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„Molecular characterization of an *Oceanospirillales* bacterium associated to males of the marine nematode *Laxus oneistus*"

verfasst von / submitted by

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Table of Contents

Acknowledgements

In 2011 I started the work on my Diplomathesis together with Silvia Bulgheresi. At one point we ran out of samples, but Silvia had another back-up project for me. In the project from Harald Gruber-Vodicka (a former member of the Shallow Water Symbiosis Group of Vienna), the metagenome of *Laxus oneistus* nematodes was *de novo* assembled and the contigs were binned according to their coverage and GC content, which led to the identification of a distinct bin that was assigned to the order *Oceanospirillales*. Silvia thought that the characterization of this *Oceanospirillales* bacterium would make the ideal back-up project from me and I immediately agreed to work on this exciting task. At that time, I was studying both Ecology and Zoology at the University of Vienna and needed two separate projects to complete both of my studies. Therefore, I also continued working on the other project, went on a sampling trip and eventually finished first my Ecology Diplomathesis on the "Molecular identification and life cycle of the giant ectosymbiont of *Eubostrichus dianeae".* Although I finished the laboratory work for my Zoology Master thesis, I did not manage to finish all the necessary exams, write up and hand in the thesis to graduate in Zoology. Meanwhile I started my PhD project on the reproduction modes of host attached bacteria and the "*Oceanospirillales*-project" had to wait again. I got my PhD and moved to Paris to work at the Institut Pasteur for my Postdoc – finally the time had come to finish that Master in Zoology!

I really want to thank Silvia for all her patients and support for letting me finish my Zoology Master. She is a great supervisor, scientist, inspiration and meanwhile friend and I really miss to work with her (and the nematodes) – and of course the amazing sampling trips to the paradise island Carrie Bow Cay in Belize.

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Abstract

Laxus oneistus is a marine nematode coated with sulfur-oxidizing *Gammaproteobacteria* of the genus *Candidatus* Thiosymbion. Up to this study this ectosymbiont was the only bacterium known to be stably associated to this nematode. In previous sequencing efforts, the metagenome of *L. oneistus* was *de novo* assembled and the contigs were binned according to their coverage and GC content. This led to the identification of a distinct bin that was assigned to the order *Oceanospirillales*. PCR with primers specific to the 16S rRNA-gene sequence present in one of the *Oceanospirillales*-derived contigs indicated that this bacterium is mostly, if not only, associated to *L. oneistus* males. Phylogenetic analysis of the 16S rRNA-gene sequences obtained from *L. oneistus* males showed that the putative second symbiont is most closely related to bacterial species of the genus *Thalassolituus.* Fluorescence *in situ* hybridization confirmed the association to male individuals and revealed that *Oceanospirillales*-like bacterial aggregates vary in size and are irregularly distributed in the vicinity of the male reproductive organs. At the same time, the sequences obtained by PCR from female individuals could not be assigned to *Oceanosprillales*-derived contig, and we could not detect any bacterial aggregates within female nematodes. The association of *L. oneistus* with *Oceanospirillales*-like bacteria that reside in the testis region is the first description of a bacterium exclusively associated to the male reproductive tract. The exact nature of this relationship, as well as the endosymbiont transmission mode remain to be identified.

Zusammenfassung

Laxus oneistus ist ein mariner Fadenwurm, dessen Haut mit schwefeloxidierenden Gammaproteobakterien der Gattung *Candidatus* Thiosymbion bedeckt ist. Bis *dato* war dieser Ektosymbiont das einzig bekannte Bakterium, welches dauerhaft mit dem Fadenwurm assoziiert ist. In früheren Sequenzierungsbemühungen wurde das Metagenom von *L. oneistus de novo* assembliert und die Contigs wurden entsprechend ihrer Coverage und ihrem GC-Gehalt eingeteilt. Dadurch konnte ein Cluster identifiziert werden, welches der Ordnung *Oceanospirillales* zugeordnet wurde. PCR mit Primern, die spezifisch für die 16S rRNA-Gensequenz aus einem der *Oceanospirillales*-Contigs sind, zeigte, dass dieses Bakterium größtenteils, wenn nicht gar ausschließlich mit männlichen Individuen von *L. oneistus* assoziiert ist. Die phylogenetische Analyse dieser 16S rRNA-Gensequenzen von *L. oneistus* Männchen zeigte, dass der potentielle sekundäre Symbiont am engsten mit Bakterienarten der Gattung *Thalassolituus* verwandt ist. Die Fluoreszenz-*in-situ*-Hybridisierung bestätigte die Assoziation mit männlichen Individuen und zeigte, dass *Ozeanospirillales*-ähnliche bakterielle Aggregate in ihrer Größe variieren und in der Nähe der männlichen Fortpflanzungsorgane unregelmäßig verteilt sind. Gleichzeitig konnten die mittels PCR von weiblichen Individuen gewonnenen Sequenzen nicht dem *Oceanosprillales*-Contig zugeordnet werden, und wir konnten auch keine bakteriellen Aggregate in weiblichen Nematoden nachweisen. Die Assoziation von *L. oneistus* mit *Ozeanospirillales*-ähnlichen Bakterien, die sich in der Testis-Region befinden, ist die erste Beschreibung eines Bakteriums, das ausschließlich im männlichen Reproduktionstrakt zu finden ist. Die genaue Art dieser Beziehung sowie der Übertragungsmodus der Endosymbionten müssen noch ermittelt werden.

Introduction

Over the past few years, lineages from the order *Oceanospirillales* (*Gammaproteobacteria*) have been repeatedly associated with diverse marine invertebrates. In shallow-water habitats, they are commonly found in the tissues and mucus of temperate and tropical gorgonian and scleractinian corals (Bayer et al., 2013b, 2013a; Bourne et al., 2013; Chen et al., 2013; La Rivière et al., 2013; Sunagawa et al., 2010), as well as in marine sponges (Flemer et al., 2012; Kennedy et al., 2008; Nishijima et al., 2013; Thiel et al., 2007). They have also been detected in the gills of several shallow-water bivalves, such as grooved carpet shell clams (Costa et al., 2012) and in invasive oyster species (Zurel et al., 2011), as well as in the tentacles and the coelom wall of sea anemones (Du et al., 2010; Schuett et al., 2007). Likewise, they are present in the tunic of ascidians (Martínez-García et al., 2007), in the gastro-intestinal tract of a nudibranch (Kurahashi and Yokota, 2007) and on the skin of starfish (Choi et al., 2010). In deep-sea habitats, *Oceanospirillales* have been found inhabiting the gills of hydrothermal vent and hydrocarbon seep bivalves (Beinart et al., 2014; Jensen et al., 2010; Zielinski et al., 2009). Further, they have been observed in organisms that are specifically found at whale-falls, residing in the ovisac and root system of the polychaete worm *Osedax* (Goffredi et al., 2005; Verna et al., 2010) and in the gill tissue of the gastropod *Rubyspira* (Johnson et al., 2010). *Oceanospirillales* are not only associated to marine invertebrates, but they also exist as endosymbionts of whiteflies (Baumann, 2005).

To date, very little is known about the nature of these animal-bacterial relationships. *Oceanospirillales* inhabit diverse niches, they are able to grow in aerobic, microaerophilic to facultative anaerobic environmental conditions. All cultivated members are heterotrophs known for deriving their energy from the degradation of complex organic compounds (Garrity et al., 2005). Therefore, several hypotheses have been proposed about the functional role of the animal-associated *Oceanospirillales* ranging from beneficial symbionts that assist in the nutrition of the host (*e.g.* in *Osedax* ; Goffredi et al., 2005) or sulfur cycling (*e.g.* in scleractinian corals; Raina et al., 2009) to parasites, which invade the nuclei of bathymodiolin mussels and utilize their chromatin as nutrition (Zielinski et al., 2009).

