Molecular Phylogenetics and Evolution 130 (2019) 406–415

Contents lists available at ScienceDirect

Molecular Phylogenetics and Evolution

journal homepage: www.elsevier.com/locate/ympev

Novel *Cardinium* strains in non-marine ostracod (Crustacea) hosts from natural populations

Isa Schön[a,b,*], Takahiro Kamiya[c], Tijs Van den Berghe[a], Lynn Van den Broecke[a], Koen Martens[a,d]

[a] Royal Belgian Institute of Natural Sciences, OD Nature, Freshwater Biology, Brussels, Belgium
[b] University of Hasselt, Research Group Zoology, Diepenbeek, Belgium
[c] School of Natural System, Kanazawa University, Kakuma, Kanazawa, Japan
[d] University of Ghent, Dept Biology, Ghent, Belgium

ARTICLE INFO

Keywords:
Endosymbiont
Phylogenetics
Metagenomics
16S
Freshwater

ABSTRACT

Endosymbiotic bacteria are known from many metazoan taxa, where they manipulate host biology and reproduction. Here, we used classic PCR amplification and direct DNA sequencing with universal primers for four different endosymbionts to test for their presence in more than 300 specimens of three recent non-marine ostracod superfamilies from different geographic areas and aquatic habitats. We verified these results with "high throughput" amplicon sequencing of 16S of nine selected specimens and evolutionary placement algorithms. The phylogenetic position of endosymbionts detected in ostracod hosts was compared to known endosymbionts from other metazoans. While *Wolbachia*, *Spiroplasma* and *Rickettsia* are absent, we find evidence for the general presence of *Cardinium* bacteria in natural populations of various non-marine ostracod species. Phylogenetic reconstructions based on *Cardinium* 16S data and estimates of genetic distances both indicate that *Cardinium* from ostracods are distantly related to *Cardinium* from Diptera and Nematoda but represent novel strains with a monophyletic origin. *Cardinium* bacteria from different ostracod hosts have genetic distances of up to 3.8%, providing evidence against recent and frequent horizontal transmissions amongst the three ostracod superfamilies. High throughput sequencing reveals more than 400 different 16S amplicon sequence variants in the investigated ostracods as well as the presence of different *Cardinium* strains within individual *Eucypris virens* and *Heterocypris* hosts. These results call for future, more in-depth investigations. Mapping *Cardinium* infections on COI trees of non-marine ostracod hosts shows that the occurrence of these endosymbionts is not linked to genetic species identity or phylogenetic host groups and, except for one ostracod morphospecies, prevalence never reaches 100%.

1. Introduction

Many metazoans and especially terrestrial arthropods (Ma and Schwander, 2017) are known to contain endosymbiotic bacteria (Hilgenboecker et al., 2008; Engelstädter and Hurst, 2009; Zug and Hammerstein, 2012; Zhang et al., 2016). Endosymbiotic infections can have profound effects on the microevolution and reproductive ecology of their hosts (reviewed in Engelstädter and Hurst, 2009; and Ma and Schwander, 2017) causing female-biased sex ratios through male killing and feminization or cytoplasmatic incompatibility (Hurst and McVean, 1996; Moret et al., 2001; Hunter et al., 2003; Gotoh et al., 2007; Engelstädter and Hurst, 2009;) through mating of infected males with uninfected females or with females carrying other bacterial strains. Consequently, cytoplasmic incompatibility can lead to rapid reproductive isolation and speciation (Werren, 1997). Given their cytoplasmic mode of transmission, endosymbionts can also shape patterns of mitochondrial genetic diversity (reviewed in Hurst and Jiggins, 2005) by linking infection patterns with certain haplotypes (Kambhampati et al., 1992) or phylogenetic clades (Sun et al., 2011), or by causing loss of mitochondrial diversity through selective sweeps (see for example Jiggins, 2003; Graham and Wilson, 2012; Jäckel et al., 2013). Investigating such possible links between endosymbiont transmission and mitochondrial diversity is of high importance, given the universal application of mitochondrial DNA sequence data (Hurst and Jiggins, 2005) for, amongst others, DNA barcoding initiatives (Hebert et al., 2003), the detection of cryptic diversity (Fontaneto et al., 2015), reconstructions of population histories (Avise, 1994) or age estimates (e. g. Wilke et al., 2006; Schön and Martens, 2012; Li et al., 2017; Schön et al., 2018). The currently available data on the presence of endosymbionts in invertebrates are biased towards studies in insects,

* Corresponding author at: Royal Belgian Institute of Natural Sciences, OD Nature, Freshwater Biology, Brussels, Belgium.
E-mail address: isa.schoen@naturalsciences.be (I. Schön).

https://doi.org/10.1016/j.ympev.2018.09.008
Received 4 June 2018; Received in revised form 6 September 2018; Accepted 10 September 2018
Available online 21 September 2018
1055-7903/

especially Hymenoptera (Ma and Schwander, 2017) and mites (Zhang et al., 2016), and probably underestimate the real prevalence of these bacteria in other groups (see, for example, Pagès et al., 2017).

Non-marine ostracods are a good model group for evolutionary studies (Martens, 1998), including evolutionary dynamics of endosymbionts and their hosts, given their excellent fossil record, which allows real time estimates of evolutionary and phylogenetic events (see for example Schön and Martens, 2012). The three superfamilies of non-marine ostracods have split from marine ancestors more than 400 myr ago (Martens, 1998). The most diverse group of living non-marine ostracods is the family Cyprididae (Martens, 1998), which comprises half of the more than 2000 known extant non-marine ostracod morphospecies (Martens et al., 2008). The real diversity of this group is probably much higher given the high incidence of cryptic species (Bode et al., 2010; Schön et al., 2012, 2014, 2017, 2018). With the exception of one study, which found no evidence for *Wolbachia* in the non-marine ostracod *Eucypris virens* (Bruvo et al., 2011), very little is known about the presence of endosymbionts in ostracods or other aquatic crustacean groups. Unidentified bacteria have been observed in the ovaries of the non-marine ostracod *Heterocypris incongruens* with transmission electron microscopy (Vandekerckhove, 1998).

Here, we applied classic PCR screening and direct DNA sequencing to both bacterial 16S and host COI regions and (limited) high throughput sequencing of bacterial 16S to verify the results of classic 16S sequencing of non-marine ostracods from all three extant superfamilies and from a wide geographic range. Our aim was to test for the presence of different endosymbionts to address the following questions: (1) Are there bacterial endosymbionts present in non-marine ostracods that are also known from other invertebrates? (2) If endosymbiont bacteria are detected – are they different or closely related to known endosymbionts from other arthropods? (3) How genetically diverse are endosymbionts, if any, among the superfamilies of non-marine ostracods?

Table 1
Prevalence of *Cardinium* in non-marine ostracod species. The number of investigated genetic (= cryptic) species is provided if more than one was present per morphospecies. Data for cryptic species are derived from Bode et al. (2010) for *E. virens*, Schön et al. (in prep.) for *H. incongruens* & *H. spec.*, Cours et al. (in prep.) for *C. vidua*, and Schön et al. (2012) for *D. stevensoni* & *P. brasiliensis*. % infection per category and overall prevalence per morphospecies were calculated for morphospecies with at least nine screened individuals.

Morphospecies - number of screened genetic species	Infected specimens	Not infected specimens	Overall prevalence per morphospecies
Superfamily Cypridoidea			
Eucypris virens - 20	36	93	27.9%
E. pigra	1		
Heterocypris incongruens - 7	51		100%
Heterocypris salina	1		
Heterocypris spec.1	2	5	
Heterocypris spec.2	1	22	4.3%
Tonnacypris lutaria		10	0%
Herpetocypris chevreuxi	4		
Cypridopsis vidua - 2	5	4	55.6%
Cypria ophthalmica		6	
Superfamily Darwinuloidea			
Darwinula stevensoni - 4	10	27	27.0%
Vestalenula paglioli	2		
V. cornelia	2		
V. cylindrica		2	
V. molopoensis		1	
Penthesilenula brasiliensis - 4		5	
P. aotearoa		1	
Microdarwinula nov. spec.		2	
Alicenula inversa		1	
Superfamily Cytheroidea			
Cytherissa lacustris	4		
Cyprideis torosa		12	0%
Romecytheridea ampla		10	0%

2. Material and methods

2.1. Material

Non-marine ostracods were sampled across the world from different aquatic habitats with several sweeps of a hand net (see Table S1A) at different sampling points and stored at 4 °C in absolute EtOH until DNA was extracted from whole individual ostracods with the DNA Blood and Tissue kit (Qiagen), similarly to Bode et al. (2010) and Schön et al. (2012). We analysed 314 ostracod specimens from 22 morphospecies and 35 genetic species (Table 1, Table S1A) belonging to all three non-marine ostracod superfamilies, and originating from different habitat types and geographic regions around the world.

In many non-marine ostracod morphospecies, cryptic genetic species have been detected by the analyses of COI sequence data (Bode et al., 2010; Schön et al., 2012, 2017, 2018), which were also obtained here to identify genetic species within morphospecies (see Table S1A for details on genetic species identity).

