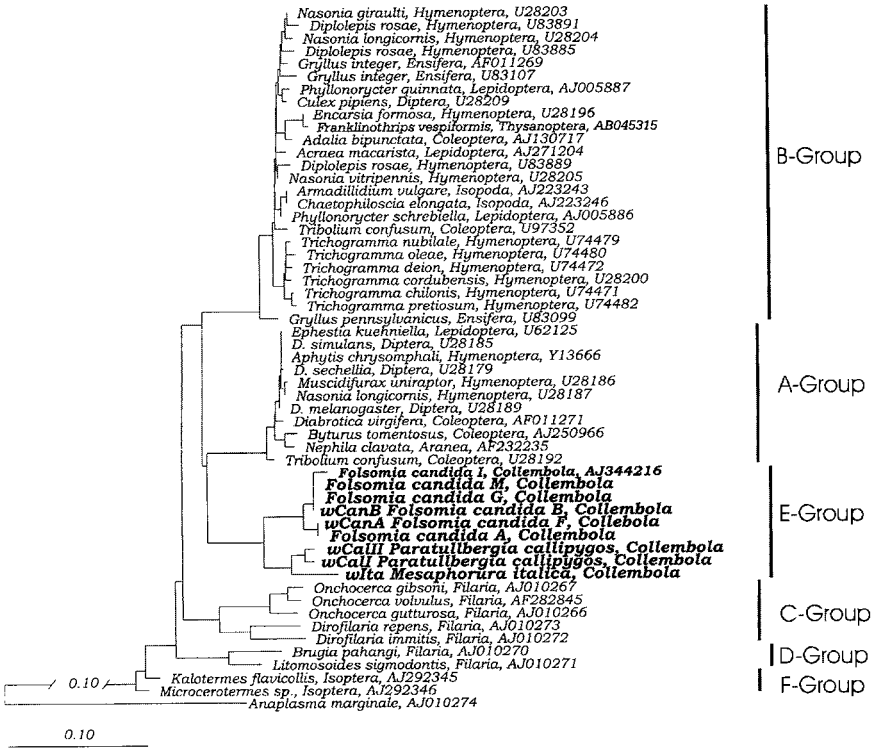

Fig. 4. Phylogenetic tree of *Wolbachia*, for the *ftsZ* gene. The tree was calculated with the Phylip Distance Method. The *ftsZ* gene of *A. marginale* was used as an outgroup. The species names in the tree indicate arthropod or nematode hosts from which the sequences were obtained. The capital letters indicate the origin of the strains from which the sequences were obtained (A, Aachen; B, Braunschweig; F, Brunoy, France; G, Goerlitz; M, East Lansing, Michigan).

bacterial probe but not with the *Wolbachia*-specific one. Because TEM can only identify bacterial cells by means of their cell wall structure and cell shape but not with a phylogenetic marker, we suspect that other RLO than *Wolbachia* were colonizing the fat tissue and the interstitial cells. In fact, in other studies we have detected in *F. candida* a 16S rRNA gene (AF327558) closely related to the Gamma-Proteobacterium *Rickettsiella grylli* which may have accounted for these observations (A.B. Czarnetzki and C.C. Tebbe, in preparation).

The phylogenetic affiliation of *Wolbachia* inhabiting Collembola as a host was, up until this study, based only on two sequences, one 16S rRNA sequence and one *ftsZ* sequence, both retrieved from the same species, *F. candida* (Vanderkerckhove *et al*., 1999; Lo *et al*., 2002). Phylogenetic analyses indicated for both genes that they were only distantly related to other known *Wolbachia* sequences and, thus, they could not be assigned to one of the four well-characterized supergroups. However, a branch which only consists of a single sequence with a low similarity to other sequences is only a weak indicator in regard to its phylogenetic root, because of problems associated with 'long-branch attraction' (Graur and Lee, 2000). In our study, we could add several *ftsZ* and 16S rRNA gene sequences of Collembola and we found that they all contributed to the postulated branch of Lo *et al*. (2002) and Vanderkerckhove *et al*. (1999). Thus, we assume that it is justified to define this branch as a new supergroup E within the *Wolbachia* phylogeny and, based on our results, this supergroup E is specific thus far for Collembola. Lo *et al*. (2002) found that the supergroup A was monophyletic with the *ftsZ* gene but not with the 16S rRNA gene. In contrast, our 16S rRNA analyses also indicated that supergroup A was monophyletic, except for the ML tree. The latter indicated a deep branching supergroup E within the supergroup A. For all inferred trees, the phylogenetic distance of supergroup E to supergroup A was lower than to supergroup B, independent of whether *ftsZ* genes or rRNA genes were analysed. Thus, we conclude that supergroup E is a sister group of supergroup A.