Here, we report a novel *Oceanospirillales* phylotype discovered after sequencing *Laxus oneistus* nematode together with their ectosymbionts, and the *de novo* metagenome assembly. *L. oneistus* belongs to a small subfamily of free-living marine nematodes, the

Stilbonematinae (Desmodoridae, Nematoda). These nematodes are characterized as being coated with ectosymbiotic bacteria in a species-specific manner (Ott et al., 2004b, 2004a, 1991). All *Stilbonematinae*-associated bacteria described so far belong to sulfur-oxidizing *Gammaproteobacteria* related to the *Chromatiaceae* (Bayer et al., 2009; Bulgheresi et al., 2011; Pende et al., 2014; Polz et al., 1994) and belong to the *Candidatus* Thiosymbion genus (Zimmermann et al., 2016; formerly Marine Oligochaete and Nematode Thiotrophic Symbionts (MONTS) cluster; Heindl et al., 2011). In the case of *L. oneistus*, the bacterial coat is formed by a monolayer of upright standing, rod-shaped bacteria, belonging to a single 16S-rRNA gene phylotype (Polz et al., 1994). However, the anterior (head) and the tip of the posterior region are symbiont-free and the nematodes cuticle reduces in diameter in correspondence of the onset of the bacterial coat (Urbancik et al., 1996) (Fig. 1).

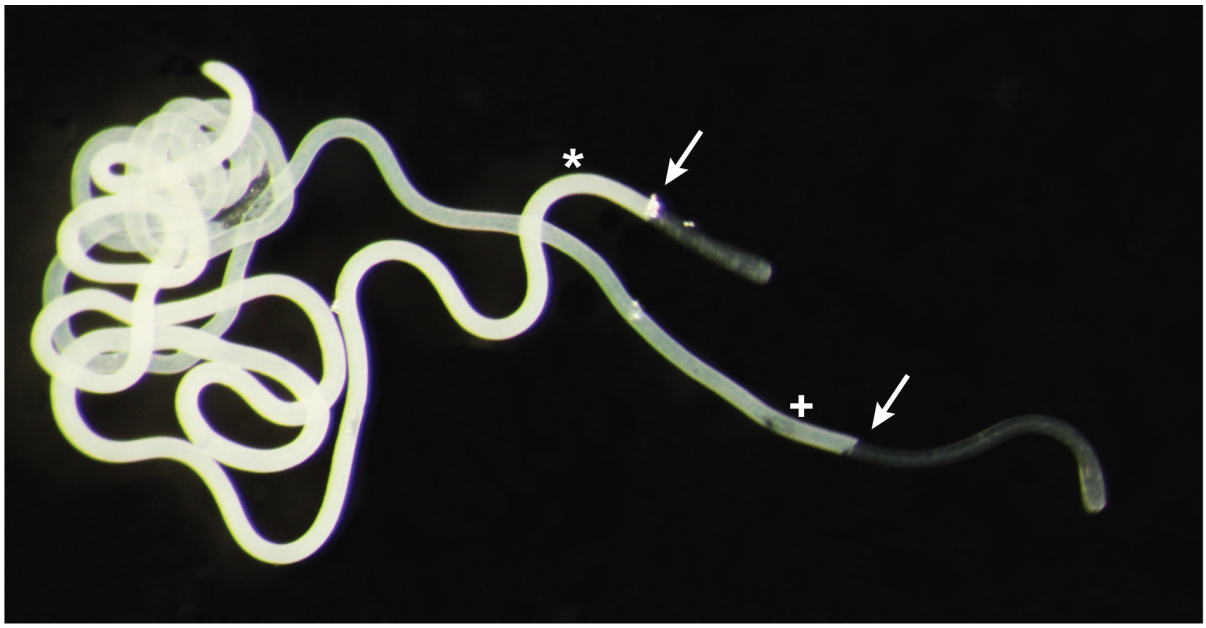

Figure 1 Light microscopy image of *L. oneistus* nematodes and their ectosymbiont. Arrows are indicating the onset of the bacterial coat, that appears bright white due to sulfur storage within the bacteria. (*) is indicating the female *L. oneistus* and (+) the male one. The symbiont free head region of the male individual is 2-3 times longer than the one of the female nematode.

Stilbonematinae are globally distributed, but are especially abundant in tropical shallow-water, sheltered, calcareous sands (Ott and Novak, 1989; Ott et al., 1991). Characteristically, in these sediments an oxidized surface layer overlays a sulfidic, anoxic one (Ott and Novak, 1989). The nematodes are thought to migrate between the sediment layers in order to provide

their bacterial symbiont with both oxygen (or alternatively nitrate) and hydrogen sulfide or other sulfur compounds (*e.g.* thiosulfate). In a process called chemosynthesis, these compounds are used for sulfur-oxidation and the resulting energy is used for carbon fixation (Hentschel et al., 1999; Ott et al., 1991). The ectosymbionts supply their hosts with nutrients (*e.g.* organic carbon molecules) and likely protect them against sulfur intoxication by converting the reduced sulfur compounds into non-toxic compounds (Hentschel et al., 1999; Ott et al., 1991).

This study describes a second, previously uncharacterized, bacterial symbiont of the marine nematode *L. oneistus*. Previous sequencing efforts led to the identification of a distinct bin that was assigned to the order *Oceanospirillales*. Amplification of the 16S rRNA-gene present in one of the *Oceanospirillales*-derived contigs indicated that this bacterium is mostly if not only present in *L. oneistus* males. We could also confirm these results by fluorescence *in situ* hybridization.

Methods

Nematode collection

Sediment samples were collected in December 2011 and January 2012 in approximately 1 m depth from a sand bar off Carrie Bow Cay, Belize (16°48'11.01"N, 88°4'54.42"W). Specimens of *L. oneistus* were extracted from the sediment by stirring the sand in seawater and pouring the supernatant through a 63-µm-pore-size mesh sieve. The retained material was transferred into a Petri dish, and single nematodes were picked by hand using fine tweezers under a dissecting microscope. For genomic DNA (gDNA) extraction, PCR, and Fluorescence *in situ* Hybridization (FISH) nematodes were fixed in methanol and transported and stored at −20 °C. All samples were deep-frozen for transportation and storage.

Distinction of *L. oneistus* male and female nematodes

Methanol-stored nematodes were rehydrated in 1xPBS. Single worms were mounted in a droplet of 1xPBS on a microscope slide. With the corners of a coverslip beeswax was scratched to create spacers of wax. When the coverslip was placed over the nematode in the 1xPBS droplet the beeswax spacer prevented the nematode from being squeezed. The slide was then examined with a Nikon Eclipse 50i microscope. The sex of each nematode was distinguished as described (Ott et al., 1995). In short, the most remarkable feature is that males have at the posterior end a spicular apparatus, while this is absent in females. Additionally, male individuals have a single testis at 39-46% of body length, whereas females have paired ovaries and extremely long ova (eggs: up to 620 µm). The symbiont free anterior region of males is 2-3 times longer than the one of females (Fig. 1). Identified male and female nematodes were stored in 100% methanol for either gDNA extraction and PCR or for FISH.

Genomic DNA extraction from single nematodes

For the construction of *L. oneistus* 16S rRNA gene libraries gDNA was extracted from single male and female nematodes as previously described (Schizas et al., 1997). In short, methanol-stored nematodes were washed 2 times in 1xPBS. Single nematodes were placed in 0.2 ml PCR reaction tubes (one nematode per tube) containing 10 µl of Dream Taq PCR reaction buffer

(Fermentas). In each reaction tube 1 µl of 10 mg/ml proteinase K solution was added and subsequently the tubes were incubated at 55°C for 3 hr in a PCR machine. In order to inactivate the proteinase K the tubes were incubated at 99°C for 5 min. Subsequently, the nematodes in the tubes were subjected to three cycles of freezing in liquid Nitrogen for 3 min and thawing at 65°C for 3 min. After this procedure, 12 µl of GeneReleaser (Eurogentec, Seraing, Belgium) were added to each tube. The tubes were incubated in a PCR machine following the GeneReleaser manufacturer's thermal cycle protocol: 65°C for 30 sec, 8°C for 30 sec, 65°C for 90 sec, 97°C for 3 min, 8°C for 1 min, 65°C for 3 min, 97°C for 1 min, 65°C for 1 min, and finally 80°C for at least 1 min. The tubes were centrifuged at 13,000 g for 1 min. The clear supernatant (gDNA) was transferred to a fresh tube and used for PCR.