2.2. Classic molecular analyses

For a pilot study of 100 specimens of non-marine ostracods, representing all three extant superfamilies, we applied PCR screening and direct DNA sequencing to test for the presence of the four known "reproduction-manipulating" bacteria from arthropods: *Wolbachia*, *Rickettsia*, *Spiroplasma* and *Cardinium*, with the following primers being specific for each genus (except for *Spiroplasma*): (i) WSP81F and WSP691R (Jeaprakash and Hoy, 2000) to amplify a 500–600 bp part of the cell surface "protein coding" gene of *Wolbachia*; (ii) CLO-f1 and CLO-r1 (Gotoh et al., 2007) to selectively amplify ~470 bp of the 16S rRNA of *Cardinium*; (iii) RICS741F and RICT1197R, which amplify a ~448 bp part of the gltA gene from *Rickettsia* (Davis et al., 1998) and (iv) SP-ITS-J04 and SP-ITS-N55 (Majerus et al., 1999) to selectively amplify the spacer region between 16S and 23S rRNA of *Spiroplasma ixodetis*. In all PCR amplifications, a negative control consisting of pure water from the HotStarTaq Master Mix kit (Qiagen) and a positive control of DNA from infected dwarf spiders *Oedothorax gibbosus* for *Wolbachia*, *Rickettsia* and *Cardinium* (Vanthournout et al., 2011) were included, while no positive control was available for *Spiroplasma*. After the pilot study, we conducted PCR amplifications and DNA sequencing of part of the 16S rRNA of *Cardinium* from an additional 214 ostracod specimens (Table 1 & Table S1A), including more non-marine ostracod morpho- and genetic species. We combined different 16S & CLO primers as in Duron et al. (2008) and Gotoh et al. (2007) to obtain bacterial 16S sequences for phylogenetic analyses that are homologous to existing 16S *Cardinium* sequences from other invertebrates. We applied three different combinations of 16S primers to amplify the 5' part (comprising V1-V2), the 3′ end (comprising V6-V9) and almost the entire 16S region of *Cardinium*, respectively, from non-marine ostracods (see Table S1A & S2 for details). Because of the limited amount of DNA from individual ostracods and the fact that we also analysed the COI gene of non-marine ostracods for phylogenetic analyses of the hosts, not all specimens could be analysed for four different endosymbionts and the various primer combinations for amplifying 16S of *Cardinium*.

PCR conditions for all 16S rRNA regions included: (1) an initial denaturation at 95 °C for 15 min; (2) 34 cycles of: denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 90 sec and (3) a final extension at 72 °C for 10 min. PCRs were performed in 25 µl volumes with the HotStarTaq Master Mix (1.5 mM $MgCl_2$ and 200 µM each dNTP), 10 pmol of each primer and 5 µl DNA (comprising 20–50 ng) in a Biometra Thermal Cycler (Westburg). Electrophoresis of the amplicons was performed on 1.5% agarose gels, followed by staining with GELRED and photographing under UV fluorescence. A random selection of PCR products was purified with the illustra™ GFX™

PCR DNA and Gel Band Purification Kit (HE Healthcare), and sequenced directly on an ABI 3130X in both directions using the PCR primers and the BigDye Terminator Sequencing mix (ABI) following the manufacturer's protocol. Sequence chromatograms were visualized with Bioedit, the Forward and Reverse strands for each sequence aligned with ClustalX (Larkin et al., 2007), and all ambiguities checked and corrected manually. Identity of the obtained bacterial sequences was confirmed by BLASTN searches (Altschul et al., 1997).

To identify the phylogenetic position of endosymbiotic bacteria from ostracods in a wider phylogenetic framework, we identified their closest relatives with BLASTN and also searched for additional sequence data from all population sets of 16S *Cardinium* sequences available to date at the ncbi website (https://www.ncbi.nlm.nih.gov/) to be included in the analyses. Table S1B provides an overview of all non-ostracod GenBank sequences of *Cardinium* that were used. The majority of the 16S rRNA sequences from GenBank comes from the 3′ end of this region, which is why this was also our focus for obtaining DNA sequence data from non-marine ostracod hosts. We additionally assembled long sequences of 16S rRNA of *Cardinium* for three non-marine ostracods (representing three morphospecies) from three individual PCRs (Table S2), comprising almost the entire 16S region (see Table S1A). For phylogenetic reconstructions, sequences were aligned with MAFFT (Katoh and Standley, 2013) on the cbrc server (https://mafft.cbrc.jp/alignment/server/). Alignments contained either only the 3′ part of the 16S region of *Cardinium*, more than 1200 basepairs (bp) of 16S from *Cardinium* or the entire 16S region from various bacteria, also including *Cardinium* (see Table S1A & B). We conducted phylogenetic analyses with and without several different outgroups (not shown). We subsequently identified the most suitable model with jModeltest 2.1.4 (Darriba et al., 2012) from all 88 models for Maximum Likelihood analyses and 24 models for Bayesian Inference. We then constructed phylogenetic trees with Bayesian Inference in Mr Bayes 3.2.0 (Ronquist et al., 2012) with 20 million generations, sampling every 100th generation and the parameters identified by jModeltest until average standard deviations of split frequencies were lower than 0.01 and the Potential Scale Reduction Factor had converged to one. We also applied the Maximum-Likelihood method in PhyML (Guindon and Gascuel, 2003) to construct phylogenetic trees with 1000 bootstrap replicates and the parameters of jModeltest.

Additionally, Bayes Factor tests (Kass and Raftery, 1995) were applied in MrBayes to check for monophyly of *Cardinium* from ostracod and other hosts with 6 million generations and two chains constraining phylogenetic groups of different *Cardinium* hosts. The means of the marginal likelihood estimates were compared between Bayesian phylogenies with and without these hard constraints in MrBayes 3.2. We also calculated mean genetic distances among *Cardinium* from different hosts in MEGA 6.0 (Tamura et al., 2013) using the model being closest to the one identified with jModeltest. For reconstructing the evolutionary history of non-marine ostracod hosts and their endosymbionts, we complemented existing mitochondrial COI DNA sequence data (Bode et al., 2010; Schön et al., 2012) with additional sequences (see Table S1A for more details) and similar methods as in these publications. The obtained COI sequences were used in Bayesian approaches to reconstruct phylogenetic trees, on which bacterial infections could be mapped.

2.3. High through put amplicon sequencing of bacterial 16S

To test for potential amplification bias of the PCR primers used above that could influence infection patterns, we also sequenced part of the bacterial 16S region with high throughput sequencing techniques. From nine DNA extractions that were used for the classic molecular screening (see above & Table S1A), we also conducted PCRs using the universal primers of Vanthournout and Hendrickx (2015) for amplifying approximately 502 basepairs (bp) of the V3-V4 region of bacterial 16S rRNA, including 22 bp tags for subsequent sample indexing (Forward primer CTTTCCCTACACGACGCTCTTCCGATCTACTCCTACGGRAGGCAGCAG; reverse primer CTGGAGTTCAGACGTGTGCTCTTCCGATCTACNVGGGTATCTAATCC). Amplifications were conducted using the HotStar Mastermix in 25 µl volumes as described above with the following conditions: 15 min at 95 °C, followed by 44 cycles with 50 sec at 95 °C, 50 sec at 52 °C, and 50 sec at 72 °C plus a terminal incubation step of 10 min at 72 °C. Amplicons were purified with AMPure XP beads, followed by an index PCR and a second purification step, and libraries were paired-end sequenced on an Illumina MiSeq running in 2x300 mode. Sequences were demultiplexed and analysed with DADA2 (Callahan et al., 2016) on the Nephele platform (Office of Cyber Infrastructure and Computational Biology (OCICB), National Institute of Allergy and Infectious Diseases (NIAID); Nephele; http://nephele.niaid.nih.gov, 2016). Barcodes and primers were trimmed, and DADA2 analyses were conducted with different parameters for filtering (see Table S3). Dereplication and denoising of sequences was followed by merging of paired reads, and removing of chimeras. Bacteria were classified from the obtained amplicon sequence variants (ASVs) by 99% comparisons to the SILVA database v.132. Results were visualized with phyloseq (McMurdie and Holmes, 2013) and ggplot2 (Wickham, 2016) in R studio. Sequences and their metadata have been submitted to the SRA of GenBank, accession number PRJNA491252.

