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Further insights in the Tardigrada microbiome: phylogenetic position and prevalence of infection of four new Alphaproteobacteria putative endosymbionts

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Data from a previous study showed that microbiomes of six tardigrade species are species-specific and distinct from associated environmental microbes. We here performed a more in-depth analyses of those data, to identify and characterize new potential symbionts. The most abundant bacterial operational taxonomic units (OTUs) found in tardigrades are classified, and their prevalence in other environments is assessed using public databases. A subset of OTUs was selected for molecular phylogenetic analyses based on their affiliation with host-associated bacterial families in tardigrades. Almost 22.6% of the most abundant OTUs found do not match any sequence at 99% identity in the IMNGS database. These novel OTUs include four putative tardigrade endosymbionts from Alphaproteobacteria (Anaplasmataceae and *Candidatus* Tenuibacteraceae), which are characterized by 16S rRNA gene analysis and investigated for their infection rates in: *Echiniscus trisetosus*, *Richtersisus coronifer* and *Macrobiotus macrocalix*. These putative endosymbionts have an infection prevalence between 9.1% and 40.0%, and are, therefore, likely secondary symbionts, not essential for tardigrade survival and reproduction. Using fluorescence *in situ* hybridization (FISH), we detected bacteria on the cuticle and within the ovary of *E. trisetosus*, suggesting possible vertical transmission. This study highlights the great contribution in biodiversity discovery that neglected phyla can provide in microbiome and symbiosis studies.

KEYWORDS: Rickettsiales – Holosporales – Anaplasmataceae – *Ca*. Tenuibacteraceae – *Echiniscus trisetosus* – *Macrobiotus macrocalix* – *Richtersius coronifer* – FISH.

INTRODUCTION

Tardigrada (water bears) are mostly known for their ability to undergo cryptobiosis (i.e. ametabolic states of life in response to adverse environmental factors) under which they are able to withstand extreme conditions (for reviews see: Guidetti *et al.*, 2011; Møbjerg *et al.*, 2011). Tardigrades are also a key taxon for the evolution of Panarthopoda (Arthropoda, Onychophora, Tardigrada; Campbell *et al.*, 2011; Mayer *et al.*, 2013; Smith & Goldstein, 2017), having an ancient origin during the Precambrian (Rota-Stabelli *et al.*, 2013; Guidetti *et al.*, 2017), and are almost ubiquitous, being widespread around the world in all continents, colonizing many different habitats of marine, freshwater and terrestrial environments. Recently, researchers have explored the relationships of tardigrades with microorganisms, both in relation to the presence of specific microbiomes (Vecchi *et al.*, 2018) and to the possible high level of horizontal gene transfer (HGT) from microorganisms (Boothby *et al.*, 2015). Although the high level of HGT initially detected (Boothby *et al.*, 2015) is likely due to contaminating sequences in the assembly

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(Bemm *et al.*, 2016; Koutsovoulos *et al.*, 2016), several different symbionts (i.e. organisms living in close and/or long-term biological interaction) have been found in relationships with tardigrades, from fungi, to protozoans and bacteria (for reviews see Kinchin, 1994; Vecchi et al., 2016, 2018). In particular, the analyses of the microbiomes of six limnoterrestrial tardigrade species belonging to several phylogenetic lineages, in tandem with the bacteria present in their respective substrates, indicated that the tardigrade microbiomes are highly species-specific and well differentiated from the environment (Vecchi *et al.*, 2018). The tardigrade microbiota is dominated by Proteobacteria and Bacteroidetes (Vecchi *et al.*, 2018). Using 16S rRNA gene analyses, operational taxonomic units (OTUs) belonging to the host-associated bacteria families Anaplasmataceae (Alphaproteobacteria; Rickettsiales) and *Ca*. Tenuibacteraceae (Alphaproteobacteria; Holosporales) were identified as tardigrade symbionts (Vecchi *et al.*, 2018). Hereafter, *Ca.* Tenuibacteraceae will be referred to as Tenuibacteraceae (*Ca.* stands for *Candidatus*, a bacterium that cannot be maintained in a microbiological culture collection). Both Anaplasmataceae and Tenuibacteraceae have been reported thus far in only few phyla within Ecdysozoa, i.e. nematodes, priapulids and arthropods, as well as spiders, insects and ticks (Sironi *et al.*, 1995; Ponnusamy *et al.*, 2014; Ceccarelli *et al.*, 2016; Kroer *et al.*, 2016; Mohammed *et al.*, 2017).

The objectives of this study are to better characterize selected members of Anaplasmataceae and Tenuibacteraceae in the microbiomes of six tardigrade species belonging to different evolutionary lines and living in different environments, analysing the bacterial OTUs obtained in the previous study of Vecchi *et al.* (2018), to identify their infection prevalence and to localize the putative symbionts within one of these tardigrade species.

MATERIAL AND METHODS

Analysis of tardigrade microbiome OTU sequences

The tardigrade microbiome OTU sequences analysed in the present study are those previously obtained by Vecchi *et al.* (2018) and defined by them as 'common OTUs' (those with a minimum abundance of at least 5% in any of the samples). These common OTUs were obtained by Vecchi *et al.* (2018) from five to six replicates of groups of specimens belonging to six species (and from their substrates), which belong to different evolutionary lineages and living in different environments: *Richtersius coronifer* (Richters, 1903) and *Macrobiotus macrocalix* Bertolani & Rebecchi, 1993, both from the same moss substrate; *Paramacrobiotus areolatus* (Murray, 1907) and *Echiniscus trisetosus* Cuénot, 1932, both from the same moss substrate; *Ramazzottius oberhaeuseri* (Doyère, 1840) from two different lichens on trees; *Acutuncus antarcticus* (Richters, 1904) from different substrates and from a laboratory culture (Table 1; for more details see: Vecchi *et al.*, 2018). An outline of the protocol followed by Vecchi *et al.* (2018) to obtain the data analysed in this paper is reported here. Each animal replicate consisted of 50 specimens washed in sterile ddH_2O, while substrate replicates consisted of 500 µL of substrate suspension. DNA was extracted with an Epicenter MasterPure DNA Purification Kit (Epicenter, Madison, WI, USA) protocol. Amplification and sequencing were performed following Earth Microbiome protocols for the V4 region of the 16S rRNA gene (Caporaso *et al.*, 2012) and sequenced with an Illumina MiSeq with 250 paired-end cycles. Bioinformatic analyses were performed with the software MOTHUR (Schloss *et al.*, 2009) and the R package 'phyloseq' (McMurdie & Holmes, 2013).