PCR and cloning of 16S rRNA genes

As template, 2 µl of gDNA were used in each 50 µl PCR reactions. 1,499 nt-long fragments of the 16S rRNA-gene were amplified by PCR with bacterial primers 616V (5"-AGAGTTTGATYMTGGCTC-3´; (Juretschko et al., 1998) and 1492R (5´-GGYTACCTTGTTACGACTT-3´; (Kane et al., 1993). Further, specific *Oceanospirilalles* 16S rRNA-gene primers, designed based on the metagenomic-derived 16S sequence, O833F (5'-CAAAGGATCAAGTCCCCCG-3') in combination with 616V and O833R (5'-CGGGGGACTTGATCCTTTG-3') together with 1492R were used to amplify 836 nt-long fragments and 666 nt-long fragments, respectively. Specific *Oceanospirilalles* 16S rRNA-gene primers were designed according to the 16S rRNA-gene sequence obtained from the *L. oneistus* metagenome assembly and annotation.

The cycling conditions for the 16S rRNA amplification were as follows: 94°C initial denaturation for 4 min, followed by 35 cycles with each 45 sec denaturation at 94°C, 30 sec annealing at 50°C and 1 minute and 45 sec elongation time at 72°C, and a final elongation of 10 min at 72°C. PCR products with the expected fragment size of approx. 1.5 kb, 850 bp and 700 bp for the 16S rRNA-gene and the *Oceanospirillales* specific 16S rRNA-gene fragments, respectively were cut out of the gel. Gel fragments were purified and directly sequenced in both directions. All sequences were aligned and compared by using the CodonCode Aligner 3.7.1 software. By aligning the sequences obtained by the O833 forward and reverse primers a 1484 nt-long sequence was generated.

16S rRNA gene-based phylogenetic analysis

A bacterial 16S rRNA-gene dataset was assembled by adding sequences from GenBank with >95% identity to the *Oceanospirillales* sequence using BLASTN (Altschul et al., 1990) and selected members of *Oceanospirillales*, as well as *Thiotrichales* and *Cardiobacteriales* (based on a tree from Kim et al., 2007) as outgroups. The sequences were aligned with MAFFT LINSI (Katoh and Standley, 2013). The alignment was trimmed to keep only phylogenetically informative sites to a 1419 nt-long sequences with BMGE-1.1 (Criscuolo and Gribaldo, 2010). To reconstruct the phylogenetic relations, maximum likelihood (IQtree, GTR+F+I+G4 model was selected as it includes the most parameters and fits best the evolutionary and biological proces; Nguyen et al., 2015) and Bayesian inference (PhyloBayes, CAT+GTR+G4 model, burnin of 25%; Lartillot et al., 2009) based algorithms were used. The node stability was evaluated by performing a bootstrapping analysis for IQtree (100 replicates; 80% support was considered significant) and posterior probabilities for PhyloBayes (0.9 support was considered significant). Trees obtained by the two algorithms were visualized in iTOL (Letunic and Bork, 2019) and merged to a single tree with indication of the node stability evaluated by both methods mentioned above, in Illustrator CC (Adobe Systems, USA).

16S rRNA gene alignment

The sequence of the *Thalassolituus marinus* strain IMCC1883 16S ribosomal RNA gene (Sequence ID: HM569768.1) had the highest sequence identity with the *Oceanospirillales* 16S rRNA-gene amplified and sequenced from *L. oneistus* male nematodes. The two 16S rRNA-genes nucleotide sequences were aligned using Clustal Omega. Relevant information was indicated in the alignment by using Illustrator CC (Adobe Systems, USA).

Fluorescence *in situ* hybridization (FISH)

By using the arb PROBE_DESIGN tool (the arb software package Ludwig et al., 2004), we designed the FISH probes (O833) specific to the *Oceanospirillales* sequence identified in the *L. oneistus* 16S rRNA-gene libraries. We confirmed the specificity by comparing it with the Arb-Silva SSU 132 database (Pruesse et al., 2007). The O833 probe had 0/5/5/178 non-target hits allowing for 0/1/2/3 mismatches. The probe was fluorescently labelled on its 5' end (Thermo

Fisher Scientific, Ulm, Germany). FISH was performed on male and female *L. oneistus* nematodes according to the study by (Manz et al., 1992), with slight adaptations. To determine stringent hybridization conditions, a formamide series was conducted for all the probes (10%, 20%, 30%, 40% and 50%; refer to Tab. 1 for optimal incubation time, formamide percentage and probe concentrations). In short, single male and female nematodes were placed in different wells of Teflon coated slides and air dried. Afterwards 20 µl of hybridization buffer was added to each well of the slide together with 2 µl of each fluorescent probe. Slides were incubated over night at 46°C. On the next day each well was rinsed 3 times for 3 min with corresponding 48°C warm washing buffer and one time with ice cold water, and quickly air dried under a weak air stream. Nematodes were mounted in antifading medium Vectashield (Vector Labs, Burlingame, CA, USA).

Table 1. Probes used for FISH

Probe	Specificity	Sequence/5' modification	Target RNA	Position*	Formamide %/incubation time (h)/probe concentration (ng µl $^{-1}$)	Reference
GAM42a	*Gammaproteobacteria*	5'-GCC TTC CCA CAT CGT TT-3' Fluorescein	23S	1027-1043	20%/12/3.8	(Manz et al., 1992)
MONTS-1	Marine Oligochaete and Nematode Thiotrophic Symbionts	5'-CTC AAA TGA GCC CAA CGG CTA GT-3' Cy3	16S	824-846	20%/12/2.4	This study
O833	*L. oneistus* endosymbiont	5'-CAA AGG ATC AGG TCC CCC G-3' Cy5	16S	833-852	20%/12/2.4	This study
O833mis	1 mismatch to *L. oneistus* endosymbiont	5'- CAA AGG ATC TAG TCC CCC G-3' Cy5	16S	833-852	20%/12/2.4	This study

*16S rRNA position, *E. coli* numbering (Brosius et al., 1978)

Fluorescence microscopy

Slides containing 10 male and 10 female nematodes stained with FISH probes were imaged using a Nikon Eclipse NI-U microscope equipped with a MFCool camera (Jenoptik). Images were acquired using the ProgRes Capture Pro 2.8.8 software (Jenoptik). Additionally, images of unstained male *L. oneistus* were taken by Leica TCS-SP2 confocal laser-scanning microscope combined with an inverted DM-IRE2 microscope (Leica Microsystems, Heidelberg, Germany).

Microscope images were processed using the public domain program Fiji, for representative images, the background subtraction function of Fiji was used on all three fluorescent channels and brightness was maximally enhanced for the far-red and brightness and contrast were adapted in the red channel images. Figures were compiled using Illustrator CC (Adobe Systems, USA).

Results

Oceanospirillales-like bacterial phylotype is found in gDNA extracts of male *L. oneistus* nematodes

In a previous study, the draft genome of *L. oneistus* nematodes together with their bacterial symbionts was assembled and annotated (Leisch et al., 2012). *L. oneistus*-derived contigs were distinguished from *Ca.* T. oneisti-derived ones by binning contigs based on GC content and coverage (Leisch et al., 2012). Interestingly, a potential second symbiont was identified with low coverage, but high GC content (Fig. 2a; unpublished data from H. Gruber-Vodicka). A MEGAN-based 16S rRNA-gene analysis of the putative second symbiont assigned it to an *Oceanospirillales*-like and more specifically to an *Oceanobacter*-like bacterium (Fig.2b; unpublished data from H. Gruber-Vodicka). To molecularly identify the bacteria found by the MEGAN analysis, we designed specific primers for this order of bacteria.