2.4. Evolutionary placement of short 16S sequence data

We applied the Evolutionary Placement Algorithm (EPA; Berger et al., 2011) of the Randomized Axelerated Maximum Likelihood (RAxML; Stamatakis, 2014) method to map all shorter 16S sequences of bacteria in ostracod hosts from both classic and high through put techniques on a bacterial 16S reference tree. To find suitable long 16S reference sequences besides *Cardinium* (see above) for this reference tree, we submitted all 16S bacterial ASVs from our DADA2 analyses to the Alignment, Classification and Tree Service (ACTS) on the silva website (https://www.arb-silva.de/aligner/) using all available databases and RAxML for tree construction online. Non-*Cardinium* bacteria were located in the ACTS tree and their nearest neighbours checked manually. Because the focus of this study is on *Cardinium*, we selected one representative of each non-*Cardinium* phylogenetic clade with taxonomic identification, downloaded these 13 bacterial 16S sequence data from the ncbi website (see Table S1B for more details) and aligned these sequences together with the long 16S *Cardinium* sequences from classic analyses (see above and Table S1B) in MAFFT. The 16S bacterial reference tree was constructed in RAxML-Workbench for Windows 1.0 with RAxML v.7.2.8 and the GTRG model. Subsequently, the short 5′ and 3′ 16S *Cardinium* sequences from ostracods (Table S1A) and all high throughput 16S sequences (Table S1A) were separately mapped with EPA and single gene alignment in RAxML-Workbench for Windows 1.0 to the 16S reference tree with heuristic searches. Results were visualized with a modified version of Archaeopteryx (Han and Zmasek, 2009) in RAxML-Workbench for Windows, and the best values for the Expected Distance between Placement Locations (EDPL; Matsen et al., 2010) and RAxML weights (Berger et al., 2011) selected for each mapped 16S sequence.

3. Results

3.1. Presence of endosymbionts in non-marine ostracods

From the high-throughput 16S amplicon sequencing, we obtained a total of 784,479 reads. After testing different parameters for the filtering and trimming in DADA2, the parameters with the best equilibrium between the number of reads and quality were identified as maximum expected errors of 4, a maximum number of mismatches in the overlapping area of paired reads of 3 and a truncation quality score of 2 (Table S3). With these parameters, 328,777 reads remained, resulting in 30,092 paired reads, an average of 21,750 reads per sample,

Table 2
Abundances of Candidatus *Cardinium* from high throughput sequencing of 16S amplicons. The identity of the amplicon sequence variants (ASVs) classified with DADA2 was verified by mapping all 16S sequences from high throughput sequencing onto the reference 16S phylogeny (Fig. S2A) with EPA. For detailed results of the EPA analyses, see Table S4A. Ds = *Darwinula stevensoni*; Cv = *Cypridopsis vidua*; Ev = *Eucypris virens;* Hi = *Heterocypris incongruens*. See Table S1A for more details on the investigated specimens and Table S4A on details of all classified ASVs. yes = *Cardinium* present according to classic PCR screening. no = *Cardinium* absent according to classic PCR screening. See also Fig. S1B for the presence of *Cardinium* among the top 20 ASVs in ostracod hosts.

Morphospecies	Sample ID	absolute number of ASVs classified as *Cardinium*	Result classic PCR
Darwinula stevensoni	Ds.no	0	Negative
	Ds.yes	2846	Positive
Heterocypris incongruens	Hi.yes1	25,889	Positive
H. salina	Hi.yes2	3914	Positive
H. nov. spec. 1	Hi.no	0	Negative
Cypridopsis vidua	Cv.yes	4490	Positive
Eucypris virens	Ev.no1	0	Negative
	Ev.yes	25,720	Positive
	Ev.no2	0	Negative

and 417 bacterial ASVs (see Table S4A). Applying EPA confirmed the identities of all 16S bacterial sequences as *Cardinium* or non-*Cardinium*, respectively, from the original ASVs classifications with DADA2 (see Table S4A). The cumulative bar charts (Fig. S1A) show that Bacteriodetes and Proteobacteria are most common bacteria in non-marine ostracods. *Cardinium* was found among the 20 top ASVs (Fig. S1B), but only in those ostracods in which we also obtained amplicons for this endosymbiont with classic PCR techniques (Table 2). Neither *Wolbachia* nor *Rickettsia* or *Spiroplasma* were detected with either classic genetic analyses (Table S1A) or high-throughput sequencing of 16S amplicons in any of the screened ostracods, and the absence of other endosymbionts is further confirmed by mapping with EPA (see Table S4A & B).

Cardinium is found in 12 out of 22 morphospecies of ostracods from all three non-marine ostracod superfamilies (Table 1; Table S1A).

3.2. The relationship of Cardinium from ostracods with Cardinium from other hosts

All primer combinations for amplifying parts of the 16S rRNA region of *Cardinium* yielded amplicons with very high amplification rates. For phylogenetic analyses and estimates of genetic distances, we mainly used unique ostracod sequences, comprising 16 sequences from the 5′ end of *Cardinium* 16S (376 basepairs), which were only used for initial analyses and for the EPA mapping, 37 sequences from the 3′ end (403 bp) and three sequences of almost the entire 16S region (1276 bp; see Table S1A & S2 for more details). *Cardinium* from an oribatid mite (see Table S1B for more details) turned out to be the most suitable outgroup while 16S sequences of *Cardinium* from for example Amoeba were very distant (not shown) and less suitable as outgroups.

Our phylogenetic reconstructions show that *Cardinium* from ostracods is monophyletic and clusters separately from *Cardinium* strains known to occur in other arthropods and nematodes with high posterior probabilities (Fig. 1 A) and bootstrap support (Fig. S3A & B). Because of low statistical support, the closest phylogenetic group to ostracods can currently not be identified with certainty; however, *Cardinium* from Diptera with aquatic larvae and from a terrestrial nematode form a well-supported phylogenetic clade together with *Cardinium* from ostracods (Fig. 1A & S3A). *Cardinium* bacteria from other hosts do not cluster according to the taxonomic identity of these hosts (Fig. 1A); thus, phylogenetically different *Cardinium* strains can occur in the same host. Although the Bayesian tree based on the shorter 3′ end of 16S rRNA is to some extent unresolved (Fig. 1B), *Cardinium* from ostracods again forms a monophyletic group by itself, and clusters with endosymbionts from Diptera and a nematode (Fig. 1B & S3B).

Bayes Factor tests on phylogenetic topologies from both 16S rRNA trees strongly support three monophyletic groups of *Cardinium*: (1) from ostracod hosts, (2) from ostracod hosts and Diptera, and (3) from ostracod hosts, Diptera and a nematode (Table 3).

Matching the topologies of the two 16S trees (Fig. 1A & B & S3A & B), the *Cardinium* sequences found in the screened non-marine ostracods appear to be genetically different from known *Cardinium* from other invertebrate hosts with mean genetic distances of 5% to 7% (DNA sequence data based on full length of 16S; Fig. 2A), respectively, and 4.5–7.2% when analysing sequence data from the shorter part of the 3′ end of 16S (Fig. 2B). Also the phylogenetic mapping of 16S sequences with EPA confirms that *Cardinium* from ostracods are novel strains. *Cardinium* from ostracods clearly differ from *Cardinium* in the copepod *Nitroca spinipes*, the only other known aquatic crustacean host (Edlund et al., 2012) as is obvious from phylogenetic reconstructions, the EPA mapping and the large genetic distance (Fig.s 1A & 2A and Table S4A).

The EPA results fully support the identity of the shorter *Cardinium* 16S sequences obtained with classic and high throughput sequencing from non-marine ostracod hosts: all ASVs not classified as *Cardinium* are correctly mapped on non-*Cardinium* branches in the reference tree (Fig. S2A & Table S4A). Likewise, all ASVs classified as *Cardinium* map to the ostracod branches in the 16S RAxML reference tree with one exception (Fig. 2A; Table S4A): *Cardinium* from *Cypridopsis vidua* is placed on an internal dipteran branch.