In the present study, the relative abundances of four specific OTUs (OTU6, 7, 22, 30) within the microbiomes of the six tardigrade species, obtained from the common OTU abundance table in the supplementary material of Vecchi *et al.* (2018), were averaged over the different tardigrade species in an EXCEL spreadsheet. All the common OTU sequences found in the six tardigrade species and their substrates were searched against the full Integrated microbial next generation sequencing (IMNGS) database to determine their prevalence in all the microbiome samples present. The IMNGS is a platform that uniformly and systematically screens for, and processes, all prokaryotic 16S rRNA gene amplicon datasets available in sequence read archive (SRA) and uses them to build sequence databases for each biological sample present in SRA (Lagkouvardos *et al.*, 2016). For IMNGS database querying, similarity thresholds of 99% and 97% were selected to target conspecific sequences (97%) and potential sequences from the same strains (99%). A minimum overlap size of 200 base pairs between query and target sequences was imposed to obtain reliable identity estimates. Common OTUs from Vecchi *et al.* (2018) were classified on the online SILVA search and classify tool with default parameters (Quast *et al.*, 2013; Yilmaz *et al.*, 2014).

Tardigrade species

For the present study, animals of *E. trisetosus*, *M. macrocalix* and *Ri. coronifer* were collected from the same mosses used by Vecchi *et al.* (2018) (Table 1). They were used to obtain full-length bacterial 16S rRNA gene sequences and to determine the prevalence of infection, and, for *E. trisetosus*, to perform

Table 1. Tardigrade species and populations (groups) used in the present study

Tardigrade taxon	Group code#	Details of the samples with tardigrade
Macrobiotus macrocalix° (Macrobiotoidea, Macrobiotidae)	S6_Mac	Moss (*Orthotrichum cupulatum*) on rock; Öland, Sweden [Lat. N 56.528867; Lon. E 16.491233]
Richtersius coronifer° (Macrobiotoidea, Richtersiidae)	S6_Ric	
Echiniscus trisetosus° (Echiniscoidea, Echiniscidae)	S7_Ech	Moss (community composed by *Grimmia montana*, *Grimmia laevigata*, and *Syntrichia ruralis*) on rock,
*Paramacrobiotus areolatus** (Macrobiotoidea, Macrobiotidae)	S7_Par	Sassomorello, Modena, Italy [Lat. N 44.424787; Lon. E 10.738364]
*Acutuncus antarcticus** (Hypsibiioidea, Hypsibiidae)	S1_Acu	Freshwater sediment (defrosted); Edmonson Point, Victoria Land, Antarctica [Lat. S 74.330733; Lon. E 165.135883]
*Acutuncus antarcticus** (Hypsibiioidea, Hypsibiidae)	S2_Acu	Freshwater sediment (dry); Terranova Bay, Victoria Land, Antarctica [Lat. S 74.709667; Lon. E 164.101433]
*Acutuncus antarcticus** (Hypsibiioidea, Hypsibiidae)	S3_Acu	Laboratory culture
*Ramazzottius oberhaeuseri** (Hypsibiioidea, Ramazzottiidae)	S4_Ram	Lichen (*Xanthoria parietina*) on tree; Modena, Italy [Lat. N 44.622366; Lon. E 10.943552]
*Ramazzottius oberhaeuseri** (Hypsibiioidea, Ramazzottiidae)	S5_Ram	Lichen (*Xanthoria parietina*) on tree; Monte Cenere, Modena, Italy [Lat. N 44.312667; Lon. E 10.759817]

The group codes are the same as in Vecchi *et al.* (2018); ° new individuals analysed; * bioinformatics analysis of data from Vecchi *et al.* (2018).

fluorescent *in situ* hybridization (FISH). Tardigrades were extracted from mosses by sieving, according to the method reported in Guidetti *et al.* (2014).

16S rRNA amplification and cloning

In the present study, the DNA used for 16S rRNA gene amplification and cloning was extracted from the above-mentioned species (*E. trisetosus*, *M. macrocalix* and *Ri. coronifer*), because in these species the four OTUs of interest were found in high abundance. The DNA was extracted from a pool of 50 individuals of each species (carefully checked with microscope for taxonomic identification) with an Epicentre Masterpure Kit in a 50 µL lysis buffer volume.

To focus on the symbiotic families Anaplasmataceae (Alphaproteobacteria; Rickettsiales) and Tenuibacteraceae (Alphaproteobacteria; Holosporales), the alphaproteobacterial 16S rRNA gene was amplified with Alphaproteobacteria-specific primers (16Sα_F19b, 16S_R1522a) and thermal cycling conditions according to Szokoli *et al.* (2016a). The PCR product was purified with a Wizard SV Gel and PCR Clean Up System (Promega) or Qiagen PCR cleanup kit (Qiagen) and then cloned into either the pGEM-T Easy vector or the pCR-TOPO cloning kit (Invitrogen) and used to transform *Escherichia coli* JM109 (Promega) competent cells. One clone for each sequence type (i.e. one for each of the corresponding four OTUs of interest) belonging to the families Anaplasmataceae and Tenuibacteraceae was sequenced with M13 primers with Sanger technology using a BigDye Terminator Cycle Sequencing Kit 1.1 (Applera) and run on a ABI Prism 3100 (Applera).

