



DIPLOMARBEIT

**„The search for *Candidatus Endoriftia persephone*
during the development of *Riftia pachyptila*“**

„Die Suche nach *Candidatus Endoriftia persephone*
während der Entwicklung von *Riftia pachyptila*“

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Abstract (Deutsch)

Die Röhrenwürmer der Familie der Vestimentifera zählen zu den am besten untersuchten Modellsystemen für Symbiosen der Tiefsee. Der Riesenbartwurm *Riftia pachyptila* zählt ebenfalls zu der oben genannten Polychaetenfamilie und kommt ausschließlich an heißen Quellen im Ost-Pazifik vor. Er wird als der am schnellsten wachsende momentan bekannte Invertebrat angesehen. Anstelle von Mund und Darm stützt sich der Wurm für seine Ernährung auf seine chemoautotrophen, schwefel-oxidierenden Endosymbionten *Candidatus Endoriftia persephone*, welche zu den Gammaproteobakterien zählen und im Trophosom, einem speziellen Organ des Wirts leben. Horizontale Transmission findet in jeder Generation aufs Neue während einer kurzen Phase während der Larvalentwicklung von *Riftia*, durch die Infektion der Röhre und der Haut gleichzeitig mit der Infektion vieler verschiedener Mikroben statt. In späteren Entwicklungsstadien von *Riftia* sind die Symbionten auf das Trophosom beschränkt und fehlen in der Röhre und der Haut. Daher geht das momentane Modell des Transmissionsmodus von einem einzelnen kurzen Infektionsereignis aus. Nichts destotrotz wurde bis jetzt nicht genau untersucht ob eine spätere Transmission möglich ist, wie im Falle der Symbiosen zwischen Leguminosen und Rhizobien. Aus diesem Grund wurde im Zuge dieser Studie die genetische, mikrobielle Diversität der Röhren und der Haut von adulten und juvenilen *Riftia* Individuen von zwei unterschiedlichen Standorten mit verschiedenen molekular-und mikrobiologischen Methoden untersucht. Röhren-, Haut- und Trophosomproben von adulten *Riftia* Individuen von unterschiedlichen Körperregionen und halbierte juvenile Würmer wurden mit 16S rRNA PCR, TRFLP (Terminal Restriction Fragment Length Polymorphism), DGGE (Denaturing Gradient Gel Electrophoresis) und FISH (Fluorescent *in situ* Hybridization) untersucht. Endoriftia wurde außer im Trophosom in der Haut von adulten und juvenilen *Riftia* Individuen und auch in den Röhren von adulten Würmern gefunden. Das lässt die Annahme zu, dass entweder der Transmissionmodus nicht nur auf ein spezielles Larvenstadium beschränkt ist oder dass die Symbionten fähig sind Ihren Wirt zu verlassen. Überraschenderweise konnte in der Haut und der Röhre von adulten Individuen auch eine völlig neue 16S Sequenz eines Mollicutes Bakteriums entdeckt werden. Diese Bakterien sind bekannt durch ihre pathogene aber auch nützliche Rolle in Assoziationen mit Insekten, Mollusken aber auch Mammalia. Die genaue Rolle und die Auswirkungen dieses neuen Mollicutesbakteriums im *Riftia*-Symbiosesystem muss noch eingehend untersucht werden. Die Präsenz von Mollicutes in über 80% der Würmer lässt aber dennoch auf eine eher nützliche Rolle schließen. Neben Endoriftia und Mollicutes wurden die Röhren auf die Zusammensetzung und Häufigkeit ihrer mikrobiellen Gemeinschaft untersucht. Verglichen zu Trophosom und Haut beherbergen die Röhren die diverseste bakterielle Gemeinschaft, wobei diese sich hinsichtlich der zwei verschiedenen Standorte und der unterschiedlichen Körperregionen gruppiert. Das könnte ein Hinweis dafür sein, dass die mikrobielle Gemeinschaft der Röhren durch Unterschiede der Temperatur und der chemischen Bedingungen an den heißen Quellen beeinflusst wird.

ABSTRACT

Vestimentiferan tubeworms are one of the best studied model systems for symbiosis in the deep sea. One of them is the giant tubeworm *Riftia pachyptila*, which can be found at deep-sea hydrothermal vents in the East Pacific Ocean. It is considered to be the fastest growing invertebrate we know of. Instead of a mouth and a gut, the animal relies on its chemoautotrophic, sulfur-oxidizing endosymbiont *Candidatus Endoriftia persephone*, which is a Gammaproteobacterium housed in a special organ, the trophosome. Horizontal symbiont transmission happens in each generation anew during a short period in the developing host larvae through infection of tube and skin, analogous to many pathogen infections. In later stages of the host's life cycle, the symbiont is restricted to the trophosome, but is missing in the skin and the tube so that the current model illustrates a single, short transmission event. However, it never has been investigated thoroughly whether or not later transmission is possible such as in the rhizobia - legume symbiosis. Therefore, the genetic microbial diversity in the tube and the skin of adults and developmental stages of *Riftia pachyptila* from two different sites was investigated with different molecular and microbial techniques. Tube, skin and trophosome samples of adult worms from three different body regions and half specimens of juveniles were screened by 16S rRNA PCR, TRFLP (Terminal Restriction Fragment Length Polymorphism), DGGE (Denaturing Gradient Gel Electrophoresis) fingerprinting and FISH (Fluorescent *in situ* Hybridization). Beside of the trophosome, *Endoriftia* was consistently detected in the skin of adults and juveniles and it was also found in the tube of adult specimens. These results suggest that either transmission of symbionts is not limited to larval stages as previously suggested or that symbionts are capable of leaving the host.

Surprisingly, we also found a new 16S phylotype of a Mollicutes bacterium in the skin and tube of adult *Riftia*. Such bacteria are well known from other animals including insects, mollusks but also mammals, sometimes as pathogens but also as beneficial microbes. The role and impact of Mollicutes on the *Riftia* model system remain to be investigated. However the presence of this phylotype in more than 80% of hosts rather points to a beneficial role.

Beside *Endoriftia* and Mollicutes the tube of *Riftia* harbors a more diverse microbial community compared to skin and tube. Interestingly, bacterial sequences found in the tubes cluster according to the two different sampling sites and the three sampling positions of the tube, suggesting that the differences in the microbial community are driven by changes in chemistry and temperature of the vent environment.

INTRODUCTION

For a long time the vastness of the deep sea has been considered inhospitable and hostile to life. Even scientists could not imagine that anything can live without light and under such high pressures. During the last third of the 19th century this was proven wrong and first evidence for life in the Deep was found during several oceanographic cruises such as the famous “Challenger”-expedition, providing first proof also for deep-sea volcanoes in general.

It took more than hundred years more until the first hydrothermal vents with their highly special and unique fauna were detected on the Galapagos Rift in 1977 (Lonsdale, 1977). Deep-sea hydrothermal vents are one of the most fascinating but extreme places where one can find life on earth. Highly toxic fluids with minimal pH values of around 4, extremely fast and steep changing temperature gradients 5° to 50° C within seconds, a total temperature range of more than 400°C, and pressures of more than 250 bar create a habitat which it is not easy to thrive in (Kelley et al. 2002; Elderfield & Schultz, 1998;). In the darkness of the vents the food chain is not based on photosynthesis, but on a process called chemosynthesis where chemical compounds such as hydrogen sulfide or methane gushing from the hot and warm vents are used as an energy source to produce energy rich carbon compounds (Ruby et al. 1981; Van Dover, 2000). The chemosynthetic primary producers at hot vents are chemolithoautotrophs. Chemolithoautotrophic organisms use a special form of chemosynthesis where only inorganic chemical compounds, e.g. from hot vent fluids, are used as electron donors and where only inorganic carbon is used as a source to synthesize organic carbon (see Dubilier et al., 2008).

Living under these extreme conditions organisms needed to adapt not only their physiology and feeding behavior but also their interspecific and intraspecific interplay. Therefore it is not surprising to easily find symbiosis in the confined spaces of these deep-sea oases of life. The term symbiosis is used here not only as it is defined by *De Bary* (1879) as “*the living together of unlike organisms*”, but more specifically as mutualism which is “*an association between different species from which all partners benefit*” (Douglas, 2010). Many large vent species rely on a mutualistic relationship with one or more clades of highly specialized chemosynthetic bacterial symbionts (Van Dover et al. 2002, Vrijenhoek, 2010). This study deals with the symbiosis between the vestimentiferan tubeworm *Riftia pachyptila* (Polychaeta, Siboglinidae, short *Riftia*) and its thiotrophic bacterial symbiont named *Candidatus*

Endoriftia persephone (short Endoriftia). Identical 16S phylotypes are also identified as the main symbionts of the co-occurring vent Vestimentifera *Tevnia jerichonana* and *Oasisia alviniae* (Di Meo et al. 2000; Nelson & Fischer, 2000).

Symbiosis in these tubeworm species is nutritionally based on chemolithoautotrophic bacteria, which use a special form of chemosynthesis called thiotrophy. In contrast to photoautotrophic organisms, which use light as energy source thiotrophic ones use reduced sulfur compounds like sulfide as electron donors. Long time it was a mystery how *Riftia* acquires these bacteria or how they are transmitted. Transmission is the process of how symbionts infect their host.

One of the key questions in symbiosis research is how symbionts are transmitted from one host generation onto the next. There are different possibilities of transmission but one can basically distinguish between two main transmission modes: the so-called horizontal and the vertical transmission but also mixes of these two modes in different degrees can often be found in nature (see Bright & Bulgheresi, 2010). Horizontal transmission occurs when symbionts which also have a free-living population in the surrounding environment are transferred to the host from the environment e.g. *Aliivibrio fischeri* in the bobtail squid *Euprymna scolopes* (see Nyholm & McFall-Ngai, 2004) or Endoriftia in *Riftia* tubeworms (see Bright & Lallier, 2010). Vertical transmission happens when the symbionts are directly transmitted at least from one parent to the offspring e.g. within gametophytes of the mother or the father or both (see Bright & Bulgheresi, 2010,). But there can also be a mix of these two transmission modes, where usually vertical transmission is completed by additional horizontal transmission events as found in gutless oligochaetes with different bacterial symbionts (Blazejak et al. 2010), bryozoans with *Endobugula* spp. (Lim-Fong et al. 2008), solemyid clams with Gammaproteobacteria (Krueger et al. 1996) and insects with *Wolbachia* spp. (Kikuchi & Fukatsu, 2003; Reuter & Keller, 2003).

All these different transmission modes can appear in different locations or organs of the host during different developmental stages. Usually it is a surface inside or outside of the host's body where the first direct contact between host and symbiont happens, e.g. tissues like the skin, the gut epithelium or surfaces of special organs of an organism (Nussbaumer et al. 2006; Singley, 1980, Savage, 1977).

The infection site is often not the final location of the symbionts and they have to migrate to their final place of residence. The process of symbionts moving from the site of first con-

tact/infection to the one where they finally reside is called translocation. For example rhizobia, which are infecting legume roots, have to migrate from cracks in the epidermis of the roots to their final residing place in the deep cortical layer upon which they proliferate and build root nodules (Goormachtig et al. 2004). In *Riftia* the symbionts have to move from the epidermis to their final location in the symbiont-housing organ named trophosome inside of the trunk region of the worms (see Bright & Lallier, 2010).