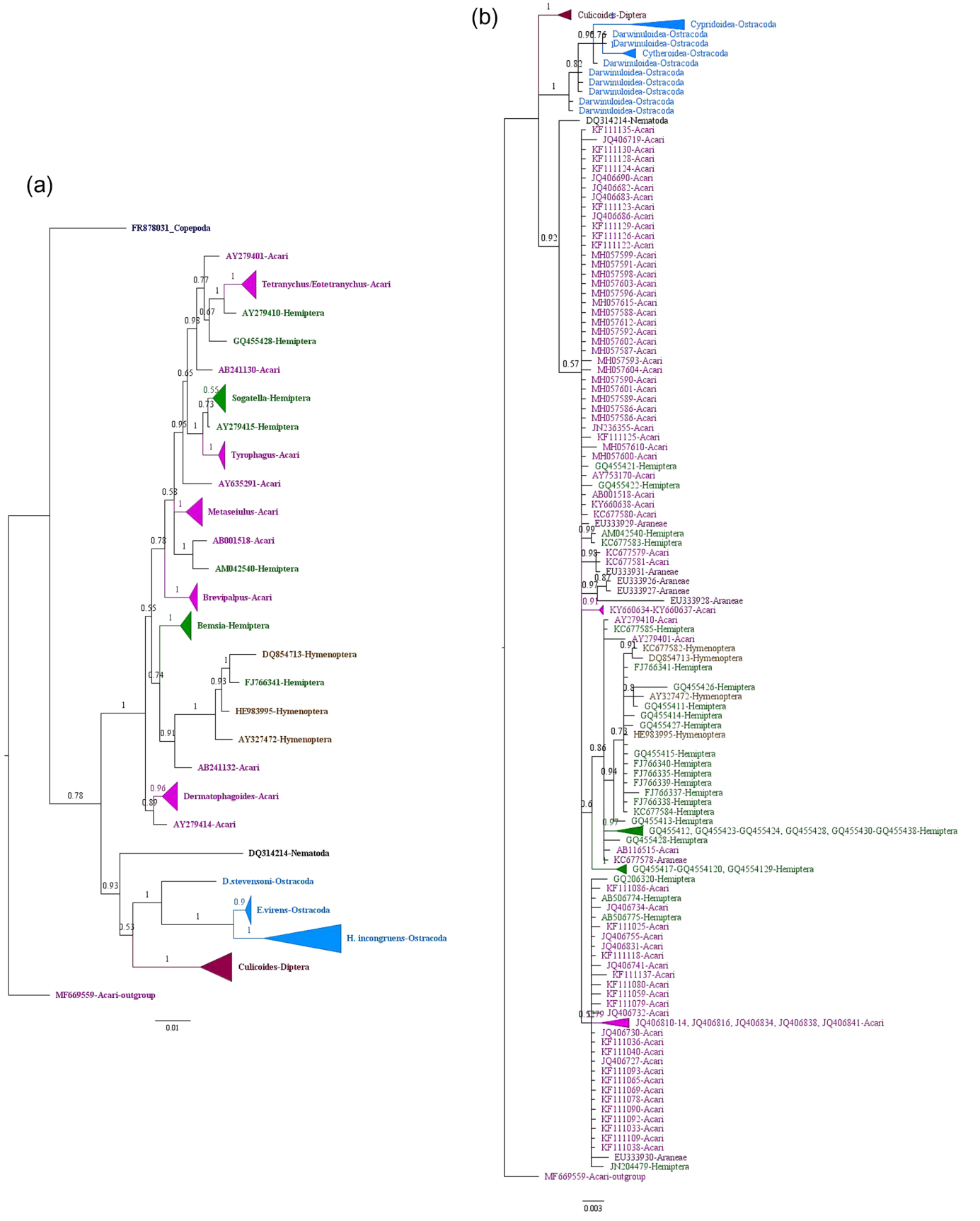
3.3. Genetic diversity of Cardinium in non-marine ostracods

The total prevalence of *Cardinium* in all investigated ostracods is 37.8%. *Cardinium* appears to have a patchy distribution within each of the non-marine ostracod superfamilies (Table 1). Its presence is confirmed in most investigated species of the Cypridoidea, in several species of the Darwinuloidea and in one species of the Cytheroidea (Table 1).

If we assess the presence of *Cardinium* in ostracod morphospecies with at least nine screened specimens (Table 1), prevalence ranges from 4.3% (*Heterocypris* spec. 2), 27% (*D. stevensoni*), and 28% (*Eucypris virens*) up to 55.6% (*Cypridopsis vidua*), respectively (Table 1), but reaches 100% only in one morphospecies, *Heterocypris incongruens*.

A monophyletic origin of *Cardinium* from non-marine ostracods *per se* and also from the superfamily Cypridoidea is supported by the 16S tree topologies (Fig. 1A & S3A) and Bayes factor tests (Table 3). The monophyly of *Cardinium* from darwinulid ostracod hosts is less clear from the phylogenetic clustering (Fig. 1B & S3B) but still supported by Bayes Factor tests (Table 3). Among non-marine ostracod hosts, 16S of *Cardinium* has an average genetic distance of 3.8% (entire 16S region; Fig. 2A), exceeding estimates of genetic distances for *Cardinium* from different insects or Acari. The 3′ end of 16S of *Cardinium* shows with 1.7% lower genetic distances among non-marine ostracods, and less within the three superfamilies of non-marine ostracods (0.2–0.9%; Fig. 2B).

The high throughput sequencing method detected a much higher diversity of *Cardinium* within individual ostracod hosts than could have been expected from classic PCR techniques and direct DNA sequencing, with a maximum of 40 different *Cardinium* ASVs in *E. virens* and 28 in *Heterocypris*, respectively, with varying abundances (Table S4A). When comparing the outcome of the EPA mapping with ostracod host identity, 74 of the 75 sequence variants classified as *Cardinium* with DADA2 map to internal or external *Cardinium* branches of ostracods in the 16S bacterial reference tree (Fig. S2A & B), and the majority of these ASVs furthermore map to the branches of ostracod hosts from the same superfamily. There is some incongruence of mapping at the species level, especially for ASVs from *H. incongruens* and *E. virens* (Table S4A). When placing the short 5′ and 3′ 16S *Cardinium* sequences of ostracod hosts

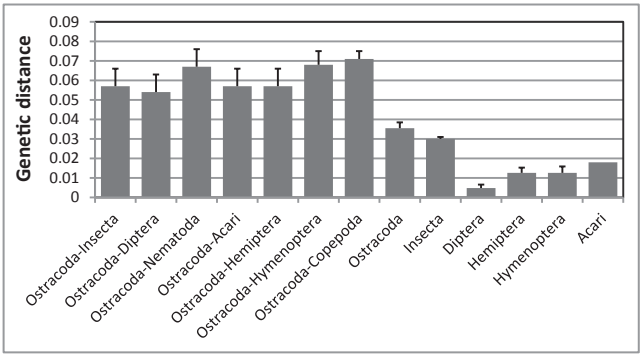


(caption on next page)

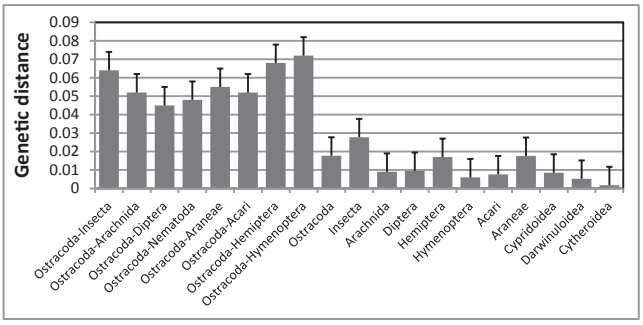
Fig. 1. 16S tree of *Cardinium* from non-marine ostracod hosts and other metazoan hosts. Both trees were constructed with MrBayes and the GTR + I + G model; numbers above branches are posterior probabilities. The matching Maximum Likelihood trees are shown in Figs. S3A &B. See Tables S1A & 1B for more details on 16S sequence data. Sequences of each statistically supported, phylogenetic group are collapsed. The height of each triangle is proportional to the number of sequences in this group. Host species are colour-coded: dark red = Diptera; brown = Hymenoptera; green = Hemiptera; pink = Acari; purple = Araneae; dark blue = Copepoda; lighter blue = Ostracoda; black = Nematoda. A. This Bayesian tree was constructed from an alignment of 1276 basepairs (bp) of 16S rRNA sequences of *Cardinium*. B. This Bayesian tree was constructed with partial 16S rRNA sequences of *Cardinium* from the 3′ end, for which most sequence data were available from GenBank. See also Fig. S4 for details of this tree illustrating the phylogenetic relationships of *Cardinium* within each non-marine ostracod superfamily. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Results of the topology tests with Bayes Factor tests for constrained or unconstrained phylogenetic groupings in the 16S phylogeny of *Cardinium* from various metazoan hosts (Fig. 1A & B). Hard constraints were defined in MrBayes as indicated in the first column and runs of 6 million generations conducted with the stepping stone method. The marginal likelihood estimates (MFE) of the model (M) with the hard constraint (monophyletic group) and with the negative constraint (no monophyletic group), respectively, were compared. According to Kass and Raftery (1995), any Bayes Factor (BF) between 3 and 5 is strong evidence that the model with the higher MFE is the better one, a BF > 5 indicates very strong evidence. The model with the higher MFE of each comparison is indicated in bold, so is any BF > 3. The different models were ranked according to MFE of the accepted model, starting with the highest.

Phylogenetic group	Dataset	MFE constraint M0	MFE unconstrained M1	Rank of model	ln BF	BF
Ostracoda-Diptera-Nematoda	Long 16S rRNA	**−5817.26**	−5877.33	2	60.07	**4.10**
Ostracoda- Diptera	Long 16S rRNA	**−5809.59**	−5880.50	1	70.91	**4.26**
Ostracoda	Long 16S rRNA	**−5860.80**	−5889.07	3	28.32	**3.34**
Ostracoda-Diptera- Nematoda	3′ of 16S rRNA	**−2133.07**	−2239.32	2	106.25	**4.67**
Ostracoda- Diptera	3′ of 16S rRNA	**−2131.78**	−2244.90	1	113.12	**4.73**
Ostracoda	3′ of 16S rRNA	**−2143.70**	−2255.91	3	112.21	**4.72**
Darwinulidae	3′ of 16S rRNA	**−2213.25**	−2244.87	5	31.62	**3.45**
Cypridoidea	3′ of 16S rRNA	**−2159.65**	−2255.74	4	96.09	**4.57**



A.



B.