Sequence analyses and phylogenetic reconstructions

The four sequences obtained from the cloning were used to search the NCBI nucleotide collection (nr/nt) using the BLASTn algorithm. The first ten matches, ordered by similarity for each sequence, were retained for the phylogenetic reconstruction together with 16S rRNA sequences from named representatives of Holosporales, Rickettsiales, and outgroups. Sequences from the four clones were also searched against the full IMNGS database (similarity threshold 97%, minimum size = 200). The downloaded IMNGS matching reads were included in the phylogenetic reconstruction.

A reference alignment was built with all sequences except the short IMNGS reads and with additional reference sequences from Alphaproteobacteria and Betaproteobacteria using the MAFFT alignment online tool (Strategy: G-INS-1, default parameters). The short IMNGS reads were then added to this alignment with the MAFFT–addfragments online tool (Direction of nucleotide sequences: Adjust direction according to the first sequence; Keep alignment length: No;

Strategy: multipair accurate). The best substitution model was tested with JModelTest2 (Darriba *et al.*, 2012) on the Cipres science gateway (Miller *et al.*, 2010). The BI phylogenetic tree was computed with the software MrBayes (Ronquist & Huelsenbeck, 2003) (nst: 6, rates: invgamma, ngen: 20000000, mcmcdiagn: yes, Diagnfreq: 1000, burninfrac: 0.10, Stoprule: yes, Stopval: 0.005, nruns: 2, nchains: 4) on the Cipres webserver (Miller *et al.*, 2010).

Based on this tree, the IMNGS sequences not belonging to Rickettsiales and Holosporales were discarded along with the reference sequences from Alphaproteobacteria and Betaproteobacteria and a new alignment and tree were built as described above. This double round of phylogenetic reconstruction, in order to discard some sequences from IMNGS, was due to the presence of false positives (i.e. sequences matching the identity to query criteria but pertaining to different bacterial orders) among them. The complete list of accession numbers is given in Supporting Information, Table S1.

Sequences of obtained clones were also matched with OTUs from Vecchi *et al.* (2018) by computing a p-distance matrix on MEGA7 of an alignment (obtained with Muscle algorithm) comprising the cloned sequences and the common OTU sequences from Vecchi *et al.* (2018) (Pairwise distance, Rates: uniform rates, Gap treatment: complete deletion). The p-distances 95% confidence intervals were calculated with the BinomCI function in the R package DescTools (Signorell, 2016).

Diagnostic PCR to detect the infection prevalence

The DNA was extracted from single tardigrades belonging to *E. trisetosus*, *M. macrocalix* and *Ri. coronifer* with a modified HotSHOT protocol (Truett *et al.*, 2000; Vecchi *et al.*, 2018). In brief, single animals were suspended in 20 µL of alkaline lysis solution and heated at 95 °C for 15 min. The solution was then cooled down to room temperature and neutralized with 20 µL of neutralizing solution. To determine the infection prevalence, diagnostic primers for the bacteria were designed on the corresponding 16S rRNA sequence with the help of the NCBI Primer Blast online tool (NCBI, 2017). For the two putative symbionts identified in *E. trisetosus* (called ETS1 and ETS2), it was possible to design only a couple of primers that identified both bacteria (as they were phylogenetically closely related), so they were analysed jointly (and referred to as ETS1-2 in the results below). Therefore, it was not possible to distinguish which of the two bacteria was present in each tardigrade. In total, 22 *E. trisetosus*, 20 *M. macrocalyx* and 20 *Ri. coronifer* animals were screened for the presence of the corresponding putative endosymbionts. The genomic DNA of these animals yielded positive amplification for tardigrade 18S rRNA. The primers and PCR conditions used are those reported in Bertolani *et al.* (2014) for *M. macrocalix* and *Ri. coronifer*, and in Vicente *et al.* (2013) for *E. trisetosus*. Reactions were performed in 10 µL volumes (1X DreamTaq Buffer, DreamTaq DNA polymerase 0.25 U, 0.2 mM of each dNTP, 1 µM of each primer, Bovine Serum Albumine 2 µg/mL) with 2 µL of genomic template. Negative and positive controls were included. Primers, cycles and controls used are listed in Table 2. Annealing temperatures for diagnostic primers were determined empirically by their ability to maximize the amplification of the positive controls without amplifying the negative controls.

Whole mount fluorescent *in situ* hybridization (FISH)

To determine the presence of bacteria on and/or within specimens of *E. trisetosus* the whole mount FISH technique with a DNA probe was used. An Alexa Fluor 594 conjugated DNA EUB338 (Alm *et al.*, 1996) targeting nearly all Eubacteria and an DAPI stain (4′,6-diamidino-2-phenylindole, binding to DNA regions rich in A-T) were used. The FISH protocol from Vandekerckhove *et al.* (2002) was taken as the starting point, but it was modified to obtain results on tardigrades. All the FISH steps were performed in spin columns and in agitation (4 Hz). If not specified otherwise, steps were performed at room temperature. Forty adult animals were fixed for 90 min in paraformaldehyde solution 4% in PBS 1X, then they were washed for 15 min three times in 0.1% Tween20 in PBS 1X. Animals were then sonicated for 45 s at 35 KHz in PBS 1X and hybridized overnight at 46 °C in hybridization solution (NaCl 1800 mM, trisHCl 40 mM, SDS 0.02%, 4 ng/µL probe). After the overnight hybridization, animals were washed at 48 °C for 45 min in washing solution (NaCl 900 mM, trisHCl 40 mM, SDS 0.01%, 5 mM EDTA). The 40 animals were then mounted singly on glass microscope slides with DABCO-glycerol mounting medium (90% glycerol, 2.5% DABCO in PBS) with 100 ng/µL DAPI and the coverslip was sealed with transparent nail polish. The slides were observed with a LEICA TCS SPZ confocal microscope at 'Centro Interdipartimentale Grandi Strumenti' (CIGS) of the University of Modena and Reggio Emilia.