There is also a difference of symbiont location in hosts because there are some which harbor their symbionts in special symbiont-housing organs and cells like the trophosome in *Riftia* (Cavanaugh et al. 1981; Felbeck 1981; Jones 1981) and there are some which have their symbionts outside of the body and also on special structures like the setae of the yeti crab *Kiwa hirsuta* (Goffredi et al. 2008). Therefore, symbionts can be distinguished as ectosymbiotic residing on the outside of a host body or endosymbiotic living inside the host. Symbiont location can be defined even more in context to host cells: There are extracellular symbionts outside of host cells, intercellular ones between host cells (which are in fact also extracellular, outside of the cell) and intracellular symbionts living inside of a host cell (see Bright & Bulgheresi, 2010). In marine chemosynthetic symbiosis there are examples like the thiotrophic symbiotic bacteria of the stillbonematine nematode *Laxus oneistus*, that are attached to the skin of the worm without a special housing structure being ectosymbiotic and extracellular (Ott et al. 1995), whereas in the symbiosis between many chemosynthetic bivalves of the genus *Calyptogena* or *Bathymodiolus* and their sulphur-oxidizing and/or methane-oxidizing symbionts, the bacteria are strictly intracellular and endosymbiotic in the gills of the bivalves (see Dubilier et al. 2008). Extracellular endosymbionts can be found in the two genera of gutless marine oligochaetes *Olavius* and *Inandrilus*. Their symbionts form a thick layer in the extracellular space under the cuticle and above the epidermal cells of these worms (Giere et al. 1995).

Interactions between different organisms usually are described as antagonistic. In contrast to that organisms have responded to predation, abiotic stresses such as low nutrient availability by forming alliances a lot of times during the history of life resulting in advanced fitness and ecological success. Most alliances are founded on reciprocity which means that an organism only helps a different organism if the favor is returned (Douglas, 2010). This is also the case in the *Riftia* symbiosis. The endosymbiotic, chemolithoautotrophic and sulfur-oxidizing Endoriftia provides its host *Riftia* with carbohydrates which are produced by the

symbiont through chemosynthesis. In return, *Riftia* provides a safe environment for the symbiont, namely the trophosome – and also a constant supply with hydrogen sulfide, oxygen and carbon dioxide for the symbiont (see Bright & Lallier). Oxygen, sulfide and carbon dioxide are taken up by the host from the surrounding waters and transported via the blood to the symbionts in the trophosome. Then the symbionts oxidize sulfide to sulfate. Via this oxidation reaction energy is created in form of ATP and used in the Carbon-Benson-Bassham (CBB) and the reverse tricarboxylic acid (TCA) cycle to produce C4 sugars (succinate and malate) which are also given back to the host (see Stewart and Cavanaugh, 2005).

In the symbiosis between the gut- and mouthless siboglinid subfamily of vestimentiferan tubeworms, which also *Riftia* belongs to and its thiotrophic symbiont only horizontal transmission with intracellular endosymbionts has been found so far (see Bright & Lallier, 2010). It is known that *Riftia*'s symbiotic partner has two different populations: a symbiotic one inside of the host trophosome and a free-living environmental one thriving in surrounding water masses of the East Pacific Rise and attached to different surfaces and not only restricted close to hydrothermal vent sites (Harmer et al. 2008). This environmental population is thought to be the main source for new transmissions and infections of juvenile tubeworms. Former studies focused on the onset of transmission in the *Riftia* model system. Long time it was thought that the larvae of *Riftia*, which disperse in the water column still possess a digestive system and take up their sulfur-oxidizing symbionts via the oral opening and the gut (Jones and Gardner, 1988; Southward, 1988). Nussbaumer et al. (2006) have found cells of *Endoriftia* infecting the epidermis, adjoining muscles and the underlying undifferentiated mesodermal tissue of *Riftia* larvae. They also detected symbiotic bacteria in the dorsal mesentery. No bacteria could be found in the gut lumen or gut cells, the mouth or the anus of infected larvae. In contrast to that they found massive apoptosis in the epidermis, muscles and undifferentiated mesoderm only in juvenile specimens but not in *Riftia* larvae. Based on these findings they conclude that symbionts are taken up by the host larvae via the developing tube and the skin shortly after settlement on basalt rock in a vent. They migrate from the epidermis to mesodermal tissue from which the trophosome is formed. A further uptake of symbionts is then terminated by massive apoptosis of host tissue surrounding the already developing trophosome.

Mouth and gut are completely reduced and the trophosome with the autotrophic symbionts inside keeps on growing until it comprises almost the whole volume of the trunk region (see Bright & Lallier, 2010).

This means that there is only a short time frame for transmission of symbiotic bacteria during a specific developmental stage of the host larvae. This is somewhat consistent with an older study by Lopez-Garcia et al. in (2002), which dealt with the general diversity of bacteria associated with the tube of adult *Riftia*. Using scanning electron microscopy and Sanger sequencing they found no evidence of the presence of the 16S phylotype associated with *Endoriftia* in or on the adult tube. Neither the symbiont nor any other bacteria have been found on adult skin so far, simply because there are not enough studies about this topic. It is also thought that all remaining body openings like gland openings plus channels and genital openings of adult *Riftia* are more or less sterile and harbor no bacteria. In contrast to that the free living-form of the symbiont can be found widely dispersed in the vent and surrounding deep-sea environment (Harmer et al., 2008), but in *Riftia* it has only been found in the trophosome of juvenile and adult stages and in the skin and developing tube of larvae so far (Nussbaumer et al. 2006). The hypothesis for this study: Are there no bacteria associated with skin and tube of adult *Riftia* or is there a mechanism in the immune system of the host which regulates the presence and absence of the symbiont during the whole life of *Riftia*? More knowledge about the presence or absence of bacteria in general and in specific of *Endoriftia* in the tube and skin of *Riftia* during different developmental stages could help to a better understanding of the transmission mode itself and the impact of it on the whole system. Because of the past findings and lack of knowledge this study focuses for the first time on possible differences of the symbiont presence between different tissues during different developmental stages and different sampling sites of *Riftia pachyptila*.

Therefore there are three working hypotheses: "Is *Endoriftia* really absent in tubes of adult *Riftia*? Is it only present in the trophosome especially in adults? Are there no other bacteria colonizing the host tissues?" For all these three main questions we additionally ask if there are any differences regarding the geographic location, the developmental stages and the different adult host tissues. Different molecular and microbial biology techniques such as PCR, DGGE, TRFLP and FISH on a set of 61 samples from six adult and six juvenile specimens from two different sampling sites at the 9° North spreading center on the East Pacific Rise were used to answer these questions.

MATERIALS AND METHODS

Sample collection:

Juvenile and adult *Riftia pachyptila* were collected during two different cruises from several sites at the 9° 50' N region of the East Pacific Rise with R/V L'Atlante and DSV Nautille in 2010 and with R/V Atlantis and DSV Alvin in 2011. Adult specimens of *Riftia* were sampled at P-Vent North (9° 50.2816' N, 104° 17.4732' W, 2500 meters depth, 2011) and TICA (9° 50.404 N, 104° 17.495 W, 2500 meters depth, 2011). Additionally also juvenile *Riftia* specimens were sampled at P-Vent South in 2010 (N, W2500 meter depth, 2010). Tubeworms sampled in 2011 were immediately dissected on board of R/V Atlantis and divided into three body regions (upper, middle and lower part of the worms) and three tissue types (the tube, the skin and the trophosome). Small pieces, not larger than 1 cm³ were cut out from the trophosome, the skin and the tube on the three different body regions, fixed and stored separately in 100% ethanol at 4°C. For every sample sterile scissors and forcepses were used to avoid contamination. Juveniles were collected as whole specimens and then cut alongside the longitudinal axis after fixation and storage in 100% ethanol. For this study three adult specimens from P-Vent North, three adult specimens from TICA, and six juveniles from P-Vent South were used (see Table 1).

DNA extraction

For genomic DNA extraction three different extraction kits were compared, according to manufacturer's instructions: UltraClean® Soil DNA Isolation Kit (MOBIO), DNeasy® Blood & Tissue Kit (QIAGEN) and FASTDNA® SPIN Kit for Soil (MP Biomedicals). The MP Biomedical Kit yielded the highest DNA concentration and purity and was therefore used in this study. The extractions were stored at -20°C for further processing.

Symbiont detection with PCR

To check for presence or absence of the endosymbiont, the 16S rRNA gene from genomic DNA extracts was amplified by PCR. Four different primers were used: the 16S symbiont specific RifTO44 forward (ThermoScientific), RifTO445 reverse (ThermoScientific), general bacte-

rial 16S primers, 27F forward (ThermoScientific), and 1492R reverse (ThermoScientific; see table 2). First samples were screened with general bacterial 16S primers to check for bacterial presence. When they showed a positive signal, specific primers were used to detect the symbiont. PCR reactions were prepared with PCR grade water (Fresenius Austria GmbH), Taq-buffer (Fermentas), dNTPs (Fermentas), MgCl₂ (Thermo Scientific), RifTO 44 primer, RifTO445 primer, BSA (Bovine albumin serum, Fermentas), Taq DNA polymerase (Fermentas) and 1 µl of extracted DNA (see table 3). PCR was performed in a PCR cycler (Eppendorf Master Cycler Pro S, Eppendorf) using the following program with 35 cycles: 95°C for 3 min 30 sec; 95°C for 30 sec, 50°C for 30 sec and 72°C for 45 sec; 72°C for 10 min and hold at 4°C. Amplified PCR products were visualised on 1% Agarose gels in 1 x TBE buffer [10 x TBE stock solution: Tris (tris(hydroxymethyl)aminomethane, 162 g/500ml, 1.33 M, Sigma-Aldrich Co.), boric acid (27.5 g/500ml, 0.44 M, Sigma-Aldrich Co.) and EDTA (ethylenediaminetetraacetic acid - Dinatrium-Dihydrate, 9.3 g/500ml, 0.025 M, VWR)]. Gels were run on a gel electrophoresis apparatus (BIO-RAD) at 90 V for 55 min and then stained with SYBR-Gold (Invitrogen®). For visual analysis a Molecular Imager® Gel Doc™ XR+ System (BioRad) connected to a Windows PC with Quantity One® (BioRad) software was used. Later the RifTO 445 primer was also used as a symbionts specific probe for fluorescence *in situ* hybridization (FISH).

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE was used for screening tube and skin of both tubeworm species to detect the inhabiting microbial community. PCR amplification using a 341_F_ GC-clamp forward primer (ThermoScientific) and a 907_R reverse primer (ThermoScientific) was carried out with the same program as described above (see table 3). The PCR products were analyzed for quality with gel-electrophoresis as described above. DNA content was measured with Nanodrop (Thermo Fisher Scientific Inc.) to ensure that the same amount of DNA was loaded onto one gel. PCR amplicons (2000-6000 ng DNA) were loaded onto a non-denaturing loading acrylamide gel [6-8 ml of 0% denaturing Acrylamide (6%, BIO-LAB Ltd.), polymerization chemicals: 60-80 µl of APS (Ammonium persulfate), Carl Roth GmbH and 6-8 ml of TEMED (Tetramethylethylenediamine), Sigma-Aldrich Co.) on top of an denaturing acrylamide gel [denaturing gradient from 30% to 70%. 24 ml of 30% mix: 9 ml of 80% denaturing Acrylamide (6%) + 15 ml of 0% denaturing Acrylamide(6%); 24 ml of 70% mix: 21 ml of 80% denaturing Acrylamide (6%)

+ 3 ml of 0% denaturing Acrylamide (6%); polymerization chemicals: 60-80 µl of APS and 6-8 ml of TEMED] in a DGGE apparatus (Ingeny) filled with 0.5 x TAE buffer (50 x stock solution: Tris (Sigma-Aldrich Co.), acetic acid (Carl Roth GmbH) and EDTA (VWR) and run at 60°C, 100 V for 16 h. PCR products were separated according to their AT and GC content (from labile to more stable) along the 30-70% denaturation gradient. The gel was stained with SYBR-Gold (Invitrogen®) and analysed with a Molecular Imager® Gel Doc™ XR+ System (BioRad) connected to a Windows PC using Quantity One® software. High abundant bands as well as bands of interest were cut out of the gel with a scalpel and stored in 50 µl PCR grade water at -20°C. DNA was again amplified with PCR using a 341_F primer (no GC-clamp, ThermoScientific) and a 907_R primer. Re-amplified DNA was purified with a DNA purification kit (PCRExtract Mini Kit, 5Prime) for sequencing with a Capillary Sequencer.