Fig. 2. Average genetic distances of *Cardinium* within and between ostracod and other metazoan hosts. Average and standard deviation of genetic distances were calculated as TrN and with a gamma parameter of 1.67 in MEGA. For details on the identity of the DNA sequences, see Tables S1A & B. A. Estimates were based on 16S rRNA DNA sequence data of 1276 bp. B. Estimates were based on DNA sequence data from the 3′ end of 16S rRNA of 403 bp.

that were obtained with direct DNA sequencing to the bacterial 16S reference tree, all sequences map to ostracod internal or external branches, and more than 80% to external branches from the same superfamily (Table S4B).

The patchy distribution of *Cardinium* among non-marine ostracods is obvious from constructing COI gene trees of their hosts and mapping infections onto these trees. We do not observe any obvious phylogenetic grouping of infected hosts among the different morpho- and genetic species of the Darwinulidae (Fig. 3) or genetic species identity within the *E. virens* (Fig. 4), *Heterocypris* and *Cypridopsis vidua* (Fig. 5) species complexes.

4. Discussion

4.1. Presence of endosymbionts in non-marine ostracods

Our study confirms the results of Bruvo et al. (2011) on the absence of *Wolbachia* in *E. virens*. *Wolbachia* is absent in all species of non-marine ostracods screened here although these bacteria are known from terrestrial crustaceans such as Isopoda (Verne et al., 2012). We also found no evidence for *Spiroplasma* or *Rickettsia* in ostracod hosts with classic PCR techniques or high throughput sequencing of 16S amplicons with general primers (Table 1, Table 2, Table S1 & S4A; Figs. S1A & B). Our study thus illustrates that only one group of endosymbionts is present in non-marine ostracods, namely *Cardinium*. This is the first report on the presence of *Cardinium* in natural crustacean populations in general and from non-marine ostracods in particular. *Cardinium* in crustaceans has previously only been detected in a laboratory culture of a copepod following antibiotic treatment (Edlund et al., 2012).

4.2. Relationship of Cardinium from ostracods with Cardinium from other hosts - novel strains of Cardinium in non-marine ostracods

Phylogenetic reconstructions with different methods (Fig. 1A & B, S3A & B) estimates of genetic distances (Fig. 2A & B), topology tests (Table 3) and EPA mapping (Figs. S2A & B; Tables S4A & B) all provide evidence that *Cardinium* from non-marine ostracods represent novel, monophyletic endosymbiotic strains, as they are genetically and phylogenetically different from known *Cardinium* of other metazoan hosts, including those from a copepod (Edlund et al., 2012), the only other known aquatic crustacean host. Tree topologies and Bayes Factor tests show that *Cardinium* from non-marine ostracods is closest to *Cardinium* from aquatic larvae of midges (Diptera; Nakamura et al., 2009; Pagès et al., 2017) and also a terrestrial nematode. Using DNA sequence data

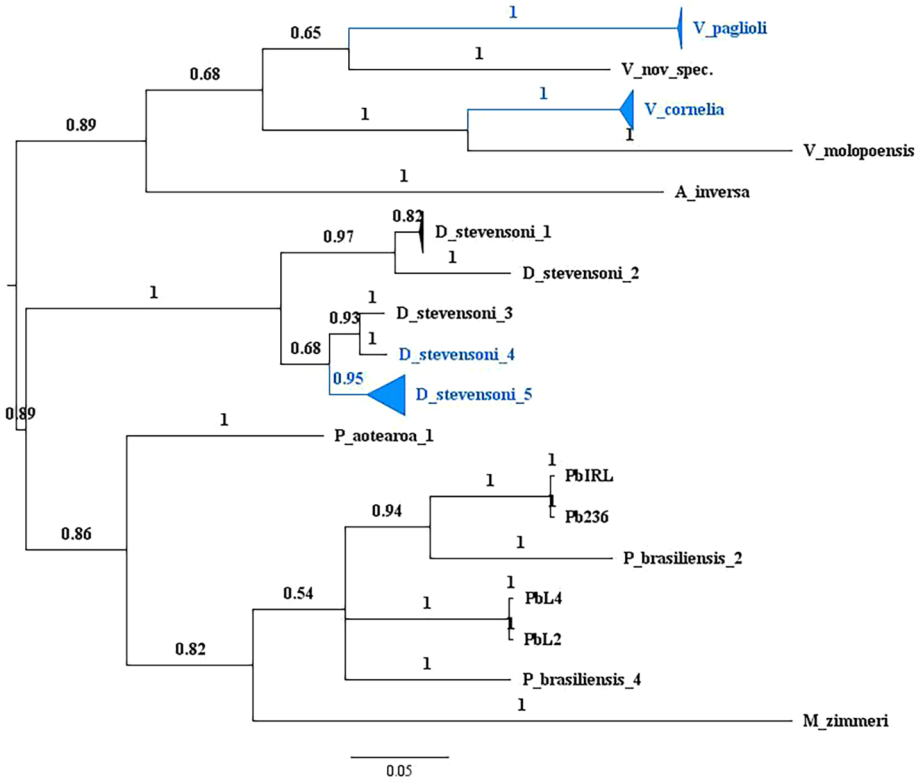


Fig. 3. COI tree of darwinulid ostracod hosts. This gene tree was constructed with MrBayes and the GTR + I + G model. Numbers above nodes are posterior probabilities of Bayesian analyses. Genetic (cryptic) species identity according to Schön et al. (2012) are indicated by Arabic numbers after species names. Individuals and morpho- and genetic species infected with *Cardinium* are indicated in blue (see Table S1A for more details).

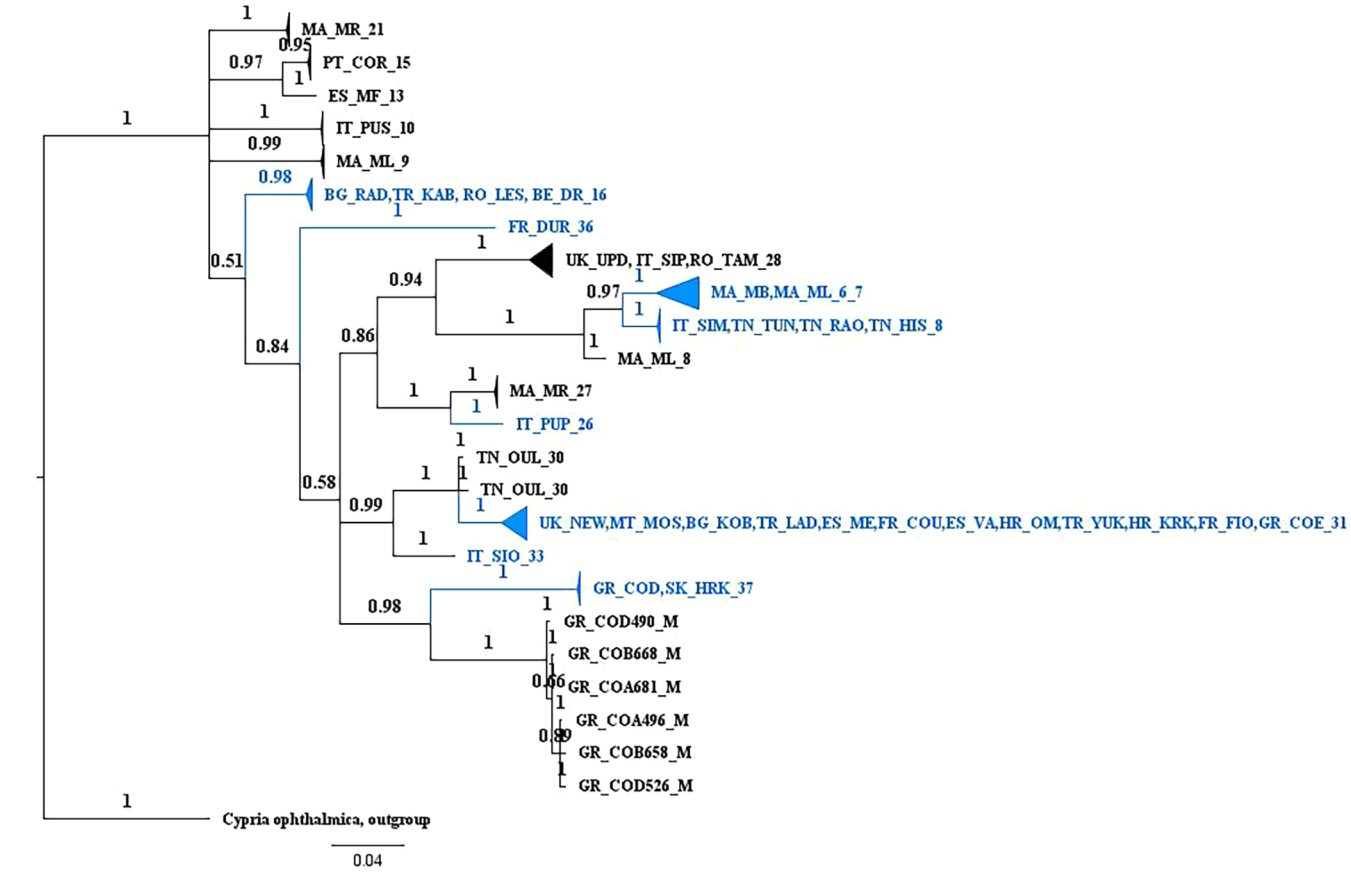


Fig. 4. COI tree of pertinent genetic species in the *Eucypris virens* species complex belonging to the Cypridoidea. This gene tree was constructed with MrBayes and the GTR + I + G model. Numbers above nodes are posterior probabilities of Bayesian analyses. Genetic (cryptic) species identity according to Bode et al. (2010) are indicated by Arabic numbers after population codes. Individuals infected with *Cardinium* are indicated in blue. See Table S1A for more details on the screened specimens. The ostracod *Cypria ophthalmica* is used as outgroup.