RESULTS

We searched the Integrated Microbial Next Generation Sequencing (IMNGS) database to identify the presence of sequences with a similarity of 97% or 99% to the most common OTUs found in *A. antarcticus*,

Table 2. Primers, PCR cycles and controls used for diagnostic PCR

Symbiont from	Primers for 16S gene	PCR cycle	Positive control	Negative control
M. macrocalix (MMS)	MMS-F417 5’-CCCGAAGAATAAGTCCCGGC-3’ MMS-R984 5’-CATGCAGCACCTGTGCAAAC-3’	1) 5’ 94 °C 2) 30” 94 °C 3) 30” 55 °C 4) 1’ 72 °C ->2 x 29 5) 7’ 72 °C	Plasmid with MMS 16S 10^{-3} ng/ µL	Plasmid with ETS1+ETS2 16S 10^{-3} ng/ µL
E. trisetosus (ETS1, ETS2)	ENLSb-F477 5’-TTCGGAATTACTGGGCGTAAAG-3’ ENLSb-R964 5’-CGAACTGAGCCTCCCTCTTCAG-3’	1) 5’ 94 °C 2) 30” 94 °C 3) 30” 55 °C 4) 1’ 72 °C ->2 x 29	Plasmid with ETS1+ETS2 16S 10^{-3} ng/ µL	Plasmid with MMS 16S 10^{-3} ng/ µL
Ri. coronifer (RCS)	RCS-f74 5’-ACTGGATGTGTCTGAGAAGA-3’ RCS-r531 5’-CCCCTTCTGTACTCAAGTTAAA-3’	1) 7’ 72 °C 2) 30” 94 °C 3) 30” 45 °C 4) 1’ 72 °C ->2 x 29 5) 7’ 72 °C	Plasmid with RCS 16S 10^{-3} ng/ µL	Plasmid with ETS1+ETS2 16S 10^{-3} ng/ µL

E. trisetosus, *M. macrocalix*, *P. areolatus*, *Ri. coronifer* and *Ra. oberhaeuseri*. We find that the tardigrade-associated microbes are mostly specific to tardigrades and/or to their substrates (Fig. 1). Among these sequences, 12 bacterial OTUs (i.e. OTUs 6, 9, 22, 26, 30, 42, 62, 105, 112, 216, 278 and 288; Supporting Information, Table S2) have no match at 99% identity. Similarly, sequences related to OTUs 6, 7, 22 and 30 have low prevalence in the IMNGS database, but are highly abundant in tardigrade microbiomes. Four of these OTUs belong to Anaplasmataceae (OTUs 6, 9 and 30) and Tenuibacteraceae (OTU22).

Four different clones were retrieved from the amplification and cloning of the nearly full length 16S rRNA gene of these four OTUs: two clones, called ETS1 (1168 bp; Genbank MK028535) and ETS2 (1374 bp; acc. n. MK028534) from *E. trisetosus*, one called MMS (1449 bp; acc. n. MK028536) from *M. macrocalix* and one called RCS (1478 bp; acc. n. MK028537) from *Ri. coronifer*. ETS1 and ETS2 matched the sequences of OTU7 and OTU6, respectively; MMS matched the sequence of OTU30; RCS matched the sequence of OTU22 (Table 3). The closest sequences to these clones found in the NCBI database by BLAST search have low identities (i.e. RCS 93% identity; MMS 88% identity; ETS1 88% identity; ETS2 87% identity). We then searched for related sequences in the IMNGS database (corresponding to NCBI Sequence Read Archive runs and looking for 97% identity matches or better; Leinonen *et al.*, 2010; Lagkouvardos *et al.*, 2016) and find RCS in four IMNGS samples, MMS in three, and ETS1 and ETS2 in seven IMNGS samples. An IMNGS sample is a collection of nucleotide sequences derived from a biological specimen (e.g. soil, water, gut content, etc.).

The phylogenetic reconstruction of bacterial sequences both selected from public databases and the new sequences of the four putative endosymbionts recovers almost all the currently recognized Rickettsiales and Holosporales clades (Szokoli *et al.*, 2016b) as monophyletic, with the exception of the family Holosporaceae (Fig. 2). The ETS1, ETS2 and MMS sequences placed these symbionts in the same family, Anaplasmataceae in the order Rickettsiales, while the symbiont with the sequence RCS belonged to the family Tenuibacteraceae in the order Holosporales (confirming the SILVA-based classification of the corresponding OTUs). The ETS1 and ETS2 clones are clearly placed in the same clade with their closest named relatives, *Neorickettsia* species and *Ca*. Xenolissoclinum pacificiensis (Fig. 2), along with sequences from IMNGS retrieved from soil, rainwater tanks, *Saxifraga* rhizosphere and bark of *Acer pseudoplatanus* L., 1753. The MMS clone clustered with sequences from IMNGS as well (retrieved from soil and Pika gut), but in polytomy with a clade comprising all Anaplasmataceae genera (i.e. *Anaplasma*, *Aegyptianella*, *Ehrlichia*, *Ca*. Neoehrlichia, *Ca*. Cryptoplasma and *Ca*. Neoanaplasma) with the exception of *Wolbachia* and