Sequencing

For sequencing of PCR products cycle sequencing was used. In this sequencing method the di-deoxy mediated chain termination method (Sanger et al. 1977) is combined with PCR (Saiki et al. 1988). Template specific primers (DGGE reverse primers) were used in a PCR with a reaction mix containing both dNTPs and ddNTPs. PCR was terminated at the 3' OH group of ddNTPs, which is necessary for the extension of the PCR product. Four different ddNTPs are used in this method and labelled with 4 different dyes, thus it is possible to identify the nucleotide at the 3' end by the colour of the PCR product. PCR amplicons are separated electrophoretically according to their length and color. The colors are detected in a capillary of the sequencer. Subsequently the nucleotide sequence can be identified by the sequence of the 4 different colours. In this study we used an ABI 3130XL Capillary Sequencer (Applied Biosystems, Lincoln, USA).

Analysis of sequences

Proof-reading of sequences

In this study the software program FINCH TV (Geospiza) was used for visualization of sequence chromatograms and proof-reading. Reading mistakes done by the sequencer during automatic nucleotide determination were corrected.

Analysis of sequences with Geneious and BLAST

Phylogenetic characterization of 16S sequences derived from DGGE bands was done with the software Geneious and BLAST (Basic Local Alignment Search Tool, Altschul et al. 1990) to find the closest related sequences. Geneious and BLAST utilise local alignments to analyse similarities in nucleotide or protein sequences due to calculations of the statistical significance of matches.

Terminal restriction fragment length polymorphism (TRFLP)

All tube samples were used for analysis with TRFLP to detect possible patterns, differences and succession stages of the bacterial community inhabiting the three different parts of the tubes (tube upper part, tube middle part and tube lower part). The 16S genes were PCR amplified using VIC and FAM fluorescence labelled general bacterial primers, 27F-6FAM forward primer (Thermo Scientific) and 1492R-VIC reverse primer (Thermo Scientific). 50 µl PCR reactions were prepared with (PCR grade water, buffer (Fermentas), dNTPs (Fermentas), MgCl₂ (Thermo Scientific), 27F primer, 1492R primer, BSA (Bovine albumin serum, Fermentas), Taq DNA polymerase (Fermentas). Then 1 µl of DNA template was added to the master mix (see table 3). Samples were run on a PCR cycler using the following programme with 30 cycles: 95°C for 4 min; 95°C for 1 min, 55°C for 45 sec and 72°C for 1min 30 sec; 72°C for 7 min and hold at 4°C. Amplified DNA was purified. The DNA amount was measured on a NanoDrop 2000 system. For digestion in a 20 µl reaction volume, 100 ng purified DNA, the restriction enzyme, BSA, NEB Buffer 4, (each from New England Biolabs Ltd.) and PCR grade water was used. DNA digestion was carried out at 37°C for 720 min followed by 65°C for 20 min to de-

activate the restriction enzyme and hold at 4°C. Purification was done in a 96 well filter plate using the Sephadex® G-50 purification system. Subsequently samples were prepared for sequencing with a master mix (HiDi formamide, Applied Biosystems, 1200 Liz Standard, Applied Biosystems, and 1.5 µl of digested sample for each sample) denatured on a PCR cyclor at 95°C for 3 min and chilled on ice for 5 min.

Analysis of TRFLP peaks

After PCR amplification and digestion with restriction endonucleases, different sequences are expected to harbor terminal restriction sites at different positions. This results in a mix of labeled DNA fragments of different lengths. This mixture is separated by electrophoresis in a capillary (usually of a sequencer), occurrence of fragments is photometrically detected and the length of the fragments is inferred by comparison with a size standard. The resulting TRFLP profiles from all tube samples were analyzed with the software “Peak Scanner” (Applied Biosystems, Lincoln, USA) and then compared to each other in a Multidimensional Scaling (MDS) plot based on Bray Curtis Similarity which was created in Primer 6 (PRIMER-E Ltd. Luton Ivybridge, United Kingdom).

Fluorescence *in situ* hybridisation (FISH)

Embedding

Small pieces of trophosome, skin, and tube, not larger than 25 mm³ (upper, middle and lower part) of adult specimens were cut using sterile forceps and dissecting scissors. Juveniles were cut in half and these pieces with varying sizes from 5 mm up to 1.5 cm length were taken for embedding. All samples were embedded in LR White low viscosity resin (London Resin Company, Reading Berkshire, UK) according to Nussbaumer et al. (2006) and Schimak et al. (2011). For dehydration samples were rinsed with 100% ethanol for 30 minutes three times. Samples transferred in a 50:50 volume mix of resin and 100% ethanol for 60 min, put into pure LR White for 30 minutes seven times and kept in LR White overnight up to 12 hours. Polymerisation was carried out in sealed and dried gelatine capsules under vacuum in a desiccator at 45°C for a minimum of 48 hours.

Sectioning

Semithin sections (1 µm) of embedded samples were done on an ultramicrotome (Leica EM UC7, Leica Microsystems, Weitzlar, Germany) using a glass knife. On each gelatine chrome alaun coated glass slide 9 to 12 sections were placed in groups of three on a drop of 20% acetone and then dried on a hotplate over night at a maximum of 46°C to ensure no ribosomes were destroyed.

Hybridization

On every slide each group was marked by a surrounding ring with a liquid blocking PAP pen (Daido Sangyo Co.Ltd). Three different probes were used on each slide: a symbiont specific probe RifTO445 (Nussbaumer et al. 2006) targeting the 16S rRNA of the symbiotic bacteria, a general bacterial probe EUB-mix (EUB-mix 338 consisting of EUB338 I, EUB338 II, EUB338 III, ThermoScientific) detecting *Bacteria* and a nonsense probe NONEUB (ThermoScientific) according to Wallner et al. 1993 (See table 4).

Different probe combinations in Cy3 and FLUOS and slides treated with or without proteinase K (AppliChem) were analyzed and compared for best fluorescent signals. Using symbiont specific probes with EUB-mix probes showed best signals when combined as follows: from left to right on the slide: First and second group with symbiont specific probe in FLUOS and EUB-mix probe in Cy3, third group with symbiont specific probe in Cy3 and EUB-mix probe in Fluos and fourth group with the nonsense probe in Cy3.

Hybridisation chambers were prepared with a paper tissue soaked in 2 ml of hybridisation buffer (5 M NaCl, Carl Roth GmbH, 1 mM Tris/HCl, Sigma-Aldrich Co., pH 8, 10% sodium dodecyl sulphate (SDS), Sigma-Aldrich Co., 0-30% formamide deionized, Sigma-Aldrich Co. and MilliQ water, Merck Milipore) and preheated at 46°C. 4.5-6 µl probe working solution (for 3-4 sections groups) were mixed with 60-80 µl hybridisation buffer and 20 µl of the mix was pipetted on each of the 3-4 groups on the slides. Hybridisation chambers with slides inside were incubated in a hybridisation oven at 46° C for a minimum of 3 h and up to 24 h. Then stringent washing buffer (5 M NaCl, 1 mM Tris/HCl, 10% SDS, 0,5 M EDTA (VWR) pH 8, MilliQ) was also preheated in a water bath at 48°C and used to remove probes with unspecific binding (incubation for 15 min). Slides were then dipped into ice cold MilliQ water and air

dried at room temperature in the dark. All sections were DAPI (Sigma-Aldrich) stained, mounted with Citiflour (Agar Scientific Ltd.), and sealed with a coverslip using nail polish.

Slide analysis

For visual analysis we used a Zeiss Imager A1 axio epifluorescence microscope equipped with a mercury lamp and linked to a Windows PC with analysis Pro software (version 3.2, Soft Imaging System, Münster, Germany). Overlays of different probe pictures where done with Photoshop (Adobe Systems Inc., San Jose, CA, USA) or GIMP2 (The GIMP Developer team, <http://www.gimp.org/>).

RESULTS

PCR

PCR screening with general bacterial primers showed positive results for all samples, thus symbionts-specific primers (RifTO 44 and RifTO445) were used in further applications. In all juvenile specimens, for which trophosome, skin, and tube were tested together in the samples, as well as in the trophosome of all adults, taken as control, *Endoriftia* was detected (see Table 5). In all adults the symbiont was found in at least one tube sample, only, in specimen 1541, the symbiont was not detectable in the tube. It was also lacking in the upper and middle tube part of specimen 1542 and in the upper tube part of specimen 1540. Taken together, *Endoriftia* was present in 76 % of the tube samples of adult *Riftia* with differences at the two sampling sites. In the three adult specimens collected at TICA, *Endoriftia* was detected in three out of nine tube subsamples, while in the specimens collected at P-Vent North, all nine tube subsamples were positive for *Endoriftia*.

DGGE

After positive results with PCR all samples were investigated more detailed with DGGE. The full 16S rRNA gene sequence of *Endoriftia* from trophosome and skin samples was used as a marker (M) on every DGGE gel (Fig. 1). Bands at the same height as the marker were considered to include the sequence of *Endoriftia* and were later sequenced to obtain a definite proof. Concerning the presence of *Endoriftia*, PCR results matched those revealed by DGGE. Comparing the different DGGE gels of skin, tube and trophosome of different adult specimens, the tubes were found to harbor a more diverse bacterial community than the skin and trophosome. DGGE gels of the trophosome revealed only *Endoriftia*, except for all three trophosome subsamples of specimen 1541 (Fig. 1a). The bacterial community detected in the skin was also little diverse. Beside *Endoriftia* being found in 100% of all skin samples, additional bands were detected in almost all subsamples of the skin (Fig. 1b). Surprisingly we found one of these bands not only in 83.3 % of all *Riftia* skin samples, but also in two tubes and in the three trophosome subsamples of specimen 1541 (Fig. 1a and table 3).

On DGGE gels of juvenile *Riftia* there were lots of smear bands. They emerged because of overloading the gel with very high amounts of DNA, to ensure visibility even of weak bands but nevertheless all bands cut out of the gel and sequenced came from *Endoriftia* and no other microbes could be detected in juveniles (Fig. 1).

Sequencing

Sequencing DGGE gels was carried out on the most abundant bands found in the tube as well on all the bands in skin and trophosome. The sequencing results matched well with those of PCR and DGGE.

Sequencing of the bands found in 83.3% of the skin but also the three bands found in the trophosome of specimen 1541 and the single bands in the subsamples of specimen 1540, 1541, 1542 resulted in the discovery of a new member of the class of Mollicutes. Its closest relative with a similarity of 94.8% was found in the gut of a deep-sea chiton (Dupperon et al. 2012). In addition to the bands revealed as *Endoriftia*, present in all skin samples and Mollicutes present in all skin samples, except those in specimen 1555, a third band (Fig. 1b) found in the skin showed that it is an artifact as a result of irregularities in the DGGE process per se. This band is a mix of the two already known sequences of *Endoriftia* and Mollicutes. As expected, the trophosome harbored the bacterial community with the lowest richness: in all subsamples only *Endoriftia* was found with the exception of a Mollicutes sequence detected in all three trophosome subsamples of specimen 1541.

Endoriftia was found in 12 sub samples of the tube. Mollicutes was found in three sub samples of the tube. In addition, also bacteria of the CFB group, Alpha-, Delta-, Epsilonproteobacteria, Verrucomicrobia, and a lot of unidentified microbes were found in adult tube samples. A more detailed overview can be seen in table 7.

In contrast to the gels of adult skin and tube subsamples all sequences coming from DGGE gel bands of juvenile *Riftia* samples showed the same 16S phylotype namely the one of *Candidatus Endoriftia persephone*.