The rRNA genes of *Wolbachia* that were detected in different breeding stocks of *F. candida* from France, the US and Germany were identical to each other and also identical to a sequence previously detected in the same host bred for three years in a Belgium laboratory (Vanderkerckhove *et al*., 1999). Interestingly, the partial 16S rRNA genes of *Wolbachia* that were characterized from the three relatively closely related species from the Tullbergiidae group were also identical. The difference between the 16S rRNA genes of *F. candida* and the Tull-

bergiidae as judged by comparison of partial sequences was 0.84%, indicating that in fact there was a certain degree of agreement between the phylogenetic relation of hosts and *Wolbachia*.

As expected, the *ftsZ* gene was more sensitive in differentiating *Wolbachia* than the 16S rRNA gene (Werren *et al*., 1995). Two *ftsZ* sequences could be differentiated from the breeding stocks of *F. candida* with identical 16S rRNA genes, one from the stocks in Braunschweig, Görlitz (both Germany) and East Lansing (Michigan, U.S) and one from Aachen (Germany) and Brunoy (France). The other known *F. candida ftsZ* sequence from Milan (Italy) was different from both sequences retrieved in our study. The *ftsZ* genes found in the Tullbergiidae group were also different depending on the host. Differences between *M. italica* and *P. callipygos* were greater than between the two *ftsZ* genes of *P. callipygos* or the differences found between the *F. candida* stocks. Thus, in accordance to the data with the 16S rRNA genes, we found that the phylogenetic distance between the *Wolbachia* corresponded to that of the hosts.

Correspondence between the phylogenies of hosts and endosymbionts is indicative of co-evolution. In the extreme case of the *Buchnera*-symbiosis, the co-evolution can be traced back to a single event of infection that happened to a common ancestor 200–250 million years ago (Ma) (Martinez Torres *et al*., 2001; Moran, 2001). For *Wolbachia* in arthropods, the case is more complicated as there is no general congruence between the phylogenetic trees of the hosts and endosymbionts. It was estimated by means of synonymous substitution rates that the *Wolbachia* of the supergroups A and B evolved from a common ancestor 58–67 Ma (Werren *et al*., 1995). Recent analysis indicates that more probably the groups separated later, 35 Ma, and that 52 Ma there was a common ancestor of all *Wolbachia* (including supergroups C and D of filarial nematodes) (Clark *et al*., 1999). During this period of evolution, the arthropods were already highly evolved and differentiated. Collembola are one of the oldest living group of terrestrial arthropods with an age of approximately 380 million years (Whalley and Jarzembowski, 1981). The orders that were analysed in our study, Poduromorpha and Entomobryomorpha, are in accordance with the molecular phylogeny of Collembola (Frati *et al*., 1997; D'Haese, 2002). These groups surely separated before *Wolbachia* radiated. Based on substitution rates suggested by Clark *et al*. (1999) for analysing 16S rRNA genes of endosymbionts, a divergence as we found it for the partial 16S rRNA gene (0.8%), if it would apply for the whole 16S rRNA gene, indicates a common ancestor of *Wolbachia* in supergroup E approximately 15 Ma. Due to the difference between the time of radiation of Collembola and *Wolbachia* from supergroup E we assume that Collembola were not colonized by *Wolbachia* earlier than other arthropods.