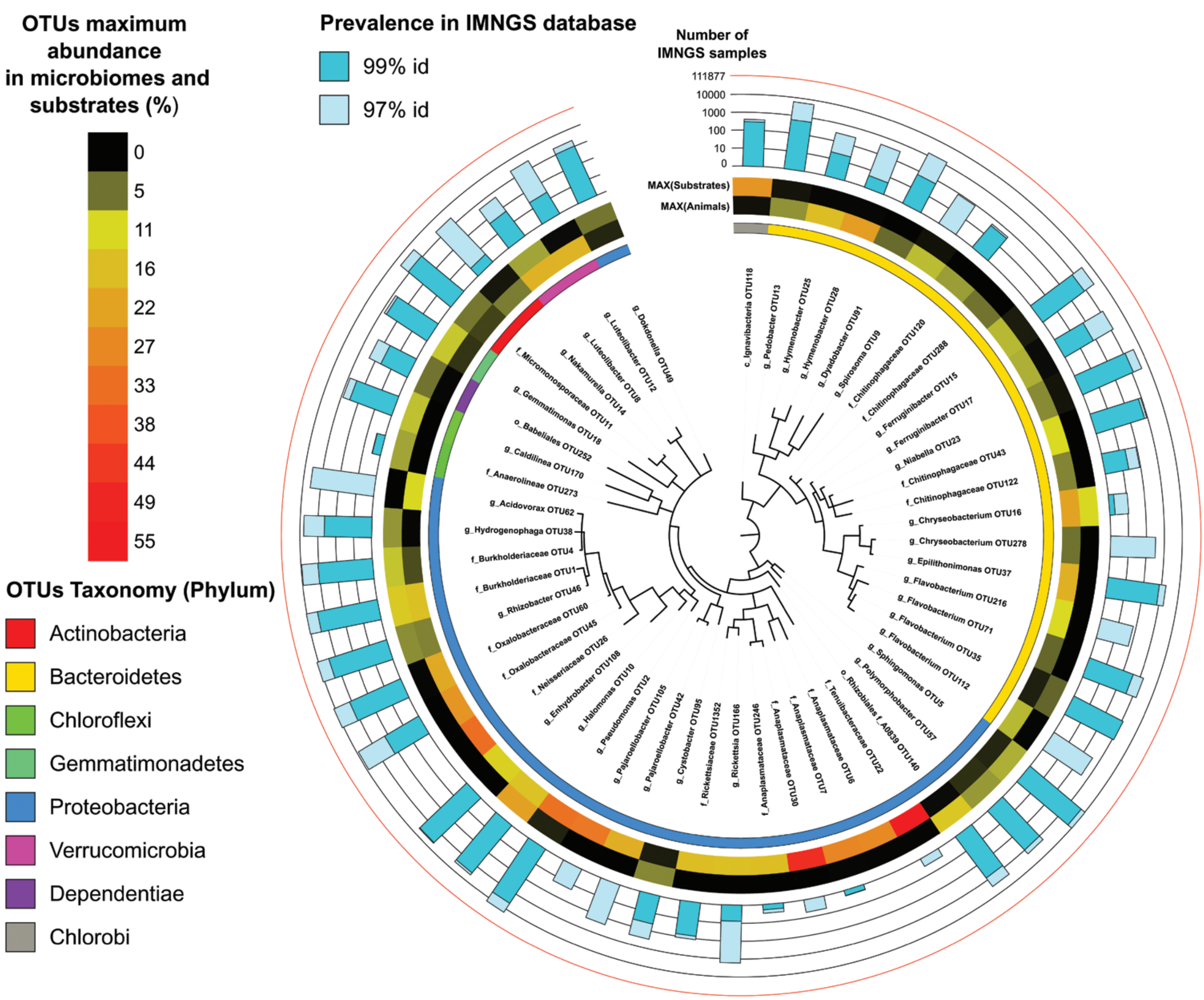

Figure 1. Taxonomic identification, abundances within animals and substrates, and prevalence in the IMNGS database of the common OTUs found in tardigrades and their environments. Single letters before each taxon name represent the systematic level (c = class, o = order, f = family, g = genus) of SILVA-based identification for each OTU. First internal circle represents the phylum of each OTU. Second and third internal circles represent the percentage of maximum abundance of each OTU in microbiomes of the animals and their environments (from Vecchi *et al.*, 2018). External histogram (light blue columns in logarithmic scale) represents the prevalence in IMNGS database of each OTU with a 99% or 97% of identity. The internal phylogenetic tree represents the evolutionary relationships identified by Vecchi *et al.* (2018) in the common OTUs.

the *Wolbachia* clade (Fig. 2). In contrast, RCS is in a clade with IMNGS sequences (from freshwater, cryoconite and the lichen *Lobaria pulmonaria* (L.) Hoffm. (1796)) and placed inside Tenuibacteraceae in the same clade containing *Ca*. Tenuibacter priapulorum and environmental bacterial sequences from rivers, lakes and from the crustacean *Bosmina coregoni* (Baird, 1857).

We infer the presence and relative abundance of these four putative symbionts (ETS1, ETS2, RCS and MMS) in the microbiomes obtained by Vecchi *et al.* (2018). The distribution of the putative endosymbionts is associated with their phylogenetic position (Fig. 3). The putative endosymbiont with the MMS sequence infects almost exclusively *M. macrocalix*, while those with the sequences ETS1 and ETS2 infects mainly *E. trisetosus*. The RCS endosymbiont is mainly found in *Ri. coronifer*, but is also identified in the other two species (*M. macrocalix* and *P. areolatus*) of the Macrobiotoidea clade. In contrast, the tardigrade species of the Hypsibiioidea clade (*A. antarcticus* and *Ra. oberhaeuseri*) are practically not infected by any of the new putative endosymbionts (Fig. 3).