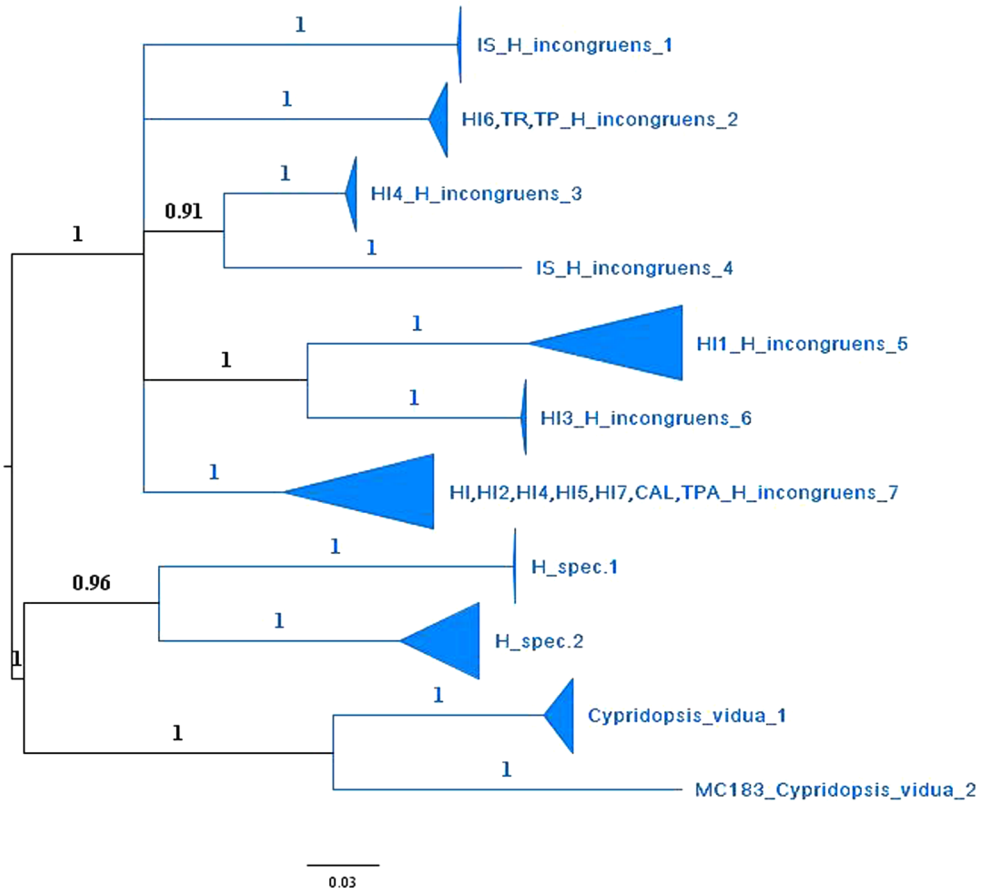


Fig. 5. COI tree of pertinent genetic species in the *Heterocypris* and *Cypridopsis vidua* species complexes belonging to the Cypridoidea. This gene tree was constructed with MrBayes and the GTR + I + G model and midpoint rooted. Numbers above nodes are posterior probabilities of Bayesian analyses. Genetic (cryptic) species identity for *Heterocypris* and for *C. vidua* are presented as Arabic numbers after species names. Individuals infected with *Cardinium* are indicated in blue (see Table S1A for more details).

of the less variable 3′ end of 16S rRNA of *Cardinium*, the genetic distance between these bacteria from non-marine ostracods and other invertebrate hosts amounts to at least 4.5% (Fig. 2B), and more than 5% with the longer and more variable 16S rRNA dataset (Fig. 2A), countering the possibility of recent horizontal transmissions between ostracod and non-ostracod hosts, even if evolutionary rates of bacterial 16S rRNA can vary between 0.025 and 0.091% per myr (Kuo and Ochman, 2009).

Also the EPA results support the absence of recent horizontally transmitted *Cardinium* between ostracods and other hosts, given that we found only one ASV out of 417 (0.02%) where *Cardinium* sequences from *Cypridopsis vidua* are placed on an internal branch among dipteran hosts fitting the phylogenetic relationships of *Cardinium* from ostracods and Diptera described above.

4.3. Genetic diversity of Cardinium in non-marine ostracods

If *Cardinium* infections in non-marine ostracods are ancient, then this would imply that most *Cardinium* strains have split off many millions of years ago within their ostracod hosts (Martens, 1998). The observed genetic distances between *Cardinium* strains of different non-marine ostracod superfamilies (Fig. 2A & B) exceed genetic distances among other *Cardinium* hosts, and match the proposed old age of both initial infections (of several dozen millions of years, calculated from genetic distances between *Cardinium* from different ostracod hosts) and the ostracod hosts themselves (of several hundred million years from the fossil record - Martens, 1998).

EPA mapping confirms not only the identity of the short 16S *Cardinium* sequences from both direct and high throughput sequencing as *Cardinium* from ostracod hosts, but also matches the host identity at the ostracod superfamily and family level for the majority of sequences. The divergence of *Cardinium* within non-marine ostracods is much higher than could be expected from initial PCR amplification and direct sequencing. This is most remarkable for *E. virens* and *H. incongruens*, with 40 and 28 different *Cardinium* strains each in a single ostracod host (Table S4A). It is therefore not surprising that some of the shorter 16S sequences map to other ostracod host species in the reference tree (Table S4A & B).

Here, we used high throughput sequencing techniques only to confirm the results from classic PCR screening regarding the presence or absence of *Cardinium*. However, our results from a limited number of specimens already illustrate the potential of high throughput sequencing methods for future studies on bacterial diversity in Ostracoda. To conclusively confirm poly- or monophyly of *Cardinium* within the different superfamilies of ostracods additional DNA sequence data of *Cardinium* from other non-marine ostracod hosts from longer, more variable 16S regions (Fuks et al., 2018) or other markers than 16S (Zhang et al., 2016) will be required to construct better resolved phylogenies and verify the results of EPA within ostracod superfamilies.

When mapping infections on mitochondrial host trees, we do not find any obvious link between phylogenetic groupings and presence or absence of *Cardinium* infections in either the Darwinulidae (Fig. 3) nor in the Cyprididae (Figs. 4 and 5) similarly to the results of von der Schulenburg et al. (2002) on lady birds, while others observed such relationships in hemipterans and pill bugs, respectively (Gueguen et al., 2010; Verne et al., 2012). This implies that *Cardinium* infections in the investigated non-marine ostracods have not caused selective sweeps nor are they in any obvious way correlated to the high cryptic genetic diversity in for example *E. virens* (Bode et al., 2010).

With the exception of *H. incongruens*, none of the non-marine ostracod species screened here show complete prevalence of *Cardinium* (Table 1). Other studies found similar incomplete infection patterns of *Cardinium* in mites (Ros and Breeuwer, 2009) and spiders (Stefanini and Duron, 2012) and described them as a state of polymorphic equilibrium

between parasites and hosts. Several mechanisms could have caused the absence of complete prevalence of *Cardinium* in most non-marine ostracod species, but not all of these are equally likely. We might have underestimated the prevalence of *Cardinium* in species with known low population densities (e.g. the Darwinulidae *Vestalenula molopoensis*, *V. cylindrica*, *V. paglioli*, or *Alicenula inversa*), but this does not apply to all investigated ostracod species (Table 1; Table S1A). We can largely rule out technical difficulties, given that results on *Cardinium* presence/absence were totally congruent between classic, PCR-based and novel 16S high-throughput amplicon sequencing techniques (Figs. S2A & B; Tables 2 & S4A & B), while also using different primers (Table S2). Likewise, genetic distances between *Cardinium* strains from different ostracod hosts (Fig. 2A & B) and the genetic variability of the hosts themselves (Figs. 3–5) provide evidence against recent, incomplete *Cardinium* infections.

For *Wolbachia*, the best studied endosymbiont, several factors and their interactions seem to influence bacterial density in host tissues (Hoffmann et al., 2015). These can include temperature (Hurst et al., 2001; Morag et al., 2012; Mouton et al., 2007; Bordenstein and Bordenstein, 2011), age (Unckless et al., 2009; Tortosa et al., 2010), reproductive mode (recently reviewed by Ma and Schwander, 2017), and the occurrence of natural antibiotics, for example from fungi (Ho et al., 2013). To what extent any of these factors could explain the patchy phylogenetic distribution of *Cardinium* in non-marine ostracods will be described and discussed in depth elsewhere.