Horizontal transmissions of *Wolbachia* are indicated by the diversity of hosts found in the phylogenetic supergroups A and B. In addition, evidence for horizontal transmissions were found at the ecological level (Kittayapong *et al*., 2003). Transfer of *Wolbachia* can happen between predators and prey, as indicated by closely related *Wolbachia* strains in parasitoids and their hosts, e.g. *Nasonia* and its fly host *Sacrophaga* (Werren *et al*., 1995), *Trichogramma* and its host *Ephestia kuehniella* (van Meer and Stouthamer, 1999) or in spiders and insects (Oh *et al*., 2000). For the Collembola forming the putative supergroup E we have no conclusive evidence for such transfer processes yet. Because Collembola serve as prey, e.g. for predatory mites, it would be interesting to screen this group of arthropods more intensively for *Wolbachia* from supergroup E. The relatively close relationship of the *Wolbachia* strains found in *F. candida* and the Tullbergiidae could also mean that, at the evolutionary scale, a recent horizontal transfer between these groups has happened and that further on, as indicated by differences in the *ftsZ* gene, a certain degree of co-evolution took place.

A co-evolution of an endosymbiont and a host more probably indicates beneficial than detrimental effects. One of the beneficial effects that has been described for *Wolbachia* host interactions is the induction of reproduction by parthenogenesis (Koivisto and Braig, 2003). The results of our study suggest that *Wolbachia* infections may lead to parthenogenesis in Collembola: we chose a total of six species from two orders and three families. *Wolbachia* was only detected in the four species with parthenogenesis. It can be argued that we missed *Wolbachia* in the two other, sexually reproducing species due to lacking 16S rRNA gene primer specificities. However, because we picked up *Wolbachia* sequences from two different orders, and within these orders failed to detect them in the sexually reproducing species, we conclude that induction of parthenogenesis is a probable effect. Conclusive evidence for parthenogenesis induction would be provided if *Wolbachia*-infected *F. candida*, *M. macrochaeta*, *M. italica* or *P. callipygos* specimens would revert from parthenogenesis to sexual reproduction after eliminating the bacteria from the host, e.g. by antibiotic treatments. This strategy has successfully been applied to eliminate *Wolbachia* from different insects (Holden *et al*., 1993; Kyei-Poku *et al*., 2003), but our attempts with antibiotic treatments of *F. candida* so far have resulted in the eventual death of the host organisms (data not shown).

Collembola and bacteria have coexisted for approximately 400 million years on our planet and, thus, there has been sufficient time to develop sophisticated and complex interactions. Our study shows that the *Wolbachia*

endosymbiosis in Collembola is in this regard rather young. However, it may turn out that this interaction between a reproduction parasite and an arthropod group that is highly abundant and functionally important in terrestrial ecosystems has ecological consequences of which we are not yet aware.

Experimental procedures

Collembola

In addition to our own breeding stock of *F. candida* (Hoffmann *et al*., 1998; Thimm *et al*., 1998), designated as breeding stock 'Braunschweig', we obtained specimens bred in Aachen (Germany), Brunoy (France), Görlitz (Germany) and East Lansing (MI, USA). Further Collembola included in this study were *Protaphorura fimatus* (formerly: *Onychiurus fimatus*) from Aachen, *Isotoma viridis* from a wild population in Braunschweig, and *Mesaphorura macrochaeta*, *M. italica* and *Paratullbergia callipygos* (all from Brunoy). The specimens of the Tullbergiidae species analysed in this study were all descendants of single females. For the taxonomic affiliation of the Collembola, see Table 1.

Microscopy and fluorescence in situ *hybridization (FISH)*

Adult specimens of *F. candida* were collected during their starvation period within a molting cycle (Thimm *et al*., 1998). The specimens were fixed in formaldehyde, as described elsewhere (Thimm and Tebbe, 2003). The fixed animals were dehydrated by subsequent washes with ethanol at increasing concentrations (Rieder and Schmidt, 1987) and then embedded in paraffin with a melting point of 58°C (Merck). Sections of 7 μm thickness were cut in a rotation microtome (Jung), mounted on poly L-lysine- (Sigma-Aldrich) coated glass slides, dewaxed by subsequent washes with 90%, 60% and 30% (v/v) of ethanol in tap water and finally dried at 37°C. Bacterial cells as controls for gene probe specificities were prepared as described by Amann *et al*. (1990).