TRFLP

The results of the Multidimensional Scaling (MDS) show that all tube subsamples differed clearly from each other. A more detailed look reveals that they cluster in three distinct bacterial communities, which are related to the three different tube sections (lower part, middle part and upper part). There was a community, which thrived on the lower part of the tubes (green dots, see figure 2), which differed from the communities living on the middle (blue dots) and on the upper part of the tube (red dots). Although there were TRFLP peaks found in the other clusters, all three groups differed significantly from each other. Surprisingly, also the bacterial tube communities of the two close sampling sites P-Vent North and TICA differed significantly from each other (Fig. 3). Juvenile tubes could not be analyzed with TRFLP because it was not possible to separate the tube from the animal without contaminating the tube with the symbiont coming from the trophosome during dissection.

FISH

Fluorescence *in situ* hybridization showed positive results for *Endoriftia* in both, juvenile and adult *Riftia* specimens. Signals of both, EUB-mix and symbiont-specific probes were detectable in trophosome and skin subsamples of adult *Riftia* (see figure 4 and 5 respectively). Generally labels of Cy3 probes were stronger but also in FLUOS probes showed good labels. Labels of symbionts in trophosome were more explicit than in the skin. *Endoriftia* could be precisely localized in the skin of an adult specimen (1540) being intracellular in the blood vessel epithelium (see figure 5), but it was also detected extracellular attached to the cuticle (see figure 6).

In juvenile *Riftia* (Sample 1467) the symbiont was detected intracellular in the epidermis of the skin (Fig. 6). The symbiont could not be detected in the tube so far, but not all samples have been screened and further investigations are necessary to confirm this result. Interestingly there was a clear and strong DAPI signal on the outer surface and inside of both, adult and especially juvenile tubes (see figure 7a). Surprisingly EUB-mix and symbiont specific probes showed no signal at the same position of the DAPI signal on the tube.

In consequence of the DGGE findings screening of adult skin and tube samples was started with a probe specifically designed for Mollicutes. No signal has been detected yet as only a few samples have been analyzed.

DISCUSSION

Endoriftia:

In symbiosis the assurance of successful transmission of symbionts is crucial for the life of a nutritionally dependent host especially in a rather unstable environment like deep-sea hydrothermal vents. After the symbiotic relationship in the giant beard worm *Riftia pachyptila* was detected (Jones, 1981; Cavanaugh et al. 1981) different models for symbiont acquisition and transmission were suggested. First it was thought that the aposymbiotic, lecithotrophic and planctivoric *Riftia* larvae take up their bacterial symbiont *Candidatus* Endoriftia persephone with other food via the mouth and gut (Jones and Gardner, 1988; Southward, 1988). A study by *Nussbaumer and colleagues* (2006) showed that the transmission of symbionts does not happen via the digestive system. In contrast to previous assumptions they found symbiont cells already within bacteriocytes in the region of the foregut in mesodermal tissue located between the dorsal blood vessel and the foregut in an early trochophore larva with specific FISH probes. In contrast to that they did not find any symbiont cells inside the gut. Based on these findings the current model for the transmission mode in the *Riftia pachyptila* system depicts an early and temporary limited uptake of the symbiont *Candidatus* Endoriftia persephone. After a short free-swimming phase with a fully developed and working digestive system the metatrochophore larvae settle on basalt rock next to hydrothermal vents in areas of diffuse flow (see Bright & Lallier, 2010). Uptake of symbiotic bacteria via the skin is thought to start simultaneously with settlement. Symbionts coming from the vent environment infect the early mucus-like tube and the skin. They also seem to accumulate in the mucus tube and from there move deeper into the epidermis. They migrate through several tissue layers until they reach the visceral mesoderm surrounding the foregut which is the final location and place where the trophosome develops. Only a small amount of bacteria is thought to serve as a starting population for the young developing trophosome because shortly after the symbiont uptake massive apoptosis of endodermal and mesodermal cells surrounding the deeper visceral mesoderm stops symbiont infection (Nussbaumer et al. 2006; Bright et al. 2012). No symbionts have been found in the skin and tube of juvenile and adult specimens of *Riftia* so far.

In contrast to that we were able to detect Endoriftia in the skin of both adult and juvenile *Riftia* and also in tubes of adult *Riftia* and *Tevnia jerichonana* with PCR, DGGE and for *Riftia* also localize it with FISH. Combined results from PCR and DGGE showed that Endoriftia was present in all skin subsamples and in twelve out of eighteen tube subsamples of adult *Riftia* (see table 5). This leads to several possible explanations: The presence of Endoriftia in adult and juvenile skin could be an evidence for a prolonged transmission mode in the *Riftia* symbiosis system with a possible symbiont infection during the whole lifespan of the tubeworm. A similar transmission mode was found in legumes where Rhizobia constantly infect the plant roots and are also released back to the environment (Gage, 2004, Hirsch 1996). More precisely there are even two possibilities for the prolonged transmission in *Riftia*: First, the same symbiont strain is infecting the tubeworms like this is suggested for most of the vestimentiferan tubeworms (see Bright & Lallier, 2010). Secondly, it is also possible that there is a cheating strain which is able to infect the worms early in life and does not provide any benefit. This was investigated for *Lotus japonicus* with both beneficial and cheating strains of native-occurring *Bradyrhizobium* symbionts. In this system the host is able to control and sanction the cheating symbionts strain which leads to lower infection rates of the cheater (Sachs et al. 2010).

From a different point of view it could also mean that the symbiont is capable of leaving its host while it is still alive. If this process is active, when bacteria migrate out of the host or passive like in the bobtail squid *Euprymna scolopes* where the symbiotic population of *Aliivibrio fischeri* in the crypts of the light organ is regulated by the squid host via symbiont release (Boettcher et al. 1996) cannot be answered. It is also possible that symbionts infect *Riftia* early in life and then stay in the skin and also in the tube and cannot migrate to their final destination and die there. It could also be a mix of both, prolonged infection and active escape. The fact that Endoriftia was not detected in every single tube sample of adult *Riftia* could point to possible environmental differences between the two sampling sites TICA and P-Vent North. In TICA the symbiont was found only in three out of nine tube samples whereas in P-Vent it was detected in all nine tube subsamples.

This could point to different environmental conditions caused by different chemical conditions of the two sampled vents which could be driving the difference of the whole microbial communities and also explain the different distribution in presence and absence of Endoriftia (see Sievert & Vetriani, 2012). Environmental influence on the microbial community asso-

ciated with the tubes is discussed more in detail later in the chapter: Bacterial diversity associated with tubes.

The presence of Endoriftia in juvenile samples found with DGGE and PCR is not surprising, because all halves of the juvenile specimens included the trophosome. The main source of symbionts detectable with DGGE and PCR was therefore derived from the trophosome tissue.

The results of FISH screening gave even more proof for the presence and exact location of Endoriftia in the skin of *Riftia*. The fact that symbiont cells were found intracellular in both adult and juvenile skin gives more evidence for a possible prolonged transmission mode. Endoriftia was found intracellular in the skin of both, adult and juvenile *Riftia* but in adults it was found much deeper in the epithelium of a blood vessel compared to juveniles where it could be detected shallower in the epidermis of the skin. The deeper tissue location in adults compared to juveniles could be the first real hint for an active migration of the symbionts. Still the other two possibilities of symbiont escape and trapped symbionts cannot be excluded, as with FISH it is not possible to show where to and if bacteria are migrating. There is good evidence for a prolonged and mixed transmission mode of multiple ribotypes of its *Oceanospirillum* symbiont strain in *Osedax*, a siboglinid which lives at whale falls and is closely related to *Riftia* (Salathé and Vrijenhoek, 2012). The symbionts of *Osedax* are located in a trophosome which has a different structure compared to those found in other members of Siboglinidae. Nevertheless, the symbionts are also located in bacteriocytes which can be found in ovisac tissue as well as in the root system of the host which is necessary for the penetration of whale bones and uptake of nutrients (Goffredi et al. 2005, Katz et al. 2010, Katz et al. 2011). There is now evidence that in this symbiosis system the transmission of multiple symbiont strains seems to happen horizontally from the surrounding environment not only once but several times during the life of *Osedax* (Salathé and Vrijenhoek, 2012). Considering that *Osedax* and *Riftia* are both members of the family of Siboglinidae (see Bright & Lallier, 2010) this strengthens the possibility for a possible prolonged transmission mode also in *Riftia*. Still, it is possible that it is the other way around and the symbiont is capable of actively leaving its host.

Mollicutes

The previous assumption concerning the skin of adult *Riftia* was that it is sterile and not colonized by any bacteria. Surprisingly, we did not only find Endoriftia but also other bacteria of which one specific 16S phylotype is strongly related to the clade of Mollicutes.

Mollicutes are bacteria and known to be the smallest self-replicating forms of life. The smallest ones like *Mycoplasma genitalum* have a size of about 0.2-0.3 μm and a minimal genome size of 580.000 kb (Trachtenberg et al. 2005). The class of Mollicutes is divided into 5 orders, which are Acholeplasmatales, Anaeroplasmatales, Entomoplasmatales, Haloplasmatales and Mycoplasmatales. Mollicutes bacteria are not only very small but show more specific traits which make them unique compared to other bacteria such as the absence of peptidoglycan, a high nutritional dependence on their hosts which leads to a high host specificity (Razin et al. 1998; Trachtenberg et al. 2005). All members of the Mollicutes clade show a reduced genome size with low G-C content (Razin et al. 1998, Trachtenberg et al. 2005) which is typical of a parasitic lifestyle but can also be found in strictly vertical transmitted symbiosis (Bright & Bulgheresi, 2010). Mollicutes are well known as parasites and commensals in plants, Annelids, Arthropods, Mollusks and Vertebrates (fish, reptiles, mammals) (Gasparich, 2004; Duperron et al. 2012; Nechitaylo et al. 2009). They can cause several severe diseases in mammals (and also in humans), such as atypical pneumonia (*Mycoplasma pneumonia*, also found in hamster and mice) and rheumatoid arthritis (Razin et al. 1998). Interestingly members of Spiroplasma (which is a Genus in the Entomoplasmatales) and Phytoplasma (which is a Genus in the Acholeplasmatales) live in plant tissue, plant fluids and in insects like leaf hoppers using them as vector for transmission from plant to plant (Gasparich, 2004; Weintraub, 2005). It was also shown that members of Spiroplasma can cause male killing in butterflies and have a comparable effect on the genome of its host as *Wolbachia* has in other insect hosts (Jiggins, 2000).

In this study the 16S phylotype of the new Mollicutes member was found in 83.3% of all adult skin samples and also in a few tube and trophosome sub samples of adult *Riftia*. With a similarity of 94.8% it is related to a sequence found in the gills and the gut of the deep-sea Chiton *Leptochiton boucheti* living on wood fragments trawled around Vanuatu Islands (Duperron et al. 2012). Interestingly, this specific 16S sequence was only found in adult *Riftia* samples and neither in samples of adult *Tevnia* nor in ones of juvenile *Riftia* in this study.

This could be an evidence for high species specificity in *Riftia*. Within adult *Riftia* they were mainly found in the skin of specimens living at TICA and also in three subsamples of the trophosome of the same *Riftia* specimen and in three tube sub samples of three different animals also from TICA. In P-Vent North Mollicutes could only be detected in the skin of two out of three specimens which might point to a possible site specificity or preference of TICA. The fact that no 16S sequences of Mollicutes could be found in juvenile *Riftia* specimens points to a high time specificity of the infection, which might be limited to adult stages of *Riftia*. Altogether, these findings fit well with the typical high host specificity of all Mollicutes bacteria (Razin et al. 1998, Citti & Blanchard, 2013). The fact that most of the 16S Mollicutes sequences were found in skin subsamples and the absence in most parts of the tube and trophosome emphasizes the importance of the skin in the *Riftia* symbiosis for the onset of bacterial infections as already shown by *Nussbaumer and colleagues* (2006). The possible high species and site specificity is in contrast to the lower one of Endoriftia, which infect the four different vestimentiferan tubeworm species *Riftia pachyptila*, *Tevnia jerichonana*, *Oasisia alvinae* and *Ridgeia piscesae* which can be found at vent sites located at the East Pacific Rise, the Galapagos Spreading Center, the Guaymas Basin (*Riftia*, *Tevnia* and *Oasisia*) the Explorer Ridge, the Juan de Fuca and the Gorda Ridge (*Ridgeia*) (Desbruyeres et al. 2006, Coykendall 2011; also see Bright & Lallier 2010).