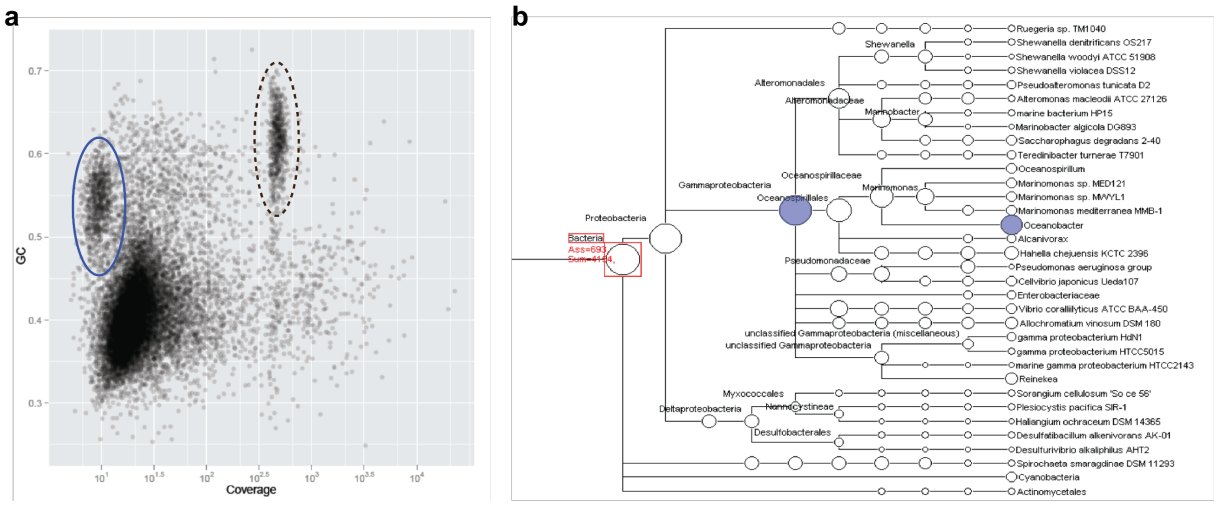

Figure 2 Metagenome assembly of *L. oneistus* and its symbiont. (**a**) Assembled contigs were binned based on their % GC content and coverage. Dotted black line ellipse is specifying the contigs from the ectosymbiont *Ca*. T. oneisti with 350X-600X coverage and 55.0 % GC content. Full blue line ellipse is indicating contigs of the putative second symbiont with less than 25X coverage and high % GC content. The rest represents contigs of the *L. oneistus* host with 20X-40X coverage and 41.3 % GC content and contaminants. (**b**) MEGAN-based identification (Huson et al., 2007) of the putative endosymbiont. Each circle represents a taxon in the NCBI taxonomy and is labeled by its name. The size of the circle is scaled logarithmically to represent the number of contigs assigned directly to the taxon. Most of the contigs were assigned to *Oceanospirillales* and more specific to an *Oceanobacter*-like bacterium (blue spots). Unpublished data from H. Gruber-Vodicka.

Single male and female nematodes of *L. oneistus* were identified by light microscopy according to the anatomic features described in (Ott et al., 1995). Their gDNA was extracted and the 16S

rRNA-gene was amplified by PCR with *Oceanospirillales*-specific primers (0833F and O833R). Fragments amplified with the *Oceanospirillales*-specific primers of male individuals gave single sharp bands of the correct size, whereas with the gDNA of female nematode we obtained several bands of different sizes indicating multiple amplification (Fig. 3). The PCR fragments from three males and three females were directly sequenced and only the 16S rRNA sequences amplified by the *Oceanospirillales*-specific primers from males corresponded to the one obtained by the metagenome analysis. The sequences from female individuals were not corresponding to the one obtained by the metagenome analysis, but to other environmental *Oceanospirillales*-like bacteria. These results indicate that the putative secondary symbiont is primarily, if not only, associated with *L. oneistus* males.

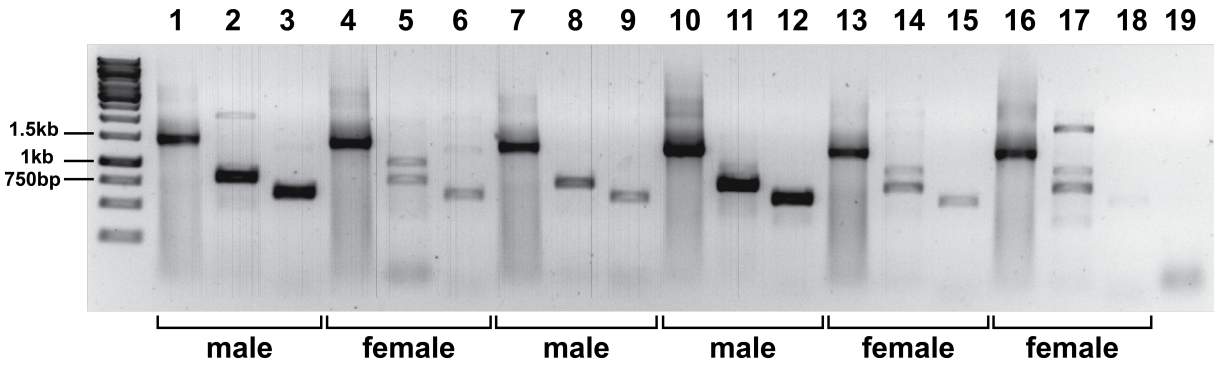

Figure 3 PCR with *Oceanospirillales* 16S rRNA-gene specific primer (0833). PCR on gDNA obtained from three single males and females. In lanes 1, 4, 7, 10, 13, 16 and 19 a 1,499nt-long fragment is shown amplified with the general 16S rRNA primers 616V and 1492R. Primers used in lanes 2, 5, 8, 11, 14 and 17 are the *Oceanospirillales*-specific primer O833F together with 616V. Primers used in lanes 3, 6, 9, 12, 15 and 18 are the *Oceanospirillales*-specific primer O833R together with 1492R. With the gDNA of male individuals an 836nt-long and a 666nt-long fragment were amplified using the *Oceanospirillales*-specific primers that was corresponding to the 16S rRNA found in the metagenome assembly. Lane 19 is the negative control (water).

Phylogenomic analysis revealed that the *Oceanospirillales*-like bacterial phylotype is most closely related to the genus *Thalassolituus*

To determine the relationship of the potential secondary symbiont of *L. oneistus* with other *Oceanospirillales*, a maximum likelihood and Bayesian inference was used to construct a 16S rRNA gene-based phylogenetic tree. The constructed 16S rRNA gene tree shows that the putative secondary symbiont 16S RNA gene falls within the well supported family of *Oceanospirillaceae* and is most closely related to diverse *Thalassolituus sp.* (Fig. 4). However, the deeper relationship between the putative second symbiont and other members within the *Oceanospirillaceae* is not clear as these nodes are not well supported. A 16S rRNA-gene blastn search sowed that *Thalassolituus marinus* strain IMCC1883 16S ribosomal RNA gene (Sequence ID: HM569768.1) has 98.99% (1469 nt/1484 nt) sequence identity over 100% coverage and no gaps with the *Oceanospirillales* 16S rRNA-gene amplified and sequenced from *L. oneistus* male nematodes (Fig. 5). Based on the common 97% sequence identity threshold for bacterial species, the bacterium associated to *L. oneistus* males could be considered as a *Thalassolituus sp.*