Table 3. p-distances (proportion of nucleotide sites at which two sequences are different) between cloned sequences (ETS1, ETS2, MMS, RCS) and the corresponding OTUs (7, 6, 22, 30) and their 95% confidence interval. Bottom-left: p-distances; Upper-right: 95% confidence interval minimum-maximum

	1	2	3	4	5	6	7	8
1. ETS1		0.1248–0.2190	0.1655–0.2686	0.2109–0.3216	0.1248–0.2190	0.2109–0.3216	0.0000–0.0158	0.1655–0.2686
2. ETS2	0.1667		0.1730–0.2775	0.2033–0.3128	0.0000–0.0158	0.2033–0.3128	0.1248–0.2190	0.1730–0.2775
3. MMS	0.2125	0.2208		0.1994–0.3084	0.1730–0.2775	0.1994–0.3084	0.1655–0.2686	0.0000–0.0158
4. RCS	0.2625	0.2542	0.2500		0.2033–0.3128	0.0000–0.0158	0.2109–0.3216	0.1994–0.3084
5. OTU 6	0.1667	0.0000	0.2208	0.2542		0.2033–0.3128	0.1248–0.2190	0.1730–0.2775
6. OTU 22	0.2625	0.2542	0.2500	0.0000	0.2542		0.2109–0.3216	0.1994–0.3084
7. OTU 7	0.0000	0.1667	0.2125	0.2625	0.1667	0.2625		0.1655–0.2686
8. OTU 30	0.2125	0.2208	0.0000	0.2500	0.2208	0.2500	0.2125	

We further characterize the prevalence of infection of the endosymbionts by identifying the presence of the bacterial 16S rRNA sequences within the DNA extracted from each single tardigrade. The infection prevalence of the bacteria with the sequence ETS1-2 (i.e. attributable to either ETS1 or ETS2) is 9.1% in *E. trisetosus* (i.e. ETS1-2 is detected in two animals of the 22 analysed), the infection prevalence of the bacteria with the sequence MMS is 10.0% (two animals out of 20) in *M. macrocalix*, and the infection prevalence of the bacteria with the sequence RCS is 40.0% (eight animals out of 20) in *Ri. coronifer*.

Finally, using whole mount FISH on *E. trisetosus*, we detect the presence of bacteria on the external surface of the cuticle of all the 40 examined specimens and within the ovary of only three animals (Fig. 4). A strong fluorescent signal of the EUB338-Alexa Fluor 594 probe is observed in the body cavity of all the *E. trisetosus* animals, and especially in the gut region, but we cannot visually resolve individual bacteria.

DISCUSSION

Tardigrade symbionts

The species-specific tardigrade microbiomes include four putative endosymbionts that are characterized by the sequences ETS1, ETS2, MMS and RCS. These endosymbionts have been found so far only in species of the phylum Tardigrada, and are good candidates to be new bacterial species belonging to new bacterial genera. In fact, their identity with the closest named 16S rRNA found in GenBank sequences exceeds the commonly used thresholds for the species (97%) and genera (95%) delimitations (Tindall *et al.*, 2010). These new Anaplasmataceae (Rickettsiales) and Tenuibacteraceae (Holosporales) bacterial taxa are the first putative symbionts in tardigrades. In fact, all known members of Anaplasmataceae (Dumler *et al.*, 2015) are known to have an endosymbiotic lifestyle, and members of Tenuibacteraceae are associated with ecdysozoans (Kroer *et al.*, 2016; Szokoli *et al.*, 2016b). Because we use a near-full length 16S rRNA gene sequence to query the IMNGS database, we are generally able to identify a higher number of corresponding reads compared to a search with short amplicons of the corresponding OTUs, expanding on results by Vecchi *et al.* (2018). The RCS clone found in association with the tardigrade species *Richtersius coronifer* belongs to the recently erected family Tenuibacteraceae (Kroer *et al.*, 2016; senior synonym of *Ca.* Hepatincolaceae in Szokoli *et al.*, 2016b). The RCS's closest named relative is *Ca*. Tenuibacter priapulorum (Kroer *et al.*, 2016), which is associated with the microvilli-lined gut of ecdysozoans. Tardigrades possess a microvilli-lined