A different transmission in Mollicutes mode could be a possible reason for the differences in species, site and even time specificity compared to the transmission of Endoriftia. A lot of the Mollicutes bacteria especially members of Phytoplasma and Spiroplasma are transmitted by an insect vector to the end host which in this case are plants (Gasparich 2004; MacLean and Hogenhout, 2013). While Endoriftia is transmitted horizontally from the vent environment it could be that this newly discovered Mollicutes member is transmitted by a species which is associated with *Riftia* or which uses it as a food source. There are several crustacean species such as *Bythogaea thermydron*, *Munidopsis subsquamosa* and some *Ceuthocetes* species, which are known to feed on *Riftia* (Fisher et al. 1994, Micheli et al 2002, Gollner et al. 2006). There is also the phyllodocid polychaete species *Galapagomystides aristata* which is thought to suck and live on *Riftia* blood (Jenkins et al. 2002). All these species are in direct contact with *Riftia* and therefore could be used as a possible transmission vector by Mollicutes comparable to transmission from plants to insects and back to plants (Gasparich, 2004).

Whether this new microbe plays a more beneficial part in this symbiosis or not, is difficult to say with the current results. Only a few semi-thin sections of adult *Riftia* skin subsamples have been screened with Mollicutes specific FISH probes showing no yields. Also no fitness parameters for *Riftia* with Mollicutes compared to *Riftia* without Mollicutes infection have been measured yet. Still the high occurrence in adult *Riftia* and the absence in juveniles and in *Tevnia* might show that this is not a normal pathogen infection but something very specific.

Bacterial diversity associated with tubes

In the past tubes of *Riftia* were investigated for the bacterial community associated with them only in one study (Lopez-Garcia, 2002) so far. This study dealt with the very thin (0.3-0.5 mm) outer layer of the tubes. In the current study small pieces of the whole tube were used for further treatment. After DGGE and TRFLP screening, sequencing and analysis of the different bands found in the tube the past findings could be partially confirmed. Surprisingly, we found the 16S sequence of Endoriftia in 76% of all adult *Riftia* tube samples which is in contrast to the findings of Lopez-Garcia and colleagues. In addition to these Endoriftia sequences new 16S sequences could be detected, which have not been found in the tubes e.g. including members of the Bacterioidetes, Cytophaga, Alphaproteobacteria and many uncultured and unspecified bacteria. For more details see table 7. In general, all sequences detected fit well in the setting of chemosynthetic habitats such as deep-sea vents, volcanoes and seeps (see table 7). The 16S sequence of the same member of Mollicutes found in the skin could also be detected in the tube. Although it is not possible with the DGGE and PCR findings to see where this new bacterium is exactly located in the tube, it shows that it is not only restricted to a special tissue like the skin. This could mean that they failed to attach to the skin and targeted the tube simply because it provides also a surface for attachment. It might also be that they migrate through different tissues as this has been shown for Endoriftia (Nussbaumer et al.2006). Another reason to associate with the tube is because of nutrition. Tubes of Vestimentiferan tubeworms are known for their high amount of chitin and in *Riftia* tubes there is also a high amount of protein (Webb, 1971; Gaill & Hunt, 1986; Shillito et al.,1995; Chamoy et al., 2000), which could be used as a food source by bacteria. There is evidence that Endoriftia possesses Chitinase genes (Lopez-Garcia, 2002) to use chi-

tin as a food source and live inside the tube. This might be also possible for other vent related bacteria. As long as there is no precise localization with specific FISH probes and there are no definitive results from genome analysis for all these different tube associated bacteria this is a highly vague assumption.

Analyses of TRFLP peaks from the tubes show a clear clustering of OTUs. They clearly cluster for sampling site, which means that there is a specific community in TICA which differs from a specific one in P-Vent North. This could be driven by chemical and physical differences between the two different vents at the sampling sites. It has been shown many times (Luther et al., 2001; Von Damm, 1990; Van Dover, 2001; Xie, 2010) that fluids from different vent sites and changes in vent chemistry can influence the composition of the microbial community, which could also be the case in this study.

More specifically there have been studies about seismic and volcanic activity at the 9° North spreading center which showed that there were differences in vent activity between the two different vent sites after a strong volcanic eruption in 2006 (Tolstoy et al. 2006). In a long term monitoring study *Robidart and colleagues* (2013) found temporal and spatial differences in physical-chemical conditions, like changing temperature and methane concentration in a cluster of Vestimentiferan tubeworms next to vents at the Juan de Fuca Ridge with diffuse hydrothermal fluids, which correlated well with the occurring shifts and differences of the microbial community.

It is also clearly visible that OTUs cluster not only for site but also for sampling height within the tube. This can be explained with different succession stages of the microbial community (Cooke 1967). Tubes of *Riftia* grow from the bottom to the top (Schulze 2003; Southward, 2005). Considering this it means that one finds the oldest microbial community on the lower parts of the tube, a transitional or mixed community in the middle parts and the youngest bacterial community is found on the top of the tubes. This also means that bacteria thriving on the bottom of the tube associated with bare basalt are the ones most exposed to heat. On the top there are bacteria which are thriving in more moderate and cooler temperatures. Beside the growth of the tube variations in temperature and chemical composition of vent fluid and therefore also of the surrounding water in close proximity of the tubes might influence the visible changes and shifts of the microbial tube community from bottom to top.

This has already been shown not for tube microbes but for free living and attached microbial communities (see Hügler & Sievert, 2010).

All these findings in former studies fit well with our results and could explain the differences in the microbial community associated with different parts of the tubeworms at different sampling sites including also the differences in *Endoriftia*. This also fits well with the ecological theory of succession, when a pioneer community, often adapted to more extreme conditions is followed or even outcompeted by several transitional stages until a climax community might but not necessarily has to be reached (Cowles, 1911; Clements, 1916; Gleason, 1926; Connell & Slayter, 1977). Although bacteria are very different compared to plants, succession has also been shown for microbes underlying the same rules as higher organized biota (Cooke, 1967).

Conclusion

Further research needs to be done to clarify the question whether *Endoriftia* actively leaves *Riftia* during its lifetime or if the symbiont is able to infect its host for a longer or even during the whole life time. More skin and tube samples from *Riftia* (and also other vent Vestimentifera) should be taken and analyzed with both FISH and state of the art sequencing methods to provide more data to clarify the transmission mode in the *Riftia* symbiosis, the exact role of Mollicutes in this system and the meaning of the microbial community associated with the tubes.

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ZUSAMMENFASSUNG

Die Röhrenwürmer der Spezies *Riftia pachyptila* an den heißen Quellen des Ost-Pazifischen Rückens zählen zu den am besten erforschten und interessantesten Symbiosen Modellsystemen heutzutage.

Obwohl extreme Bedingungen für Leben an den Quellen herrschen, haben es viele Organismen mit Hilfe von symbiontischen Bakterien geschafft dort zu leben. *Riftia* lebt in Symbiose mit *Candidatus Endoriftia persephone*, einem chemoautotrophen, schwefeloxidierenden Gammaproteobakterium, das in einem speziellen Organ, genannt Trophosome, in millionenfacher Zahl lebt. Der Röhrenwurm nimmt Sauerstoff (O), Sulfid(H₂S) und Kohlendioxid (CO₂) aus dem Wasser auf und transportiert es mittels des Blutes zu den Symbionten im Trophosome. Dort oxidieren die Bakterien Sulfid zu Sulfat. Diese Reaktion ist energiereich und es wird ATP gebildet, welches wiederum im Calvin-Benson Zyklus und Reverse-Citrate Zyklus zur Herstellung von C₄ Zuckern aus CO₂ genützt wird. Einen Teil der entstandenen Kohlenhydrate geben die Symbionten dem Wurm. Der Wurm bietet den Bakterien im Gegenzug einen sicheren, relativ störungsfreien Ort zum Leben und einen konstanten Strom an Nährstoffen.

Endoriftia wird mittels horizontaler Transmission in jeder Generation von *Riftia* neu übertragen. Dabei wird eine gewisse Anzahl an Symbionten nicht wie in der vertikalen Transmission über ein Elternteil an die Nachkommen weitergegeben sondern freilebende Symbionten infizieren die Larven von *Riftia*, d.h.: es gibt zwei Populationen des Symbionten: Eine im Wirt und eine im Umgebungswasser der Vents. Die freilebende Population dient als Quelle für Neuinfektionen von *Riftia* Larven (siehe Bright & Lallier, 2010).

In einer früheren Studie konnte gezeigt werden, dass Endoriftia Symbionten die Larven über die Haut und die sich entwickelnde Röhre infizieren und dabei durch mehrere Gewebeschichten wandern bevor sie sich im visceralen Mesoderm festsetzen und die Ausbildung des Trophosoms und die Rückbildung des gesamten Verdauungstraktes beginnt (Nussbaumer et al. 2006). In der selben Studie konnten in späteren juvenilen Stadien von *Riftia* keine symbiontischen Bakterien außerhalb des Trophosoms gefunden werden.

Daher nimmt man an, dass die Transmission relativ kurz und zeitlich begrenzt nur ein einziges Mal während der Entwicklung von *Riftia* vorkommt.

Im Gegensatz dazu konnte der Symbiont in großen Mengen sowohl im Seewasser als auch auf den Oberflächen von Probenplatten nachgewiesen werden und zwar nicht nur in unmittelbarer Nähe von Vents sondern auch ca. 100 m davon entfernt (Harmer et al. 2008).

Die Röhre von adulten *Riftia* wurde bereits auf die mit ihr assoziierte bakterielle Diversität untersucht, jedoch wurden nur die obersten Schichten und nicht die ganze Röhre dafür verwendet (Lopez-Garcia et al. 2002). Die Präsenz von *Endoriftia* konnte nicht nachgewiesen werden.

Da die Haut von adulten *Riftia* bis jetzt nicht im Detail auf mögliche vorhandene Bakterien untersucht wurde, beschäftigt sich diese Studie mit dem Vergleich der bakteriellen Diversität in der Haut und in der Röhre von juvenilen und adulten *Riftia* Röhrenwürmern.

Dazu wurden 61 Proben von 6 adulten und 6 juvenilen Röhrenwürmern von zwei Standorten, TICA und P-Vent North am Ost-Pazifischen Rücken, von Ausfahrten im Jahr 2010 und 2011 mit verschiedenen molekularbiologischen und mikrobiologischen Methoden bearbeitet. PCR (Polymerase Kettenreaktion), DGGE (Denaturierungsgradientgelelektrophorese), TRFLP (Terminaler Restriktionsfragmentlängenpolymorphismus) und FISH (Fluoreszenz *In Situ* Hybridisierung) wurden verwendet um mögliche Unterschiede in der bakteriellen Gemeinschaft zu untersuchen.

Die Ergebnisse vorangegangener Studien konnten teilweise bestätigt werden.