5. Conclusions

Discovering *Cardinium* in non-marine ostracods is only the first step towards several new research avenues that need to be pursued in the near future. Further screening with high throughput sequencing, both taxonomically and geographically, of non-marine and marine ostracods and other crustaceans is required to further explore the abundance of *Cardinium* in natural populations and the factors shaping infection patterns. Since endosymbionts can have a wide range of positive or negative effects on their hosts (Werren et al., 2008), the biological effects of *Cardinium* on its hosts will need to be carefully assessed by life history studies of infected crustaceans, including non-marine ostracods, under various environmental conditions.

Acknowledgements

This research was funded by Belgian Federal Science Policy (Belspo WI/36/I03, MO/36/015, BR/314/PI/LATTECO) and was also supported by the EU Marie Curie Research Training Network 'SexAsex' ('From Sex to Asex: a case study on interactions between sexual and asexual reproduction', contract MRTN-CT-2004-512492) coordinated by K. M. as well as the SPEEDY-project, funded by the Interuniversity Attraction Poles program of the Belgian Science Policy Office BELSPO (IAP-grant P7/04) and coordinated by Prof Luc De Meester (Leuven, Belgium).

We thank Saskia Bode, Marie Cours, Zohra Elouaazizi, Dunja Lamatsch, Gregory Maes, Valentina Pieri, Céline Poux and Andy Vierstraten for technical support. Giuseppe Alfonso, Stuart Halse, Federico Marrone, Emrys Martens, Fransesc Mezquita-Joanes, Ricardo Pinto, Valentina Pieri, Robin Smith and Alison Smith are thanked for providing ostracod samples. We thank our colleague Frederik Hendrickx (Brussels, Belgium) for providing the positive controls and initial primers for classic genetic analyses and for useful comments on an earlier version of this manuscript. Our thanks also go to the previous SexASex Consortium for important contributions to the database of European and African *E. virens* specimens. We are also grateful to Poorani Subramanian and Lewis Kim from the Nephele helpdesk for providing a script for exporting DADA2 results from Nephele into phyloseq. Two anonymous reviewers provided helpful comments on earlier drafts of the manuscript.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2018.09.008.

References

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J.H., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucl. Acid. Res. 25, 3389–3402.

Avise, J.C., 1994. Molecular Markers, Natural History, and Evolution. Chapman & Hall, New York.

Berger, S.A., Krompaß, D., Stamatakis, A., 2011. Performance, accuracy and web-server for evolutionary placement of short sequence reads under maximum likelihood. Syst. Biol. 60, 291–302.

Bode, S.N.S., Adolfsson, S., Lamatsch, D.K., Martins, M.J.F., Schmit, O., Vandekerkhove, J., Mezquita, F., Namiotko, T., Rossetti, G., Schön, I., Butlin, R.K., Martens, K., 2010. Exceptional cryptic diversity and multiple origins of parthenogenesis in a freshwater ostracod. Mol. Phyl. Evol. 54, 542–552.

Bordenstein, S.R., Bordenstein, S.R., 2011. Temperature affects the tripartite interactions between bacteriophage WO, *Wolbachia*, and cytoplasmic incompatibility. PLoS ONE 6, e29106.

Bruvo, R., Adolfsson, S., Symonova, R., Lamatsch, D.K., Schön, I., Jokela, J., Butlin, R.K., Müller, S., 2011. Few parasites, and no evidence for *Wolbachia* infections in a freshwater ostracod inhabiting temporary ponds. Biol. J. Linn. Soc. 102, 208–216.

Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. Nat. Methods 13, 581–583.

Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. jModelTest 2: more models, new heuristics and parallel computing. Nat. Methods 9 772 772.

Davis, M.J., Ying, Z., Brunner, B.R., Pantoja, A., Ferwerda, F.H., 1998. *Rickettsia* relative associated with Papaya bunchy top disease. Curr. Microbiol. 36, 80–84.

Duron, O., Hurst, G.D.D., Hornett, E.A., Josling, J.A., Engelstädter, J., 2008. High incidence of the maternally inherited bacterium *Cardinium* in spiders. Mol. Ecol. 17, 1427–1437.

Edlund, A., Ek, K., Breitholtz, M., Gorokhova, E., 2012. Antibiotic-induced change of bacterial communities associated with the copepod *Nitocra spinipes*. PLoS ONE 7, 33107.

Engelstädter, J., Hurst, G.D.D., 2009. The ecology and evolution of microbes that manipulate host reproduction. Ann. Rev. Ecol. Evol. Syst. 40, 127–149.

Fontaneto, D., Flot, J.F., Tang, C.Q., 2015. Guidelines for DNA taxonomy, with a focus on the meiofauna. Mar. Biodiv. 45, 433. https://doi.org/10.1007/s12526-015-0319-7.

Fuks, G., Elgart, M., Amir, A., Zeisel, A., Turnbaugh, P.J., Soen, Y., Shental, N., 2018. Combining 16S rRNA gene variable regions enables high-resolution microbial community profiling. Microbiome 6, 17.

Gotoh, T., Noda, H., Ito, S., 2007. *Cardinium* symbionts cause cytoplasmic incompatibility in spider mites. Heredity 98, 13–20.

Graham, R.I., Wilson, K., 2012. Male-killing *Wolbachia* and mitochondrial selective sweep in a migratory African insect. BMC Evol. Biol. 12, 204. https://doi.org/10.1186/1471-2148-12-204.

Gueguen, G., Vavre, F., Gnankine, O., Peterschmitt, M., Charif, D., Chiel, E., Gottlieb, Y., Ghanim, M., Zchori-Fein, E., Fleury, F., 2010. Endosymbiont metacommunities, mtDNA diversity and the evolution of the *Bemisia tabaci* (Hemiptera: Aleyrodidae) species complex. Mol. Ecol. 19, 4365–4378.

Guindon, S., Gascuel, O., 2003. A simple, fast, and accurate algorithm to estimate large phylogenies by maximum likelihood. Syst. Biol. 52, 696–704.

Han, M.V., Zmasek, C.M., 2009. phyloXML: XML for evolutionary biology and comparative genomics. BMC Bioinformat. 10, 356. https://doi.org/10.1186/1471-2105-10-356.

Hebert, P.D., Cywinska, A., Ball, S.L., deWaard, J.R., 2003. Biological identifications through DNA barcodes. Proc. Biol. Sci. 270, 313–321.

Hilgenboecker, K., Hammerstein, P., Schlattmann, P., Telschow, A., Werren, J.H., 2008. How many species are infected with *Wolbachia*? – a statistical analysis of current data. FEMS Microbiol. Lett. 281, 215–220.

Ho, W.H., To, P.C., Hyde, K.D., 2013. Induction of antibiotic production of freshwater fungi using mix-culture fermentation. Fungal Div. 12, 45–51.

Hoffmann, A.A., Ross, P.A., Rašić, G., 2015. *Wolbachia* strains for disease control: ecological and evolutionary considerations. Evol. Appl. 8, 751–768.

Hunter, M.S., Perlman, S.J., Kelly, S.E., 2003. A bacterial symbiont in the Bacteroidetes induces cytoplasmic incompatibility in the parasitoid wasp *Encarsia pergandiella*. Proc. R. Soc. B. 270, 2185–2190.

Hurst, G.D.D., Jiggins, F.M., 2005. Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: The effects of inherited symbionts. Proc. R. Soc. B. 272, 1525–1534.

Hurst, G.D., Jiggins, F.M., Robinson, S.J., 2001. What causes inefficient transmission of male-killing *Wolbachia* in *Drosophila*? Heredity 87, 220–226.

Hurst, L.D., McVean, G.T., 1996. Clade selection, reversible evolution and the persistence of selfish elements: the evolutionary dynamics of cytoplasmic incompatibility. Proc. R. Soc. B. 263, 97–104.

Jäckel, R., Mora, D., Dobler, S., 2013. Evidence for selective sweeps by *Wolbachia* infections: phylogeny of Altica leaf beetles and their reproductive parasites. Mol. Ecol. 22, 4241–4255. https://doi.org/10.1111/mec.12389.

Jeaprakash, A., Hoy, M.A., 2000. Long PCR impoves *Wolbachia* DNA amplifications: wsp sequences found in 76% of sixty-tree arthropod species. Insect Mol. Biol. 9, 393–405.