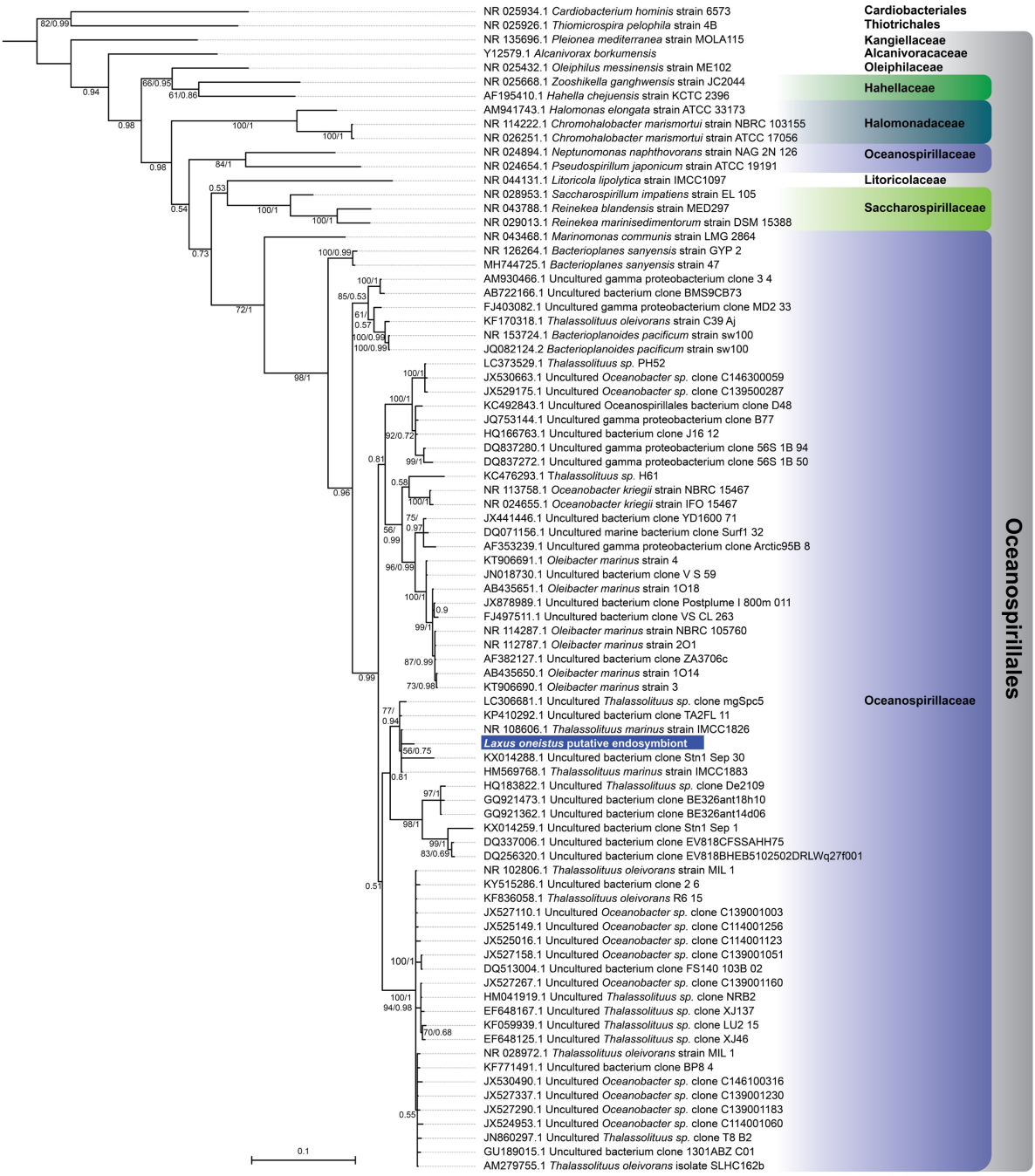

Figure 4 Maximum likelihood and Bayesian inference phylogenetic tree based on 16S rRNA sequences. The tree was built with sequences that had > 95% sequence similarity with the one obtained from the *L. oneistus* male putative second symbiont and those of other members of *Oceanospirillales*, as well as *Thiotrichales* and *Cardiobacteriales* as outgroups. To reconstruct the phylogenetic relations maximum likelihood (IQtree, GTR+F+I+G4 model) and Bayesian inference (PhyloBayes, CAT+GTR+G4 model, burnin of 25%) based algorithms were used. The node stability was evaluated by performing a bootstrapping analysis for IQtree (80% support was considered significant; number on the left) and posterior probabilities for PhyloBayes (0.9 support was considered significant; number on the right). If supports were lower than 50% bootstrapping or 0.5 posterior probabilities they were not indicated in the tree. Scale bar represents the average number of substitutions per nucleotide position.

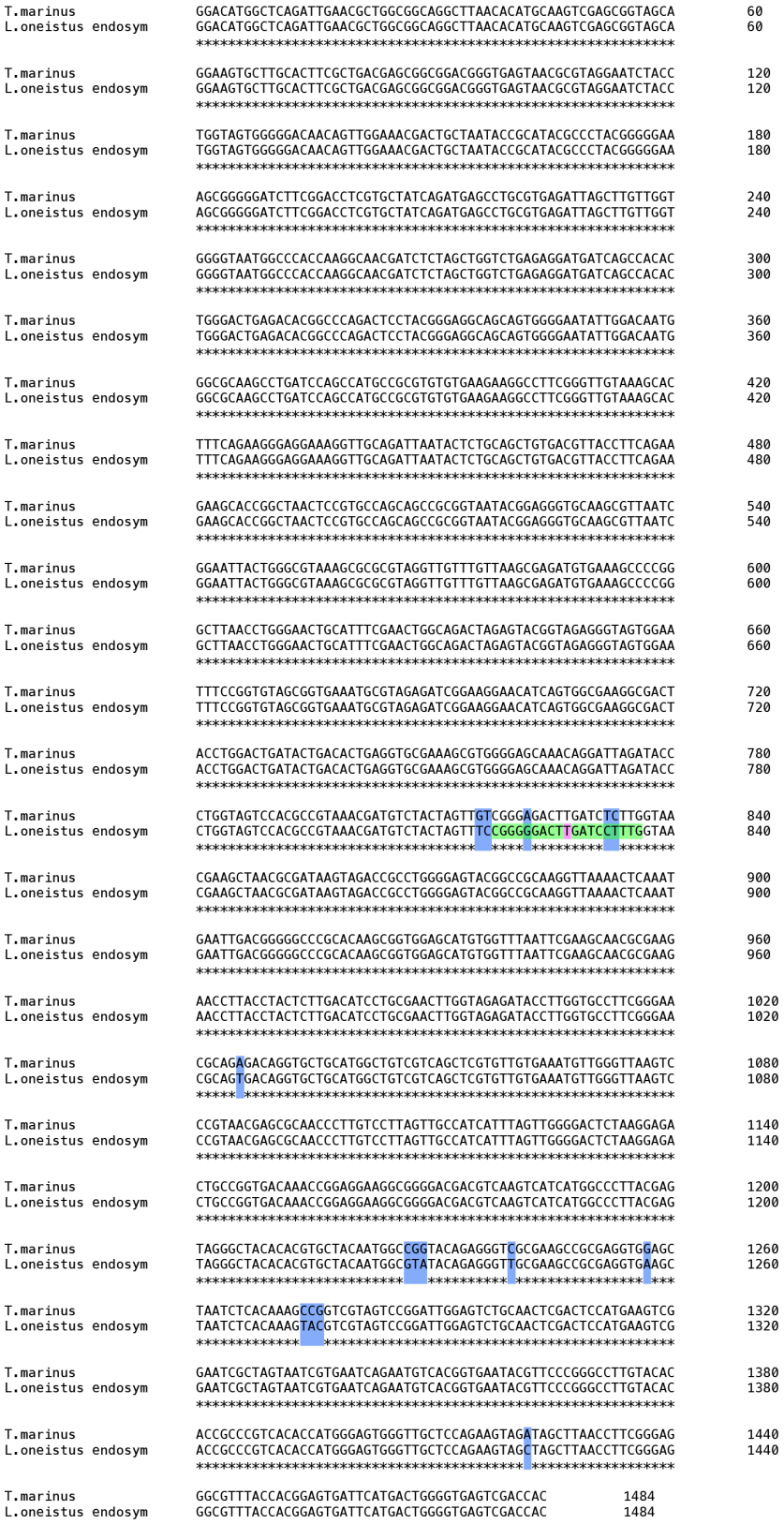

Figure 5 Nucleotide sequence alignment of the 16S rRNA-gene of *Thalassolituus marinus* and the putative second symbiont of *L. oneistus.* Asterisk (*) indicated nucleotides which are fully conserved. Blue boxes indicate nucleotides that differ between the two sequences. Green box is indicating the binding region of the *Oceanosprillales*-specific primes (O833F and R), as well as the O833 FISH-probe binding site. The single nucleotide mismatch of the O833mis FISH-probe is marked in pink.

The *Oceanospirillales*-like bacterium is associated to *L. oneistus* males

Previous studies showed that other marine invertebrates (*e.g.* bone-eating polychaetes and mussels) are commonly associated with bacteria belonging to the order of *Oceanospirillales* and that some of them are autofluorescent (Goffredi et al., 2007; Jensen et al., 2010). When observing several *L. oneistus* individuals, we were able to see slight green autofluorescence patches in male nematodes when excited with blue light (Fig. 6).

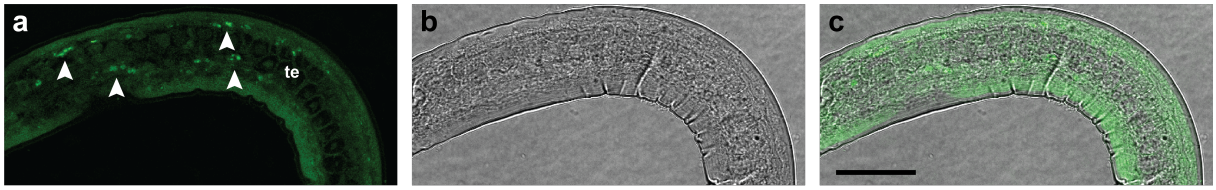

Figure 6 Laser scanning confocal microscope images of a *L. oneistus* male. A male nematode was excited with blue light and its autofluorescence was detected. (**a**) Green fluorescent channel showing auto fluorescence of the nematode with stronger fluorescent patches (arrow heads) in the vicinity of the testis region (te). (**b**) is the corresponding bright field image and (**c**) the overlay of both channels. Scale bar is 50 µm.