For FISH we chose the universal gene probe EUB338 (Amann *et al*., 1990), labelled with the fluorescence dye Cy3 and the oligonucleotide probe W2, described by Heddi *et al*. (1999) originally designed for the detection of *Wolbachia* in weevils (Insecta, Coleoptera, Curculionidae). We found that the W2 probe matched to the corresponding sequence within the 16S rRNA gene of *F. candida* (Vanderkerckhove *et al*., 1999). The W2 probe was labelled with Texas red. Both probes were supplied by MWG Biotech. The hybridizations were conducted as described elsewhere (Heddi *et al*., 1999) with 50 pmol probe ml^{-1} hybridization solution and an overnight incubation at 37°C in a moisture chamber for glass slides. At the end of the hybridization, the glass slides were carefully washed with water, dried at 37°C, embedded in Entellan (Merck) and sealed with coverslips. The sections were analysed with an epifluorescence microscope (Axioplan, Zeiss, Göttingen, Germany) using the triple bandpass filter set at 25, supplied with the microscope. Excitation wavelengths were 400, 495 and 570 nm, respectively, and emission wavelengths were 460, 530 and 610 nm. Photographs were taken with a Contax 137 Quartz camera (Yashica) on Elite Chrome 200 slide film (Eastman Kodak), with a 6 s exposure time.

DNA extraction and PCR

The specimens were either taken directly from breeding stocks or from stocks that were kept in ethanol (96% w/v) for a maximum of 1 or 2 weeks. For each extraction, one to nine specimens were transferred into a 1.5 ml microreaction tube. DNA was extracted using the MN Tissue Kit (Macherey and Nagel). To each reaction tube with the Collembola, a total of 200 μl extraction buffer, provided with the kit, was added and the specimens were crushed with a DNA-free pestle (Biozym). Subsequently, the cells were lysed by a total of four freeze-and-thaw cycles and further processed according to the protocol provided by the manufacturer, Macherey and Nagel. DNA was eluted in 80 μl of Tris-EDTA buffer, included in the kit.

All PCR were performed with a Primus 96 thermal cycler (MWG Biotech). PCR were carried out in microfuge tubes in a final volume of 100 μl, including 10 μl 10× PCR buffer (Invitrogen), 1.5 mM $MgCl_2$, 0.2 mM of each desoxynucleotide triphosphate (Amersham Biosciences) and 0.2 μM of each primer. All primers were obtained from MWG Biotech. Each PCR was started with the addition of 5 U Platinum *Taq* DNA polymerase (Invitrogen).

The primer pair 16SAf and 16SAr was chosen to detect 16S rRNA genes of *Wolbachia*, supergroup A, generating a PCR product of approximately 200 bp. Primer pair ftsZf1 and ftsZr1 amplified a fragment of 1004–1033 bp length of the *ftsZ* gene. Both primer pairs were published by Werren *et al*. (1995). In addition, we designed the reverse primer ftsZcol-r matching to the E-supergroup to amplify a PCR product of 987–988 bp length. The 5′–3′ sequence of the primer was CCTTCACTTCCCTGCTCAG. Temperature conditions for PCR were chosen as described elsewhere (Werren *et al*., 1995), except for PCR with ftsZf1 and ftsZcol-r which were optimized to an annealing temperature of 54°C.

The 16S rRNA genes (approximately 1500 bp) were amplified with the universal primers f27 and r1492, first described by Lane (1991) under the following conditions: 3 min at 94°C, followed by 10 cycles of 15 s at 94°C, 2 min at 55°C, 2 min at 72°C, and 20 cycles with 15 s at 94°C, 2 min at 55°C, 2 min 20 s at 72°C and a final extension of 10 min at 72°C. A total of 2–6 μl of DNA extracted from the Collembola was added as a template.