Erstaunlicherweise konnten aber sowohl in der Haut von juvenilen als auch in der von adulten *Riftia* 16S Phylotypen von *Endoriftia* nachgewiesen werden. Auch in der Röhre von adulten *Riftia* wurde der Symbiont gefunden. Das könnte bedeuten, dass das Zeitfenster für eine Infektion länger ist als bisher angenommen und möglicherweise findet eine Infektion während des ganzen Lebens von *Riftia* statt. Eine lebenslange Infektion bzw. Übertragung von Symbionten ist auch in Leguminosen bekannt (Gage, 2004; Hirsch 1996). Es gibt seit neuestem auch Hinweise von verlängerter und mehrfacher Übertragung sogar verschiedener Symbionten in *Osedax*, einem nahen Verwandten von *Riftia*, der auf Knochen von verschiedenen toten Wirbeltieren lebt (Salathe et al. 2012). Es könnte aber auch bedeuten dass Symbionten in der Haut der Larven steckengeblieben sind und dort mit der der Haut des Wurmes mitwachsen und sterben. Eine weitere Möglichkeit wäre, dass die Symbionten die Röhrenwürmer verlassen können und nicht hinein sondern hinauswandern und sich mit der freilebenden Population von *Endoriftia* vermischen, wie das bei *Aliivibrio fischeri*, dem Symbionten des kleinen Tintenfisches *Euprymna scolopes* der Fall ist (Boettcher et al. 1996).

Interessanterweise konnte auch eine 16S Sequenz eines bisher unbekannten Bakteriums der Mollicutes in der Haut und in der Röhre von adulten *Riftia* Exemplaren nachgewiesen werden. Sie sind bekannt als die kleinsten sich selbst replizierenden Lebensformen. Mollicutes-bakterien kommen sowohl als Pathogene als auch als nützliche Symbionten in Pflanzen, Anneliden, Arthropoden, Mollusken und Wirbeltieren vor (Gasparich, 2004; Duperron et al. 2012; Nechitaylo et al. 2009). In vielen Fällen werden sie von einem Zwischenwirt oder Vektor zum Endwirt übertragen, besonders bei Insekten zu Pflanzen (Gasparich 2004; MacLean and Hogenhout, 2013). Dies könnte jedoch auch der Fall bei diesem Vertreter der Mollicutes sein. Es ist bekannt dass, *Riftia* mit einer Vielzahl von Tieren assoziiert ist, wie Krabben, Krebse und Würmer die *Riftia* als Nahrungsquelle nutzen. Teilweise saugen sie Blut, teilweise versuchen sie Stücke der Haut zu fressen (Fisher et al. 1994; Micheli et al. 2002; Gollner et al. 2006; Jenkins et al. 2002). Dabei könnte es zu einer möglichen Übertragung von Mollicutes-bakterien kommen. Ob Mollicutes eine nützliche oder schädliche Rolle in der *Riftia* - Symbiose einnimmt kann mit den vorliegenden Ergebnissen noch nicht gesagt werden. Die Tatsache, dass der Phylotyp allerdings in 80% der adulten Hautproben gefunden wurde, deutet eher auf eine nützliche Rolle hin.

Bei der Untersuchung der bakteriellen Diversität der Röhren der sechs adulten *Riftia* Exemplare wurde erstaunlicherweise festgestellt, dass Endoriftia im Gegensatz zu älteren Studien in der Röhre vorhanden ist. Das verstärkt die Hypothese, dass Endoriftia fähig ist seinen Wirt zeitlebens zu infizieren. Die bakterielle Diversität in den Röhren war ähnlich hoch wie in älteren Studien und die vorgefunden Sequenzen stehen fast alle in Zusammenhang mit Bakterien an Tiefseevulkanen und heißen Quellen in der Tiefsee.

Die Röhren der zwei unterschiedlichen Standorte unterscheiden sich jedoch signifikant in der Zusammensetzung der mit ihnen assoziierten Bakterien. Das könnte an chemischen und physikalischen Unterschieden der beiden Orte liegen. Es ist bekannt, dass alle heißen Quellen der Tiefsee mit der Zeit immer wieder Veränderungen ihrer Stoffzusammensetzung aufweisen. Es unterscheiden sich jedoch nicht nur die Gemeinschaften der beiden Standorte sondern auch die Gemeinschaften am selben Ort nur in unterschiedlichen Höhen der Röhre. Das könnte ein Hinweis auf Sukzession der bakteriellen Gemeinschaft sein (Cooke 1967). Die ältesten und heißesten Teile der Röhre liegen unten, während die jüngsten und kühlfsten am oberen Ende zu finden sind. Abschließend kann gesagt werden, dass mehr Proben gesam-

melt werden müssen um den Transmissionsmodus von Endoriftia besser verstehen zu können und um die genaue Rolle von Mollicutes in diesem System zu klären.

LEGENDS

Table 1: Adult and juvenile specimens of *Riftia pachyptila* sampled at two different vent sites in 2010 and 2011. Adults were dissected into trophosome, skin and tube subsamples, juveniles cut in halves.

<i>Riftia</i>	Sample	Site	Layer	Partition	Abbreviation	Year
adult	1540	TICA	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
adult	1541	TICA	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
adult	1542	TICA	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
adult	1555	P-Vent North	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
adult	1556	P-Vent North	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
adult	1557	P-Vent North	Trophosome	Upper/Middle/Lower	TrU, TrM, TrL	2011
			Skin	Upper/Middle/Lower	SU, SM, SL	
			Tube	Upper/Middle/Lower	TU, TM, TL	
juvenile	1464	P-Vent South	Whole specimens		1464	2010
juvenile	1465	P-Vent South	Whole specimens		1465	2010
juvenile	1466	P-Vent South	Whole specimens		1466	2010
juvenile	1467	P-Vent South	Whole specimens		1467	2010
juvenile	1470	P-Vent South	Whole specimens		1470	2010
juvenile	1471	P-Vent South	Whole specimens		1471	2010

Table2: Primers for PCR, DGGE and TRFLP:

Primer species	Primer sequence	
PCR primers		
RifTO44 forward (symbiont specific)	5'-GGC CTA GAT TGA CGC TGC GGTA-3'	Harmer et al. (2008)
RifTO445 reverse (symbiont specific)	5'-TCC TCA GGC TTT TCT TCC-3'	Nussbaumer et al. (2006)
27F forward (general bacteria)	5'-AGA GTT TGA TCC TGG CTC AG-3'	Lane (1991)
1492R reverse (general bacteria)	5'- GGT TAC CTT GTT ACG ACTT-3'	Lane (1991)
DGGE primers		
	5'- <u>CGC CCG CCG CGC GCG GCG GGC</u>	
341_F (forward with <u>GC-clamp</u>)	<u>GGG GCG GGG GCA CGG GGG GCC</u> TAC GGG AGG CAG CAG-3'	Muyzer et al. (1993)
907_R (reverse)	5'-CCG TCA ATT CMT TTG AGT TT-3'	Muyzer and Smalla (1998)
341_F (forward, no GC-clamp)	5'-CCT ACG GGA GGC AGC AG-3'	Muyzer et al. (1993)
TRFLP primers		
27F-6FAM forward primer	FAM-5'-AGA GTT TGA TCC TGG CTC AG-3'	Suzuki et al. (1998)
1492R-VIC reverse primer	VIC-5'- GGT TAC CTT GTT ACG ACTT-3'	Fierer (2006)

Table 3: Chemicals and reagents for PCR; DGGE and TRFLP reactions

Chemical or reagent with concentration	Amount
PCR and DGGE (for 50 µl)	
PCR grade water	34.3 µl
Buffer (10x Taq buffer + KCL; -MgCl ₂)	5 µl
DNTPS (2mM)	5 µl
MgCl ₂ (20mM)	4 µl
Primers (50mM)	0.5 µl
BSA (20mg/ml)	0.5 µl
Taq polymerase (5 U/µl)	0.5 µl
Template (extracted DNA)	1 µl
TRFLP – PCR	
PCR grade water	34.3 µl
Buffer (10x Taq buffer + KCL; -MgCl ₂)	5 µl
DNTPS (2mM)	5 µl
MgCl ₂ (20mM)	4 µl
Primers (50mM)	0.5 µl
BSA (20mg/ml)	0.5 µl
Taq polymerase (5 U/µl)	0.2 µl
Template	1 µl
TRFLP – Digestion (20 µl reaction)	
Restriction enzyme Hha I (20U/ µl)	0.2 µl
BSA 100x (20mg/ml)	0.2 µl
NEB Buffer 4, 10x	2 µl
PCR grade water	Different amounts
DNA	Volume with 100ng
TRFLP - Purification	
HiDi formamide	10 µl
1200 Liz Standard,	0.3 µl
Digested sample	1.5 µl

Table 4: FISH probes with probe sequences

Probe	Probe sequence	Reference
	EUB338 I 3'-GCT GCC TCC CGT AGG	Aman et al. (1990)
	AGT-5'	
EUB-mix 338 (mix of 3 Eubacteria probes)	EUB338 II 3'-GCA GCC ACC CGT AGG	Daims et al. (1999)
	TGT-5'	
	EUB338 III 3'-GCT GCC ACC CGT AGG	Daims et al. (1999)
	TGT-5'	
RifTO44 forward (symbiont specific)	5'-GGC CTA GAT TGA CGC TGC GGTA-3'	Harmer et al. (2008)
RifTO445 reverse (symbiont specific)	5'-TCC TCA GGC TTT TCT TCC-3'	Nussbaumer et al. (2006)
NONEUB (nonsense probe)	5'-ACT CCT ACG GGA GGC AGC-3'	Manz et al.(1992)

Table 5. Results from PCR and DGGE screening, showing presence and absence of *Endoriftia* in trophosome, skin and tube of different *Riftia* specimens; Tr=trophosome, S=skin, T=tube, u=upper part, m=middle part, l= lower part; +: present, -: absent

Sample names	Tru	Trm	Trl	Su	Sm	Sl	Tu	Tm	Tl
adults									
1540	+	+	+	+	+	+	-	+	+
1541	+	+	+	+	+	+	-	-	-
1542	+	+	+	+	+	+	-	-	+
1555	+	+	+	+	+	+	+	+	+
1556	+	+	+	+	+	+	+	+	+
1557	+	+	+	+	+	+	+	+	+
juveniles					whole specimens				
1464					+				
1465					+				
1466					+				
1467					+				
1470					+				
1471					+				

Table 6. Results from DGGE screening, showing presence and absence of Mollicutes in trophosome, skin and tube of different *Riftia* specimens; Tr= trophosome, S= skin, T= tube, u= upper part, m= middle part, l= lower part; +: present, -: absent

Sample names	Tru	Trm	Trl	Su	Sm	Sl	Tu	Tm	Tl
adults									
1540	-	-	-	+	+	+	-	-	+
1541	+	+	+	+	+	+	+	-	-
1542	-	-	-	+	+	+	-	-	+
1555	-	-	-	-	-	-	-	-	-
1556	-	-	-	+	+	+	-	-	-
1557	-	-	-	+	+	+	-	-	-
juveniles					whole specimens				
1464					-				
1465					-				
1466					-				
1467					-				
1470					-				
1471					-				

Table 7. Overview of the sequences found in tube and skin of adult *Riftia pachyptila*.