To confirm that the *Oceanospirillales*-like 16S rRNA sequences obtained in our libraries originated from these autofluorescence patches detected in male individuals, we applied a FISH probe (O833 in Cy5) specifically targeting the sequence to whole mount *L. oneistus* male and female nematodes. Importantly, the probe was coupled to a far red fluorochrome to avoid bleed through from the green channel. The previously observed patches in *L. oneistus* males (Figure 6) were double stained by the *Gammaproteobacteria*-specific probe Gam42a and by the *Oceanospirillales* 16S rRNA-gene-specific probe O833 (Fig. 7a-c, arrow heads are pointing to aggregates that were enlarged in the inlays). Whereas the bacteria attached to the host surface were only double stained by the Gam42a probe and the Marine Oligochaete and Nematode Thiotrophic Symbionts probe MONTS-1 (Fig. 7). Curiously, the aggregates of *Oceanospirillales* were found in the vicinity of the male testis region (Fig. 6 and 7a-c). The number and exact localization of the aggregates varied between individuals.

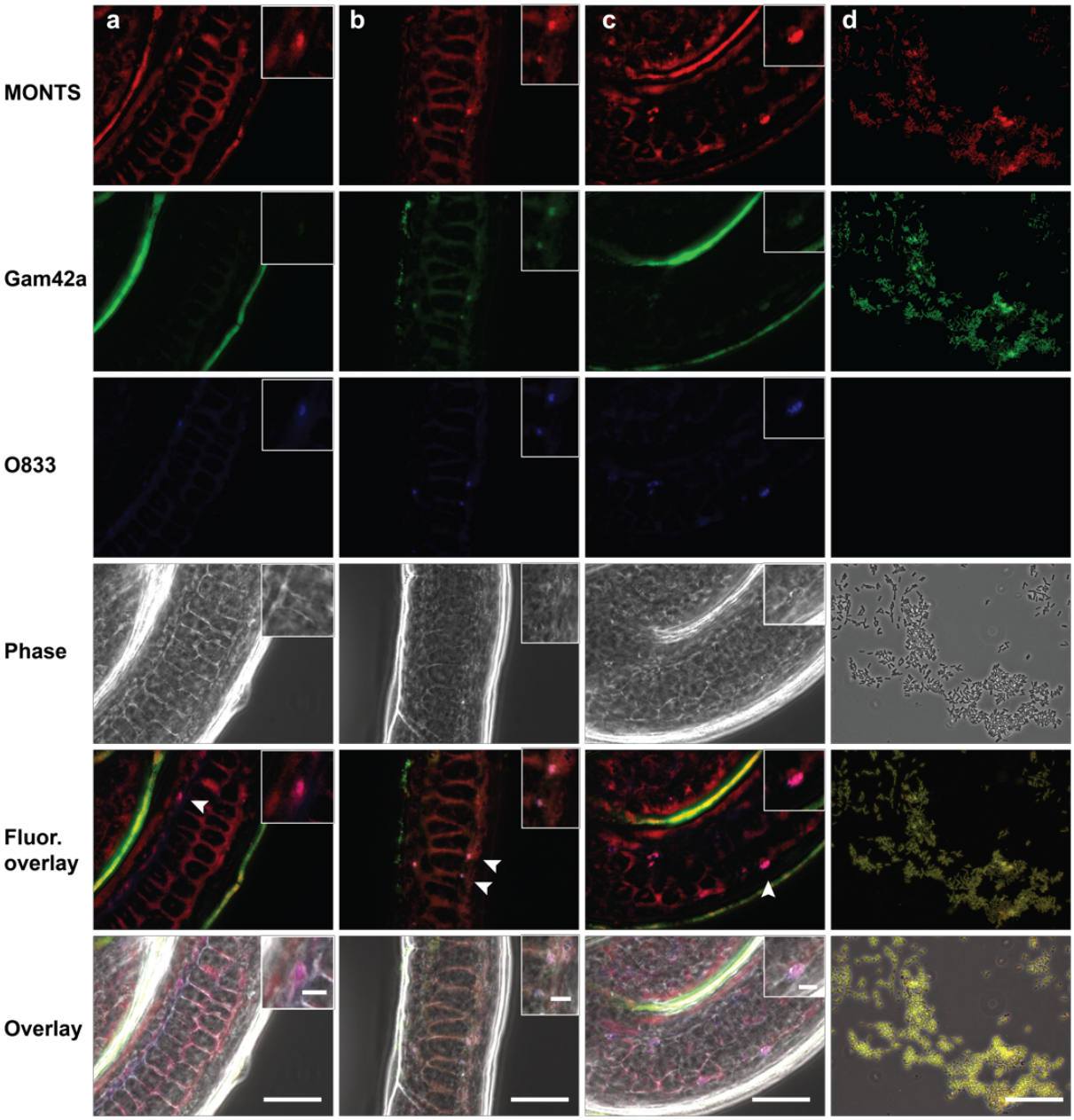

Figure 7 Epifluorescence microscope FISH images of *L. oneistus* males. Male nematodes were stained with the MONTS-specific probe (MONTS, red), a *Gammaproteobacteria*-specific probe (Gam42a, green) and the *Oceanospirillales*-specific probe (O833, blue). In the fluorescent overlay (Fluor. overlay) all three fluorescent channels are superimposed and in the Overlay the fluorescent channels and the Phase contrast channel were superimposed. (**a-c**) are showing the testis region and (**d**) are dissociated ectosymbionts. Arrow heads indicate the bacterial aggregates that were enlarged in the inlays. Scale bar in the images is 30µm and in the inlays 6µm.