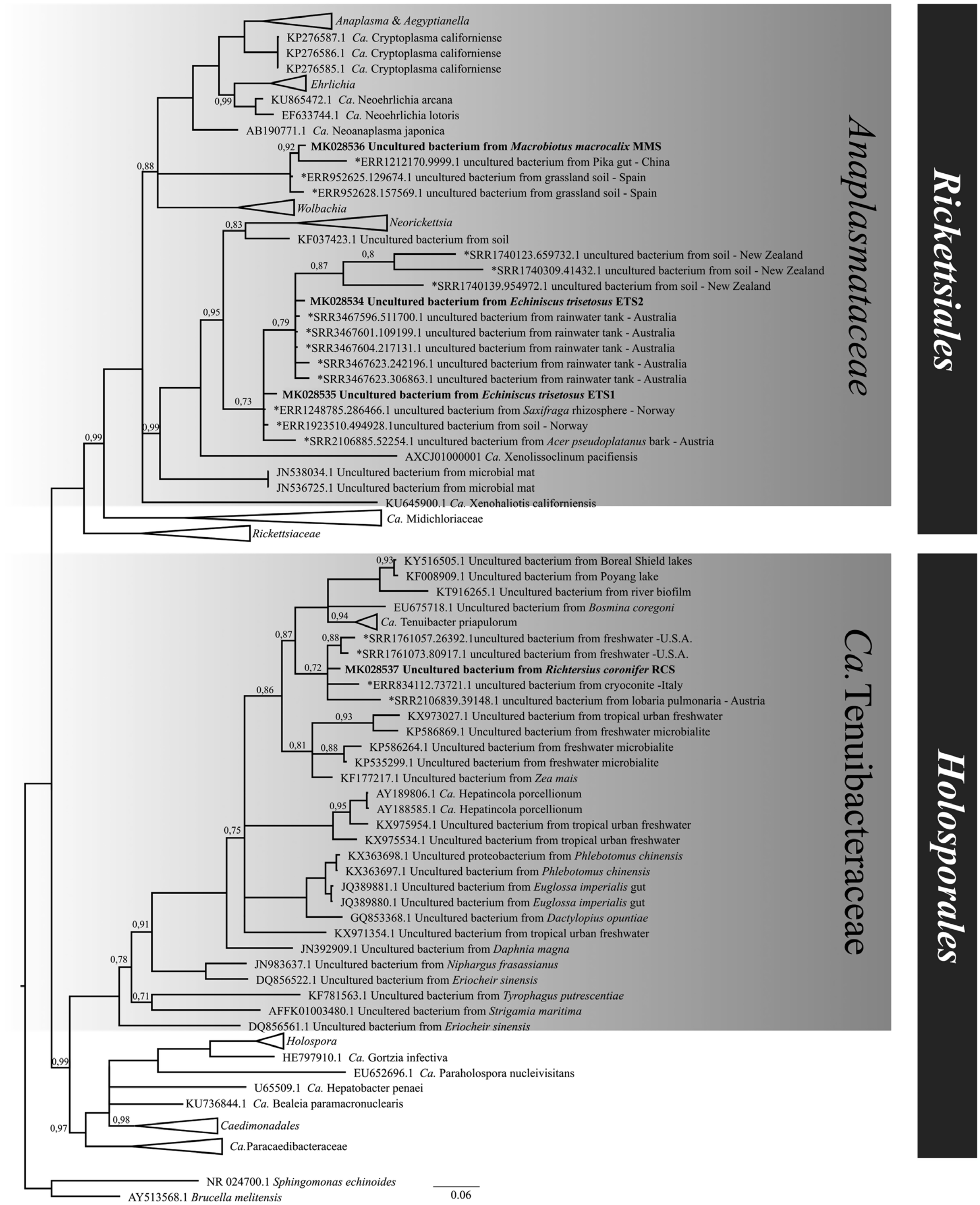

Figure 2. Phylogenetic reconstruction (Bayesian Inference) of the relationships among the cloned bacteria sequences (in bold) found in the three tardigrade species and the representatives of Rickettsiales and Holosporales. Posterior probabilities (PP) values are represented above branches (PP=1 not shown, nodes with PP < 0.75 were collapsed). Scale bar indicates number of changes per site.

gut (Dewel & Clark, 1973; Greven, 1976; Avdonina *et al.*, 2007; Rost-Roszkowska *et al.*, 2011), so RCS may reside in the tardigrade gut. The bacteria characterized by the sequences ETS1 and ETS2, both found in the heterotardigrade *Echiniscus trisetosus*, are members of Anaplasmataceae, but they cannot be assigned to any

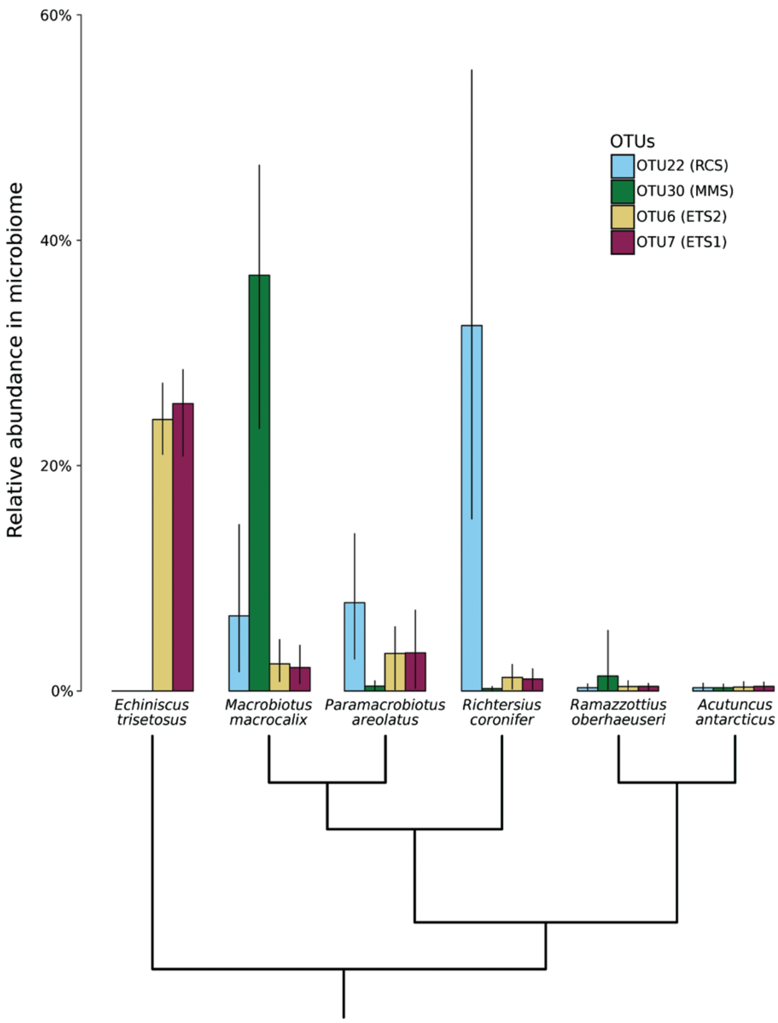

Figure 3 Presence and abundance of the four OTUs (6, 7, 22, 30) representing the four new putative endosymbionts (ETS2, ETS1, RCS, MMS) in the microbiomes of the tardigrade species. Each column represents the average of all of the species replicates analysed by Vecchi *et al.* (2018). Bars on each column indicate range (minimum–maximum). Tardigrade phylogenetic relationships according to Bertolani *et al.* (2014).

genus. It was not possible to determine if these two bacteria (ETS1 and ETS2) co-infect the same animal or are mutually exclusive in an animal, because we were not able to design primers to discriminate between the two. The MMS clone found in *Macrobiotus macrocalix* belongs to the same larger clade as *Wolbachia*, but information about this group is lacking (the closest relatives available in public databases have been found in Pika gut or in soil; Fig. 2).