Single-strand conformation polymorphism (SSCP)

SSCP analyses were conducted with a Macrophor vertical electrophoresis system, as described in more detail elsewhere (Schwieger and Tebbe, 1998). PCR products were purified with the MN Nucleo Spin Extract Kit (Macherey and Nagel) and diluted in a final volume of 40 μl buffer, supplied with the kit. A volume of 4 μl was taken and mixed with two volumes of denaturing loading buffer [95% formamide, 10 mM NaOH, 0.025% bromophenol blue, and 0.025% xylene cyanole (both from Merck)]. Samples were incubated at 95°C for 2 min and then immediately placed on ice for 3 min. Non-denaturing electrophoretic gels were prepared with 7.8 ml of acrylamide solution (MDE, FMS Bioproducts),

14.7 ml of double distilled water, 2.5 ml of 10-fold Tris buffer (Sambrook and Russell, 2001), 10 µl TEMED (N, N, N′, N′-tetraethylenediamine) and 25 µl APS (40% w/v ammonium persulphate). All chemicals, if not otherwise stated, were obtained from Roth. Electrophoreses were carried out at 21°C with 220 V and 8 mA for 14 h. Subsequently the gels were stained with silver, as described by Bassam *et al*. (1991).

DNA sequencing

For sequencing reactions, the PCR products were ligated into the vector pGEM-T (Promega) and transformed into competent cells of *E. coli* JM109 with protocols supplied by the manufacturer (Promega). The partial *Wolbachia*-specific 16S rRNA sequences obtained with primers 16SAf and 16SAr used for SSCP were verified by sequencing two clones of each transformation in both directions. Each *ftsZ* gene amplified in this study was sequenced from at least five clones of each transformation in both directions, using the vector specific primers M13-21 forward and M13-20 reverse. The almost entire 16S rRNA gene was acquired by screening clone libraries of approximately 100 transformed clones with the *Wolbachia*-specific PCR. Three *Wolbachia* clones of each library were sequenced from both sides using the primers mentioned earlier. In addition, the central regions of the cloned fragments were sequenced using the universal primers 530f and 907r (Lane, 1991).

All sequencing reactions were carried out with the Sequitherm Excel II DNA sequencing kit-LC (Epicenter Technologies). Primers labelled at the 5′ end with IRD-41, were synthesized by MWG Biotech. The sequencing was performed in a NEN Global IR2 DNA Sequencer System 4200 L (Li-Cor) and sequences were assembled and finished with the program 'Consed' (Gordon *et al*., 1998).

Sequence alignments and construction of phylogenetic trees

The alignments of both genes, 16S rRNA and *ftsZ*, were performed using the 'ARB′-program package, version July 1999 (http://www.arb-home.de). The primer sequences were deleted prior to the analysis. The 16S rRNA sequences were aligned to the sequences provided in the ARB-database (db_Dez00.arb) which was completed by the incorporation of relevant sequences imported from GenBank (http://www.ncbi.nlm.nih.gov). The alignment included information about the secondary structure of the rRNA molecules. The *ftsZ* gene sequences were aligned with the ARB-program using tools recommended for protein-encoding genes. The sequences were aligned to *ftsZ* sequences imported from GenBank (see above).

The trees were calculated with the tools provided in the ARB-program package. Where possible, we included the sequences of the same hosts for both phylogenetic analyses of the 16S rRNA and *ftsZ* genes respectively. All trees were calculated with a 50% conservation filter generated for each sequence that was included in the tree. In consideration of the fact that many *Wolbachia*-relevant 16S rRNA and *ftsZ* genes in the databases were only represented by partial sequences, we limited the calculation of trees for the 16S rRNA genes to the corresponding *E. coli* positions 86–990. For the *ftsZ* gene we chose the region between position 350 and 1050. Thus, 872 valid sites were included in the 16S rRNA phylogenetic analysis and 600 valid sites in the respective analysis of the *ftsZ* gene.

For both 16S rRNA and *ftsZ* genes we constructed trees based on distance matrix, maximum parsimony and maximum likelihood respectively. Distance matrix trees were calculated according to the method of Fitch and Margoliash (1967) using the Jukes–Cantor correction (Jukes and Cantor, 1969) with one category of substitution rates. The organism *Anaplasma marginale* was used as an outgroup and the tree constructions implied global rearrangements. The maximum parsimony trees were generated with 100 replicates for the 16S rRNA and 1000 replicates for the *ftsZ* genes.

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