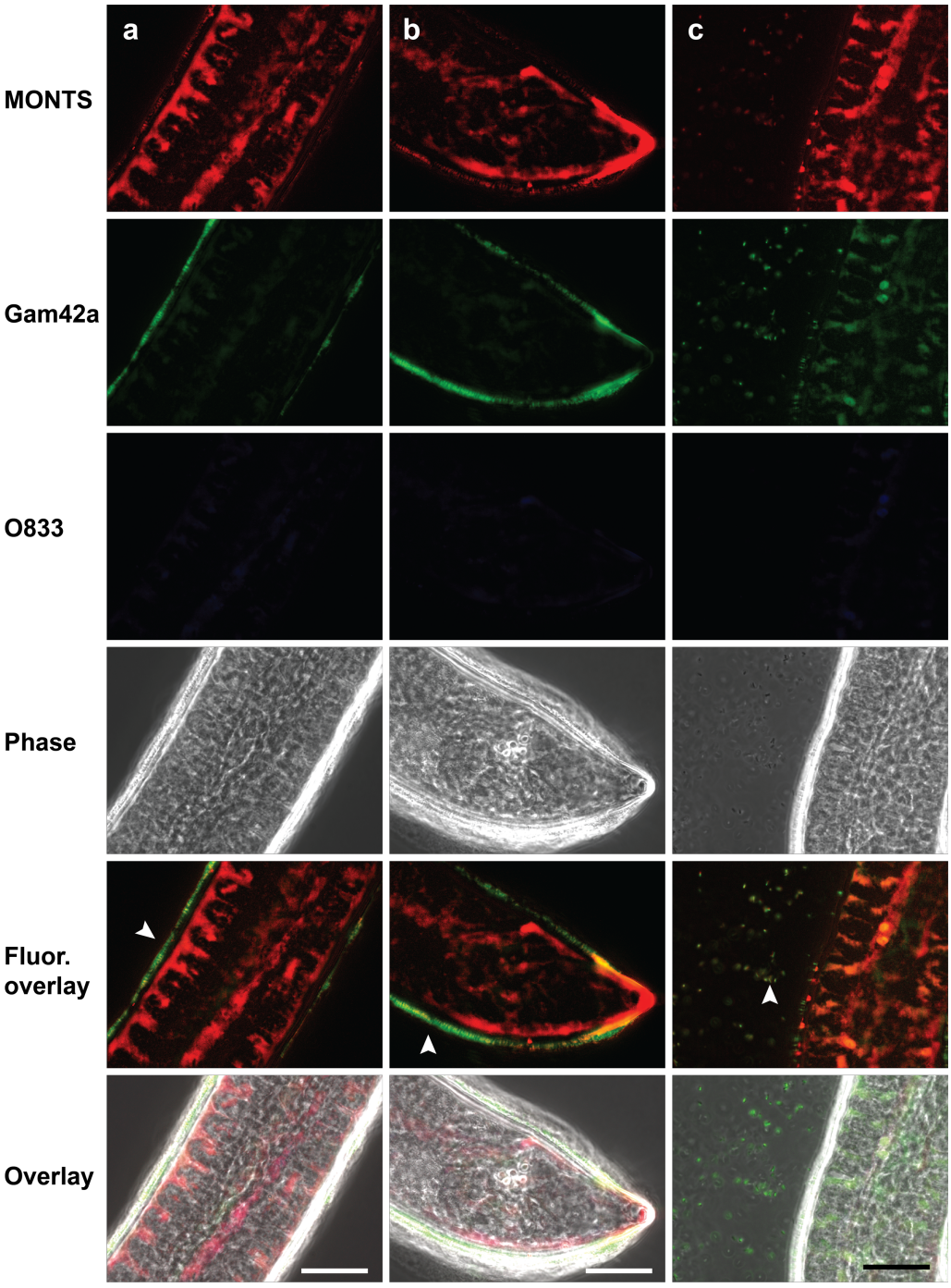

Figure 8 Epifluorescence microscope FISH images of *L. oneistus* females. Female nematodes were stained with the MONTS-specific probe (MONTS, red), a *Gammaproteobacteria*-specific probe (Gam42a, green) and the *Oceanospirillales*-specific probe (O833, blue). In the fluorescent overlay (Fluor. overlay) all three fluorescent channels are superimposed and in the Overlay the fluorescent channels and the Phase contrast channel were superimposed. (**a-c**) are showing different regions of the female nematode. Scale bar in the images is 30µm.

No staining with the O883 probe or Gam42a could be detected inside female individuals - the ectosymbiotic bacteria were double stained with the Gam42a and MONTS-1 probe (Fig. 8; arrow heads are pointing at the double labelled ectosymbionts).

To verify that the bacterial aggregates found in males were specifically stained with the O833 probe, we applied a probe carrying a single nucleotide mismatch in the middle of the sequence with respect to the specific one (O833mis in Cy5). We could detect a dim staining with the O833mis probe. The bacterial aggregates inside the males were also stained with the Gam42a probe, whereas the ectosymbiont was again only double stained with the Gam42a and MONTS-1 probe (Fig. 9). It is important to mention that the bacterial aggregates appeared not only to be autofluorescent in the green channel, but also in the red channel. Therefore, when all fluorescent channels are superimposed the *Oceanospirillales*-aggregates appear whiteish to reddish (Fig. 7a-c), but a much dimmer fluorescent signal is found when stained with the O833mis probe when compared to the specific staining (Fig.9). Taken together, these results indicate that the *Oceanospirillales*-like 16S rRNA-gene sequence obtained from the metagenome analysis and in our 16S rRNA-gene libraries originated from the bacterial aggregates in male *L. oneistus* nematodes.

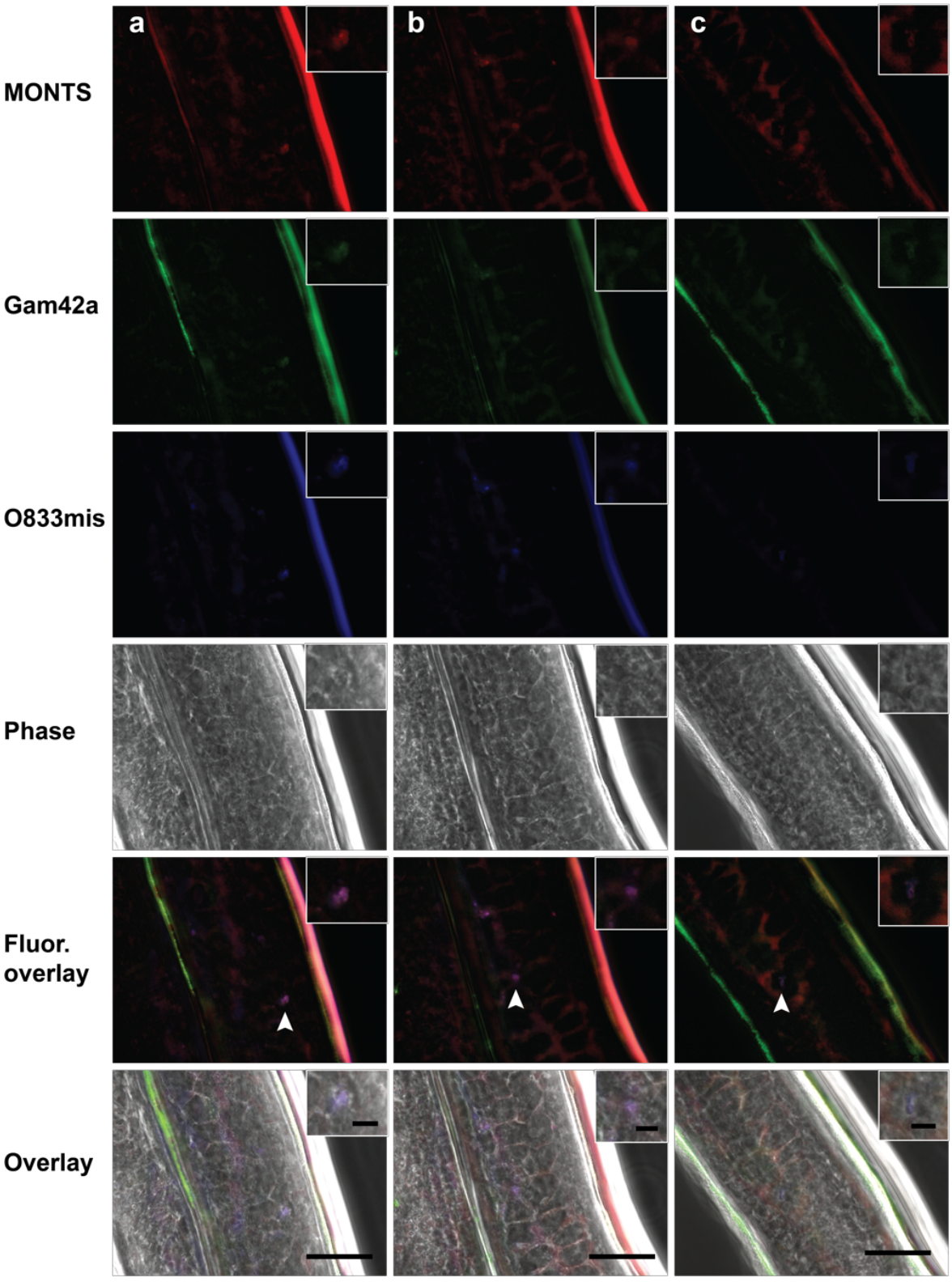

Figure 9 Epifluorescence microscope FISH images of *L. oneistus* males. Male nematodes were stained with the MONTS-specific probe (MONTS, red), a *Gammaproteobacteria*-specific probe (Gam42a, green) and the *Oceanospirillales*-mismatch probe (O833mis, blue). In the fluorescent overlay (Fluor. overlay) all three fluorescent channels are superimposed and in the Overlay the fluorescent channels and the Phase contrast channel were superimposed. (**a-c**) are showing the testis region. Arrow heads indicate the bacterial aggregates that were enlarged in the inlays. Scale bar in the images is 30µm and in the inlays 6µm.