Class	16S sequence name	Reference
Gammaproteo-bacteria	<i>Candidatus</i> Endoriftia persephone Riftia pachyptila endosymbiont 16S ribosomal RNA gene, partial sequence	Feldmam et al. 1997
Uncertain	Unc.Bacterium clone RESET_28F05	Sylvan et al. 2011
	Unc. Clone SBYW_1352	Harris et al. 2011
	Unc. sediment bacterium - clone 263-17	Schauer et al. 2011)
	Unc. bacterium clone SWB304	Fagervold et al. 2011
	Unc. bacterium clone V1tb38	Dupperon et al. 2008
	Unc. bacterium clone KZNMV-5-B94	Pachiadaki et al. 2010
	Uncultured marine bacterium clone tc3_a01 16S ribosomal RNA gene, partial sequence	Meron et al., 2012
	Uncultured bacterium partial 16S rRNA gene, clone GoC_Bac_39_D0_C0_M1	Maignien et al. 2010
Firmicutes	Uncultured bacterium partial 16S rRNA gene, clone LBDT0517-A (Mollicutes)	Dupperon et al. 2012
CFB group	Cytophaga sp. BHI80-3	Cambon-Bonavita et al. 2002
	Unc. CFB group bacterium partial 16S rRNA gene, clone P.palm C/A 42	Alain et al. 2002
	Unc. bacteroidetes clone MS-A132	Suarez-Suarez et al.2011
	Uncultured CFB group bacterium clone R103-B20	Lopez-Garcia et al. 2002
	Uncultured Bacteroidetes bacterium partial 16S rRNA gene, clone OC35	Becker et al. 2008
Delta Proteobacteria	Unc. deltaproteobacterium clone ATOS Iris 7 Rainbow 74 Uncultured delta proteobacterium clone LF8GH2b21 16S ribosomal RNA gene, partial sequence	Cambon-Bonavita et al. 2005 Forget et al. 2011
Verrucomicrobia	Uncultured Verrucomicrobia bacterium clone R76-B66	Lopez-Garcia et al. 2002
Epsilon Proteobacteria	Uncultured epsilon proteobacterium clone RT07C_BI_84	Lopez-Garcia et al. 2002
Alpha Proteobacteria	Unc. alphaproteobacterium clone TS-14	Durand et al. 2011

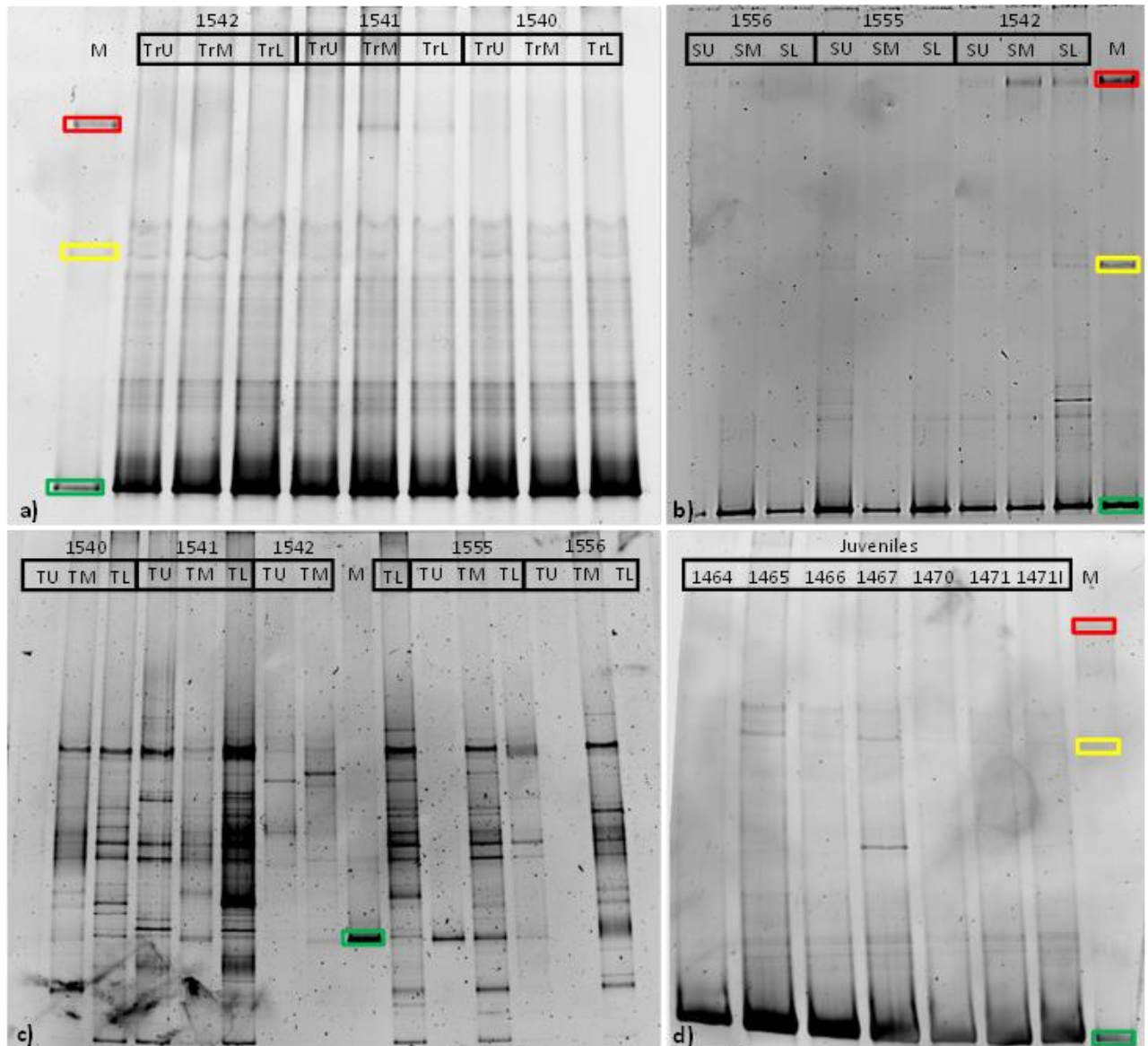


Fig.1. Pictures of DGGE gels showing trophosome subsamples of three different specimens (a), subsamples of the skin (b), tube subsamples (c) and subsamples of juvenile specimens (d). Tr= trophosome, S= skin, T= tube, u= upper part, m= middle part, l= lower part; green frames = *Candidatus* Endoriftia persephone, red frames = Mollicutes; yellow frames = mix of Mollicutes and Endoriftia in the skin and just Endoriftia in the trophosome; M = marker: Always included the three bands mentioned before except for the tube gel only with the band of Endoriftia.

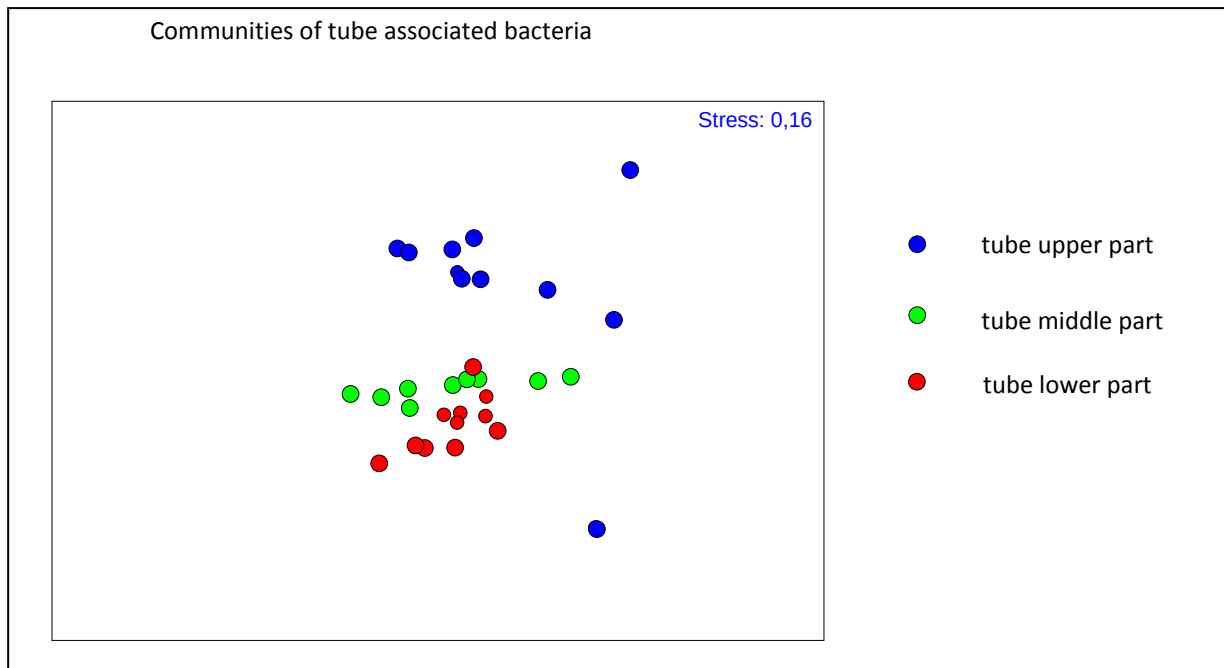


Fig. 2. MDS plot of all adult *Riftia* tube subsamples showing the bacterial communities inhabiting the three different parts of the tube. blue dots = bacteria from the upper part of the tube; green dots = bacteria from the middle part of the tube; red dots = bacteria from the lower part of the tube.

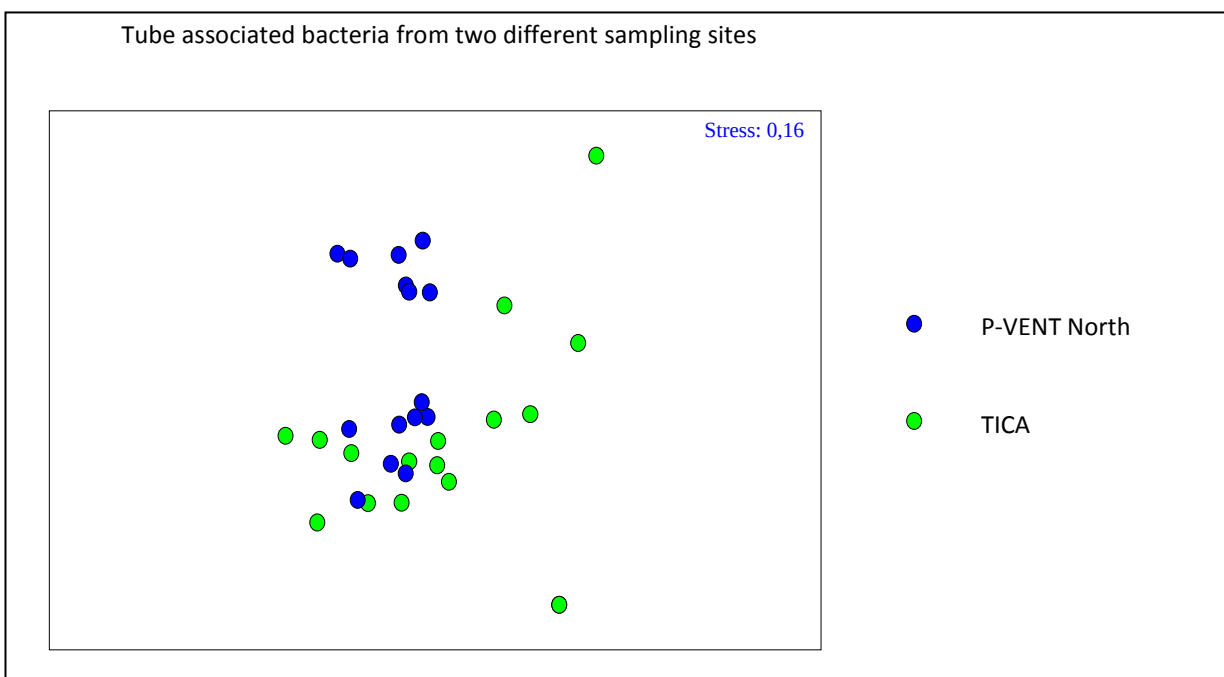


Fig. 3. MDS plot of all adult *Riftia* sub samples showing the bacterial communities from two different sampling sites P-Vent North and TICA. blue dots= P-Vent; green dots= TICA