Jiggins, F.M., 2003. Male-killing *Wolbachia* and mitochondrial DNA: selective sweeps, hybrid introgression and parasite population dynamics. Genetics 164, 5–12.
Kambhampati, S., Rai, K.S., Verleye, D.M., 1992. Frequencies of mitochondrial-DNA haplotypes in laboratory cage populations of the mosquito, *Aedes albopictus*. Genetics 132, 205–209.
Kass, R.E., Raftery, A.E., 1995. Bayes Factors. J. Am. Statist. Assoc. 90, 773–795.
Katoh, K., Standley, D.M., 2013. MAFFT multiple sequence alignment software version 7: improvements in performance and usability. Mol. Biol. Evol. 30, 772–780.
Kuo, C.H., Ochman, H., 2009. Inferring clocks when lacking rocks: the variable rates of molecular evolution in bacteria. Biol. Direct 4, 35. https://doi.org/10.1186/1745-6150-4-35.
Larkin, M.A., Blackshields, G., Brown, N.P., Chenna, R., McGettigan, P.A., William, H., Valentin, F., Wallace, I.M., Wilm, A., Lopez, R., Thompson, J.D., Gibson, T.J., Higgins, D.G., 2007. Clustal W and Clustal X version 2.0. Bioinformatics 23, 2947–2948.
Li, H., Leavengood, J.M., Chapman, E.G., Burkhardt, D., Song, F., Jiang, P., Liu, J., Zhou, X., Cai, W., 2017. Mitochondrial phylogenomics of Hemiptera reveals adaptive innovations driving the diversification of true bugs. Proc. R. Soc. B 284, 20171223. https://doi.org/10.1098/rspb.2017.1223.
Ma, W.J., Schwander, T., 2017. Patterns and mechanisms in instances of endosymbiont-induced parthenogenesis. J. Evol. Biol. 30, 868–888. https://doi.org/10.1111/jeb.13069.
Majerus, T.M.O., von der Schulenburg, J.H.G., Majerus, M.E.N., Hurst, G.D.D., 1999. Molecular identification of a male-killing agent in the ladybird *Harmonia axyridis* (Pallas) (Coleoptera: Coccinellidae). Insect Mol. Biol. 8, 551–555.
Martens, K. (Ed.), 1998. Sex and Parthenogenesis – evolutionary ecology of reproductive modes in non-marine ostracods. Backhuys Publ, Leiden, pp. 334.
Martens, K., Schön, I., Meisch, C., Horne, D.J., 2008. Global diversity of ostracods (Ostracoda, Crustacea) in freshwater. Hydrobiologia 595, 185–193.
Matsen, F.A., Kodner, R.B., Armbrust, E.V., 2010. pplacer: linear time maximum-likelihood and Bayesian phylogenetic placement of sequences onto a fixed reference tree. BMC Bioinformat. 11, 538. https://doi.org/10.1186/1471-2105-11-538.
McMurdie, P.J., Holmes, S., 2013. phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census data. PLoS ONE 8, e61217.
Morag, N., Klement, E., Saroya, Y., Lensky, I., Gottlieb, Y., 2012. Prevalence of the symbiont *Cardinium* in Culicoides (Diptera: Ceratopogonidae) vector species is associated with land surface temperature. FASEB J. 26, 4025–4034. https://doi.org/10.1096/fj.12-210419.
Moret, Y., Juchault, P., Rigaud, T., 2001. *Wolbachia* endosymbiont responsible for cytoplasmic incompatibility in a terrestrial crustacean: effects in natural and foreign hosts. Heredity 86, 325–332.
Mouton, L., Henri, H., Charif, D., Bouletreau, M., Vavre, F., 2007. Interaction between host genotype and environmental conditions affects bacterial density in *Wolbachia* symbiosis. Biol. Lett. 3, 210–213.
Nakamura, Y., Kawai, S., Yukuhiro, F., Ito, S., Gotoh, T., Kisimoto, R., Yanase, T., Matsumoto, Y., Kageyama, D., Noda, H., 2009. Prevalence of *Cardinium* bacteria in planthoppers and spider mites and taxonomic revision of 'Candidatus Cardinium hertigii' based on detection of a new *Cardinium* group from biting midges. Appl. Environ. Microbiol. 75, 6757–6763.
Pagès, N., Muñoz-Muñoz, F., Verdún, M., Pujol, N., Talavera, S., 2017. First detection of *Wolbachia*-infected Culicoides (Diptera: Ceratopogonidae) in Europe: *Wolbachia* and *Cardinium* infection across Culicoides communities revealed in Spain. Parasit. Vectors 10, 582. https://doi.org/10.1186/s13071-017-2486-9.
Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012. MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. Syst. Biol. 61, 539–542.
Ros, V.I.D., Breeuwer, J.A.J., 2009. The effects of, and interactions between, *Cardinium* and *Wolbachia* in the doubly infected spider mite *Bryobias arothamni*. Heredity 102, 413–422.
Schön, I., Higuti, J., Patel, T., Martens, K., 2018. Aquatic long-distance dispersal and vicariance shape the evolution of an ostracod species complex (Crustacea) in four major Brazilian floodplains. Mol. Phyl. Evol. 121, 86–97.
Schön, I., Martens, K., 2012. Molecular analyses of ostracod flocks from Lake Baikal and Lake Tanganyika. Hydrobiologia 682, 91–110.
Schön, I., Pieri, V., Sherbakov, D., Martens, K., 2017. Cryptic diversity and speciation in endemic *Cytherissa* (Ostracoda, Crustacea) from Lake Baikal. Hydrobiologia 800, 61–79.
Schön, I., Pinto, R.L., Halse, S., Smith, A.J., Martens, K., Birky, C.W., 2012. Cryptic species in putative ancient asexual darwinulids (Crustacea, Ostracoda). Plos ONE 7, e39844.
Schön, I., Poux, C., Verheyen, E., Martens, K., 2014. High cryptic diversity and persistent lineage segregation in endemic *Romecytheridea* (Crustacea, Ostracoda) from the ancient Lake Tanganyika. Hydrobiologia 739, 119–131. https://doi.org/10.1007/s10750-013-1581-y.
Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033.
Stefanini, A., Duron, O., 2012. Exploring the effect of the *Cardinium* endosymbiont on spiders. J. Evol. Biol. 25, 1521–1530. https://doi.org/10.1111/j.1420-9101.2012.02535.x.
Sun, X.J., Xiao, J.H., Cook, J.M., Feng, G., Huang, D.W., 2011. Comparisons of host mitochondrial, nuclear and endosymbiont bacterial genes reveal cryptic fig wasp species and the effects of *Wolbachia* on host mtDNA evolution and diversity. BMC Evol. Biol. 11, 86. https://doi.org/10.1186/1471-2148-11-86.
Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular evolutionary genetics analysis version 6.0. Mol. Biol. Evol. 30, 2725–2729.
Tortosa, P., Charlat, S., Labbe, P., Dehecq, J.-S., Barre, H., Weill, M., 2010. *Wolbachia* age-sex-specific density in *Aedes albopictus*: a host evolutionary response to cytoplasmic incompatibility? PLoS ONE 5, e9700.
Unckless, R.L., Boelio, L.M., Herren, J.K., Jaenike, J., 2009. *Wolbachia* as populations within individual insects: causes and consequences of density variation in natural populations. Proc. R. Soc. B. 276, 2805–2811.
Vandekerckhove, T.T.M., 1998. Cytoplasmic bacteria in *Heterocypris incongruens* (Crustacea, Ostracoda). In: Martens, K. (Ed.), Sex and Parthenogenesis, Evolutionary Ecology of Reproductive Modes in Non-marine Ostracods. Backhuys Publ, Leiden, pp. 119–126.
Vanthournout, B., Hendrickx, F., 2015. Endosymbiont dominated bacterial communities in a dwarf spider. PLoS ONE 10, e0117297. https://doi.org/10.1371/journal.pone.0117297.
Vanthournout, B., Swaegers, J., Hendrickx, F., 2011. Spiders do not escape reproductive manipulations by *Wolbachia*. BMC Evol. Biol. 11, 15–24.
Verne, S., Johnson, M., Bouchon, D., Grandjean, F., 2012. Effects of parasitic sex-ratio distorters on host genetic structure in the *Armadillidium vulgare–Wolbachia* association. J. Evol. Biol. 25, 264–276.
von der Schulenburg, J.H.G., Hurst, G.D.D., Tetzlaff, D., Booth, G.E., Zakharov, I.A., Majerus, M.E.N., 2002. History of infection with different male-killing bacteria in the two-spot ladybird beetle *Adalia bipunctata* revealed through mitochondrial DNA analysis. Genetics 160, 1075–1086.
Werren, J.H., 1997. Biology of Wolbachia. Ann. Rev. Entomol. 42, 587–609.
Werren, J.H., Baldo, L., Clark, M.E., 2008. *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol. 6, 741–751.
Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.
Wilke, T., Schultheiß, R., Albrecht, C., 2006. As time goes by: a simple fool's guide to molecular clock approaches in invertebrates. Am. Malacol. Bull. 27, 25–45.
Zhang, Y.K., Chen, Y.T., Yang, K., Hong, X.Y., 2016. A review of prevalence and phylogeny of the bacterial symbiont *Cardinium* in mites (subclass: Acari). Syst. Appl. Acarol. 21, 978–990.
Zug, R., Hammerstein, P., 2012. Still a host of hosts for *Wolbachia*: analysis of recent data suggests that 40% of terrestrial arthropod species are infected. PLoS ONE 7, e38544.