Discussion

Our results from the PCR and FISH experiments of *L. oneistus* nematodes indicate that the *Oceanospirillales*-like sequence identified in the metagenomic analysis is found in *L. oneistus* males. The phylogenetic placement of the *L. oneistus* putative second symbionts within the *Oceanospirillaceae* bacteria is consistent with reports of other symbionts found associated with marine invertebrates (Goffredi et al., 2005; Kaesler et al., 2008). However, the most closely related 16S rRNA-gene sequence belonged to *Thalassolituus marinus*, a non-pigmented and curved rod-shaped bacterium that was first isolated from a surface seawater sample from the Yellow Sea (Choi and Cho, 2013). Another member of this genus, *Thalassolituus oleivoran*, was isolated from sea water/sediment samples collected in the harbour of Milazzo, Italy (Yakimov et al., 2004). Both *Thalassolituus* species are free-living chemoheterotrophic and strictly aerobic. Members of this genus are hydrocarbonoclastic, which means they are obligate for hydrocarbon substrates and additionally use only a few low molecular weight organic acids (*e.g.* acetate and pyruvate) (Choi and Cho, 2013; Yakimov et al., 2004). They are often found on marine particles that contain adsorbed hydrocarbons and lipids (Overmann, J., Lepleux, 2016). Bacteria belonging to the genus *Thalassolituus*, have so far not been described to live in association with an animal and this study would be the first description of such a relationship. The natural habitat of *L. oneistus* are tropical shallow-water, sheltered, calcareous sediments (Ott and Novak, 1989; Ott et al., 1991), which already a few centimetres below the sea bottom, become high in reduced sulfur compounds and anoxic (Ott and Novak, 1989). It is possible that the nematodes proposed migration pattern between the sediment layers not only creates favourable conditions for its ectosymbiont (Hentschel et al., 1999; Ott et al., 1991), but also for the putative endosymbiont by providing access to reduced sulfur compounds found in the deeper sediment layers. Despite the low abundance and the association to potentially only male *L. oneistus*, the *Oceanosprillales*-like putative endosymbiont might play an important role in its host. Dimethylsulfoniopropionate (DMSP) is an organic sulfur compound that is produced in high concentrations by photosynthetic dinoflagellates (zooxanthellae) (Van Alstyne et al., 2009). In previous studies abundant bacteria within the *Oceanospirillales* have been identified that are able to metabolize DMSP in the coral *Acropora millepora*, suggesting important functional roles within or around their hosts (Raina et al., 2009). The potential of metabolizing sulfur compounds (such as DMSP and

dimethyl sulfide) is hypothesized to play an important role in structuring the bacterial communities in corals and has consequences on both coral and coral ecosystem health (Raina et al., 2009). The biochemicals produced by bacterial communities could influence the general health of the corals by protecting them against invasive bacterial species (Ritchie, 2006; Speck and Donachie, 2012). Additionally, it was found that the abundance of *Oceanosprillales*-like sequences in coral microbial communities correlated with the presence of photosymbionts, albeit they were also found in hosts without photosymbionts (Bourne et al., 2013). A recent review on members of the *Oceanospirillales*, more specifically, *Endozoicomonas* provided an update on the range of their hosts, global distribution and their possible function (Neave et al., 2016). *Endozoicomonas* bacteria have the remarkable ability to adapt to a broad array of hosts and environments, ranging from warm coral reefs to cold deep-sea mussels (Neave et al., 2016). Functionally they are thought to play important roles in: nutrient acquisition and provision (*e.g.* nitrogen, carbon, methane and sulfur cycling, as well as the synthesis of amino acids and other essential molecules), structuring of the host microbiome, and in host health or disease (Neave et al., 2016). In this sense, it could be hypothesized that the putative endosymbiont of *L. oneistus* potentially provides benefits to the host directly, through the breakdown of organic compounds. It could also indirectly aid the metabolism of the host and/or its ectosybiont by providing them with carbon sugars or secreted proteins. Such a scenario was proposed for the gill symbiont of *Alviniconcha*, where its *Oceanospirillales*-like endosymbiont might play a role in sulfur cycling and thereby facilitate the metabolism of another symbiont found in the gills (Beinart et al., 2014). Another possible scenario is that the putative endosymbiont of *L. oneistus* is a parasite exploiting host-produced organic compounds (*e.g.* fermentation products). Parasitic *Oceanospirillales* were described in bathymodiolin mussels, where they invade the host nuclei and utilize the chromatin as nutrition (Zielinski et al., 2009).

Not only is the role of this putative *L. oneistus* endosymbiont puzzling, but also its potentially exclusive association to male nematodes. We were able to detect *Oceanospirillales*-like aggregates in the vicinity of the male testis region, varying in size, abundance and exact localization among individual males (Fig. 6 and 7a-c). The aggregates were not only autofluorescent, but they were also stained by the specific FISH-probe. *Oceanosprillales* are known to have natural fluorescent yellow-green pigments (Garrity et al., 2005) and autofluorescent aggregates of *Oceanospirillales-like* bacteria have also been reported in

previous studies (Goffredi et al., 2007; Jensen et al., 2010). The bacterial aggregates in *L. oneistus* were also very dim stained with the O833mis probe, when compared to the specific probe O833. However, the signal from the O833mis probe could be due to the bleed-through of the autofluorescence from the other channels. In another study the hybridization of *S. pistillata* samples with a NON338 probe (non-specific probe) also resulted in a weak staining, when compared to the specific probe, of the *Endozoicomonas* aggregates (Bayer et al., 2013a). In this study it was hypothesized that the signal originated from a non-specific binding of the probe to an adhesive-type substance that may surround the aggregates (Bayer et al., 2013a). Such adhesive substances surrounding the putative endosymbiont of *L. oneistus* could explain the autofluorescent signal when excited with blue light and the weak signal of the O833mis probe. Additionally, Bayer et al., (2013a) state that the aggregates were dimly visible in no-probe control at the same intensity as autofluorescent *S. pistillata* coral tissue, suggesting that the cells are embedded within the host endoderm. The *L. oneistus* tissue is also autofluorescent (Fig. 6, 7, 8 and 9), however we were unable to see if the aggregates are embedded in the host tissue in this study.

The association of the bacterial aggregates in *L. oneistus* mostly if not only to the male testis region is peculiar from a symbiont transmission point of view. In animals with separate sexes, as it is the case for *L. oneistus* nematodes, vertically acquired symbionts are often transmitted through the female germ line and in some cases by biparental transfer (*e.g. A. pisum*) (reviewed in Bright and Bulgheresi, 2010). There are only few cases of symbionts that have been found in the sperm or paternal germ line of their hosts with a paternal symbiont transfer mode (Bright and Bulgheresi, 2010; Ebert, 2013; Moran and Dunbar, 2006; Watanabe et al., 2014). *Oceanospirillales*-like bacterial aggregates were described in a variety of host tissues (*e.g.* gills, skin, gastro-intestinal tract), but only in the polychaete worm *Osedax* they were found residing in the ovisac (and root system) (Goffredi et al., 2005). It is important to mention that the endosymbiont of *Osedax* is repeatedly acquired from the environment (horizontally transmitted) as the host tissues grows (Verna et al., 2010), and not transmitted from parent to offspring. If the *Oceanosprillales*-like bacteria, primarily found in the *L. oneistus* testis region, were transmitted through the male germline they would represent another rare case of paternal vertical transmission. Although we were not able to detect *Oceanosprillales*-like bacteria in female *L. oneistus* in this study, from an evolutionary point of view, it seems more likely that the symbionts are not only transmitted through the male germline, but are also

through the female one, as it would enhance the chances of successful transmission of the symbiont to the nematode progeny. Alternatively, it is possible that the *Oceanospirillales*-like bacteria are free-living in the sediment where *L. oneistus* is found and that they are horizontally transmitted to their host. The reason and/or advantage of the bacterial aggregates in the vicinity of the nematode's testis region as well as the impact of the endosymbiont on its host still remain elusive.

Conclusion and Perspectives

The symbiosis research field has become more and more influential and the growing awareness of the impact of microbes on organismal and environmental health and function is continually expanding this fascinating field. The association of a marine nematodes with an *Oceanospirillales*-like bacterium that resides in the vicinity of its testis region is the first description of such an unconventional relationship. The exact nature of this relationship and the transmission mode of the symbiont are still unclear and leave a lot for future research to be explored. Since the deeper nodes of the 16S rRNA-gene tree were not well resolved, a more robust tree could be calculated with several concatenated ribosomal proteins, to place the putative endosymbiont more accurately within the family of *Oceanospirillaceae*. Quantitative PCR (qPCR) could be used to determine the abundance of the putative endosymbionts in *L. oneistus.* The morphology of the bacteria and the aggregates in association to the testis could be described by using transmission electron microscopy (TEM). The signal of FISH probes could be enhanced with catalysed reporter deposition FISH (CARD-FISH) and thereby the aggregates could be better distinguished from the autofluorescent host tissue. Finally, by performing both FISH and TEM on sections of tissue and superimposing the images, ultrastructural investigation could be coupled to FISH-based bacterial identification (Halary et al., 2011; Laming and Duperron, 2016).

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