The relatively low incidence of infection of these four bacteria within tardigrade populations leads us to hypothesize that they are 'secondary symbionts' (i.e. facultative endosymbiotic microorganisms not essential for their host survival and/or reproduction, in contrast to the obligate endocellular symbionts called 'primary symbionts'; e.g. Dale & Moran, 2006). Many insect species are known to harbour various facultative symbionts, belonging to distinct lineages in Alphaproteobacteria and Gammaproteobacteria (Moran *et al.*, 2005; Sakurai *et al.*, 2005). The low prevalence of the RCS sequence in the sampled individuals of the studied tardigrade species is similar to that observed in the related *Ca*. Hepatincola porcellionum symbiont of pillbugs (Wang *et al.*, 2007), leaving the question open whether this tardigrade-associated bacterium is pathogenic, commensal or mutualistic. Finally, the bacteria with the sequences ETS1 and ETS2 are likely facultative symbionts as their closest relatives (*Neorickettsia* and *Ca*. Xenolissoclinum) also infect at low prevalence and can be pathogens (Chae *et al.*, 2003; Kwan & Schmidt, 2013).

Although most secondary bacterial symbionts are either parasitic or commensal for their hosts, in

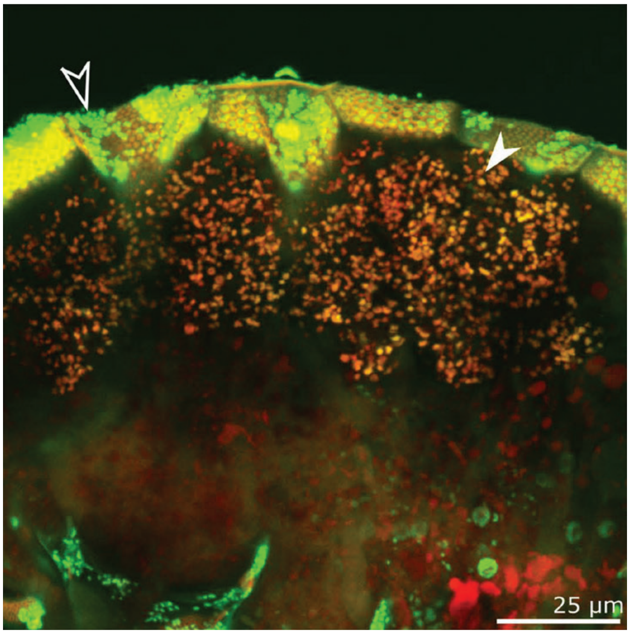

Figure 4. Whole mount fluorescent *in situ* hybridization (FISH) on *Echiniscus trisetosus* (lateral view). DAPI (green) and EUB338-I (red) channel are merged. Empty arrowhead = bacteria on the dorsal cuticle surface. Full arrowhead = bacteria in the gonad.

particular ecological contexts they can positively affect the host fitness (see: Oliver *et al.*, 2003, 2010; Haine, 2008). Therefore, it is possible that the tardigrade secondary endosymbionts can have an impact on tardigrade evolution. Indeed, in spite of the fact that the four putative symbiotic bacteria are not primary symbionts, a close evolutionary link between the bacteria and their hosts is suggested by the specificity of these bacteria to each tardigrade clade: some tardigrade evolutionary lineages have specific bacteria, while other lineages have none of them (Fig. 3). Moreover, the impact of secondary symbionts on host fitness might depend on the environment: they might be beneficial in one environment and deleterious in another (Haine, 2008).

Vertical transmission of bacteria

Evidence of the close link between tardigrades and their microbiomes is suggested by our finding of bacteria within the ovary of the parthenogenetic population of *E. trisetosus*, which hints at possible maternal transmission from mother to offspring (i.e. vertical transmission). The proportion of animals with infected gonads (7.5% found with FISH) is similar to the proportion of animals found positively infected by PCR (9.1% found with diagnostic PCR). The bacteria in the ovary have not been yet identified (Fig. 4), but it is probable that they correspond to those characterized by the sequences ETS1 and ETS2 given their abundance in tardigrade microbiomes, their phylogenetic affiliation with bacteria known to be endosymbionts infecting oocytes and the comparable infection prevalence between the individuals analysed with the specific diagnostic PCR and FISH.

In general, symbionts that are vertically transmitted must either increase the fitness of their host or manipulate host reproduction in ways that benefit their own transmission in order to be maintained in host populations (for a review see: Haine, 2008). Symbiotic bacteria that are transmitted vertically are common among arthropods. Some of these bacteria are fundamental for host survival (see: Chen *et al.*, 1999), others are facultative, but they can increase host resistance to parasitoids (see Oliver *et al.*, 2003). Others manipulate host reproduction to enhance their transmission; for example, by distorting the sex ratio of the host towards females, the sex that will transmit them to future generations. Bacterial symbionts influencing host reproduction (e.g. *Rickettsia* spp., *Wolbachia* spp. and *Cardinium* spp.) are common and widespread in arthropods (see: Werren *et al.*, 1995; Zchori-Fein & Perlman, 2004; Weinert *et al.*, 2007), and many strains are not completely penetrant, infecting only a small proportion of the host population (Jiggins *et al.*, 2001). However, phenotypes induced by vertically transmitted symbionts are often difficult to ascertain, as they can have few effects, mutualistic or pathogenic, and may not influence host reproduction (see: Haine, 2008).

Four new putative endosymbionts from the families Anaplasmataceae and Tenuibacteraceae were identified and associated with tardigrade species of different ecological niches and belonging to different evolutionary lineages. These bacteria are characterized by their 16S rRNA genes and here we provide tools for their identification and infection prevalence rates. This work highlights how accounting for 'minor' or 'neglected' phyla in microbiome and symbiosis studies can lead to the discovery of new diversity in biotic relationships and unexplored bacteria biodiversity.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site.

Table S1. GenBank accession numbers of the bacterial sequences used for the phylogenetic analysis.
Table S2. Prevalence of common OTUs in tardigrade microbiomes (found by Vecchi *et al.*, 2018) in the IMNGS database (queried November 2017). Numbers of OUTs as in Figure 1.