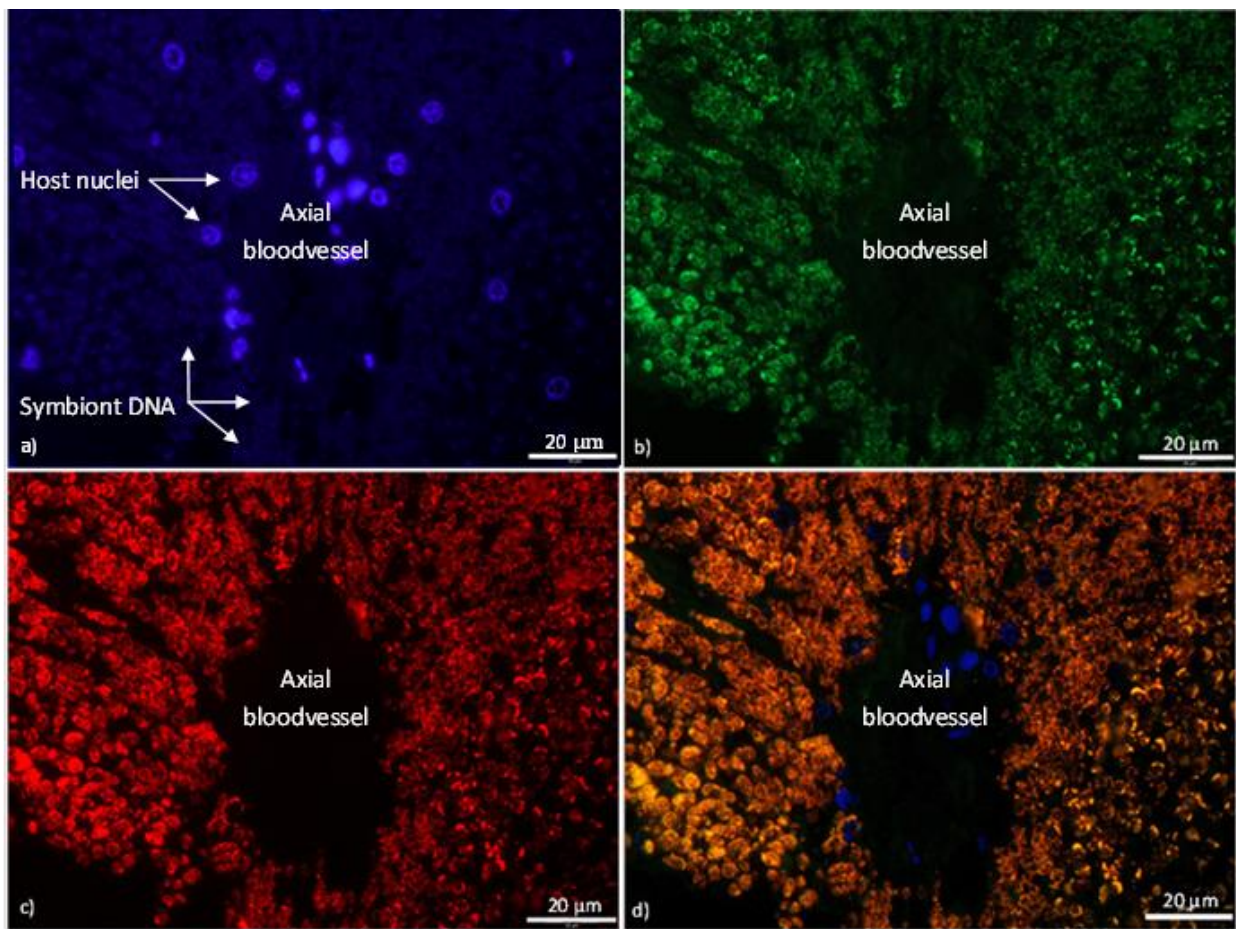


Fig. 4. Epifluorescence images of trophosome sections of an adult *Riftia* sample (1540 TO) with symbionts located in bacteriocytes: DAPI staining of DNA, big blue rings and dots are host nuclei, smaller ones symbionts cells (a), EUB-mix probe in FLUOS (b), symbiont-specific probe in Cy3 and the overlay of the EUB-mix probe, symbiont-specific probe and DAPI staining (d).

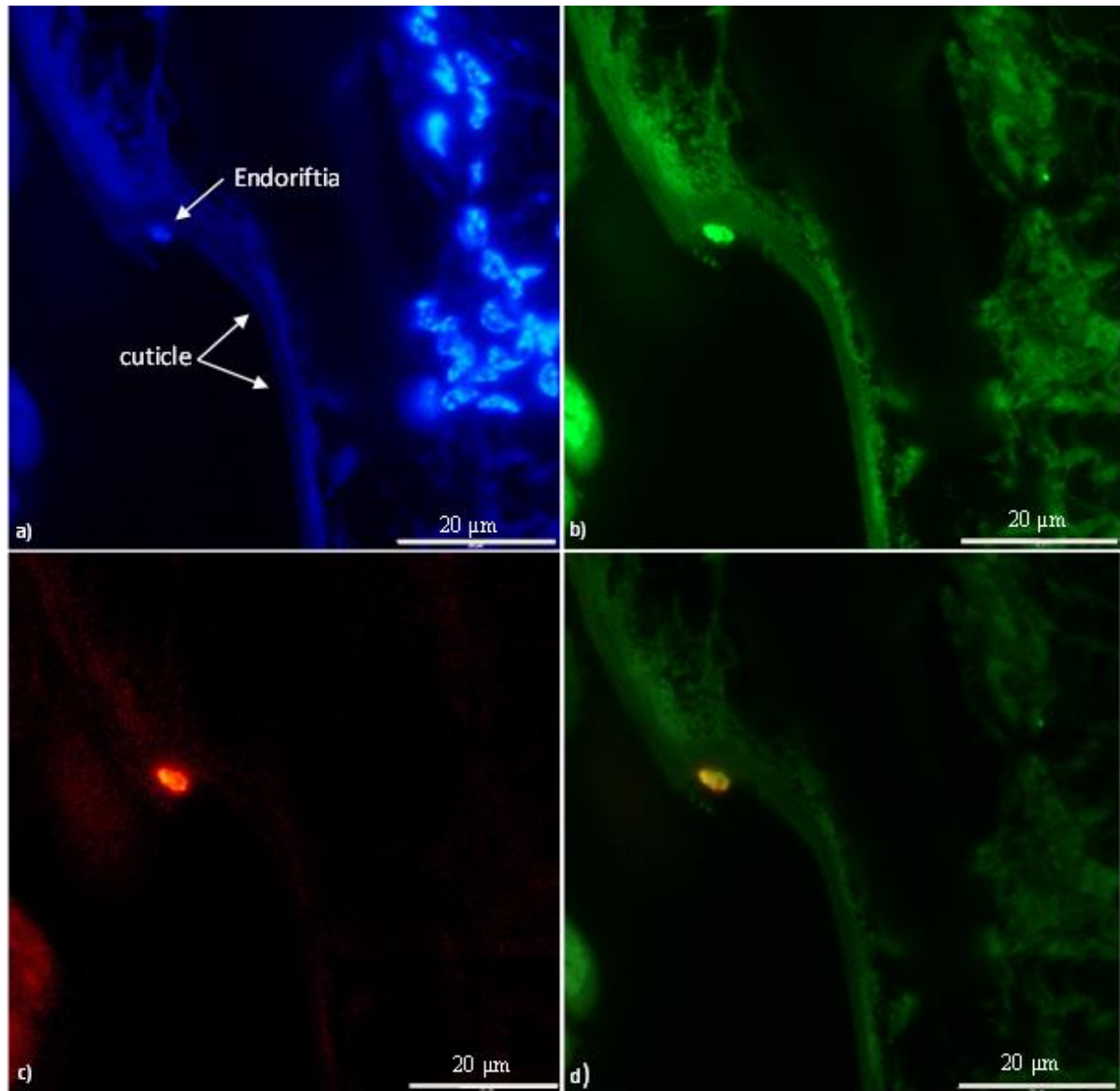


Fig.5. Epifluorescence images of symbionts located outside of the skin on the cuticle of a subsample of an adult *Riftia* (1540 SM). DAPI staining of all DNA (a), EUB-mix probe in FLUOS for general Eubacteria (b), symbiont specific probe RifTO 445 in Cy3 (c) and the overlay symbiont-specific and EUB-mix probe.

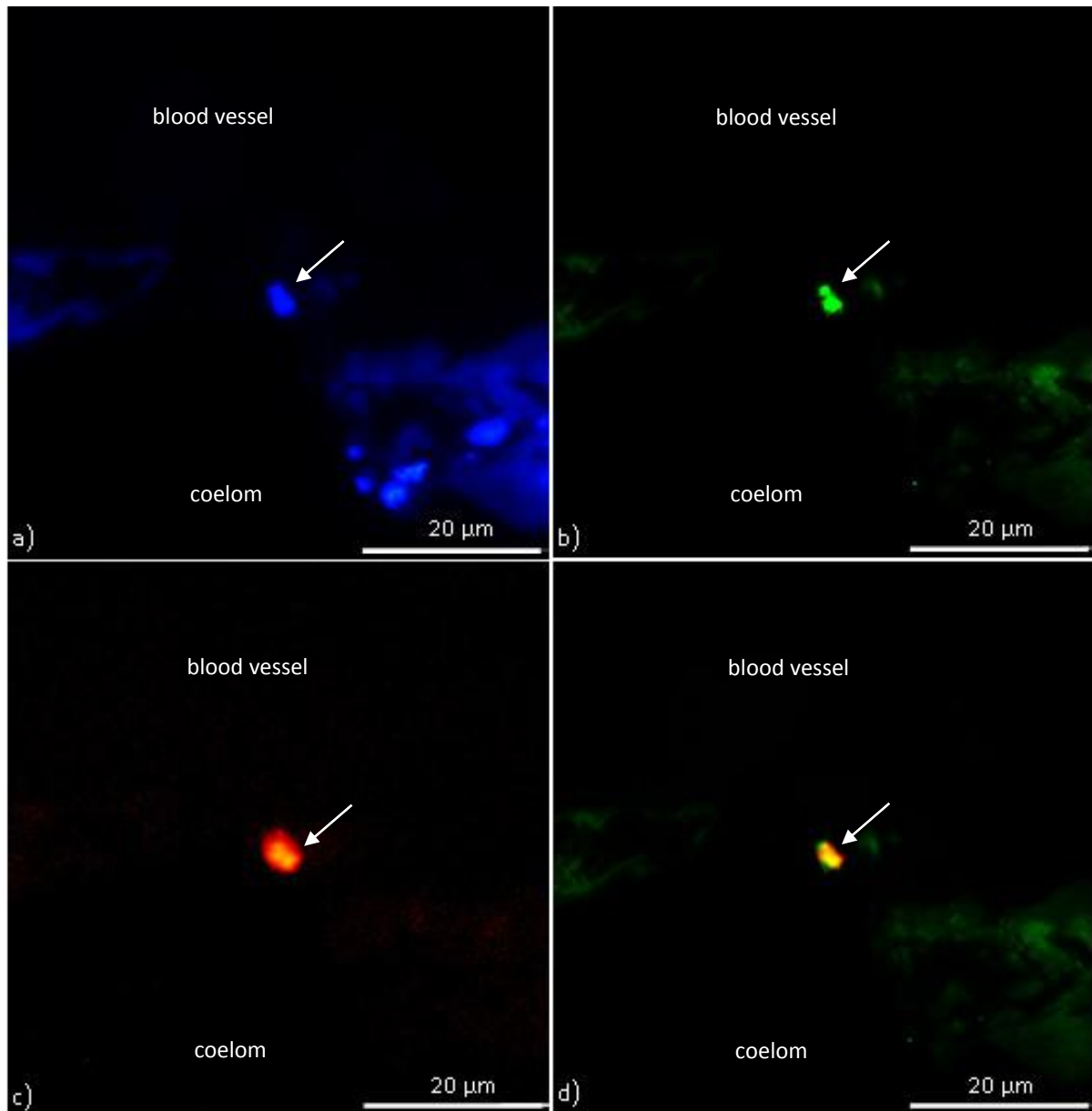


Fig. 6. Epifluorescence images of skin sections of one adult *Riftia* sample (1540 SL) with symbiont located in host tissue being intracellular in the epithelium of a blood vessel: DAPI staining of DNA (a), EUB-mix probe in Cy3 (b), symbiont-specific probe in Cy3 (c), and overlay of EUB-mix and symbiont-specific probe (d). Arrows indicate the symbiont. Arrows indicate the signal of *Endoriftia* with the different probes/DNA staining.

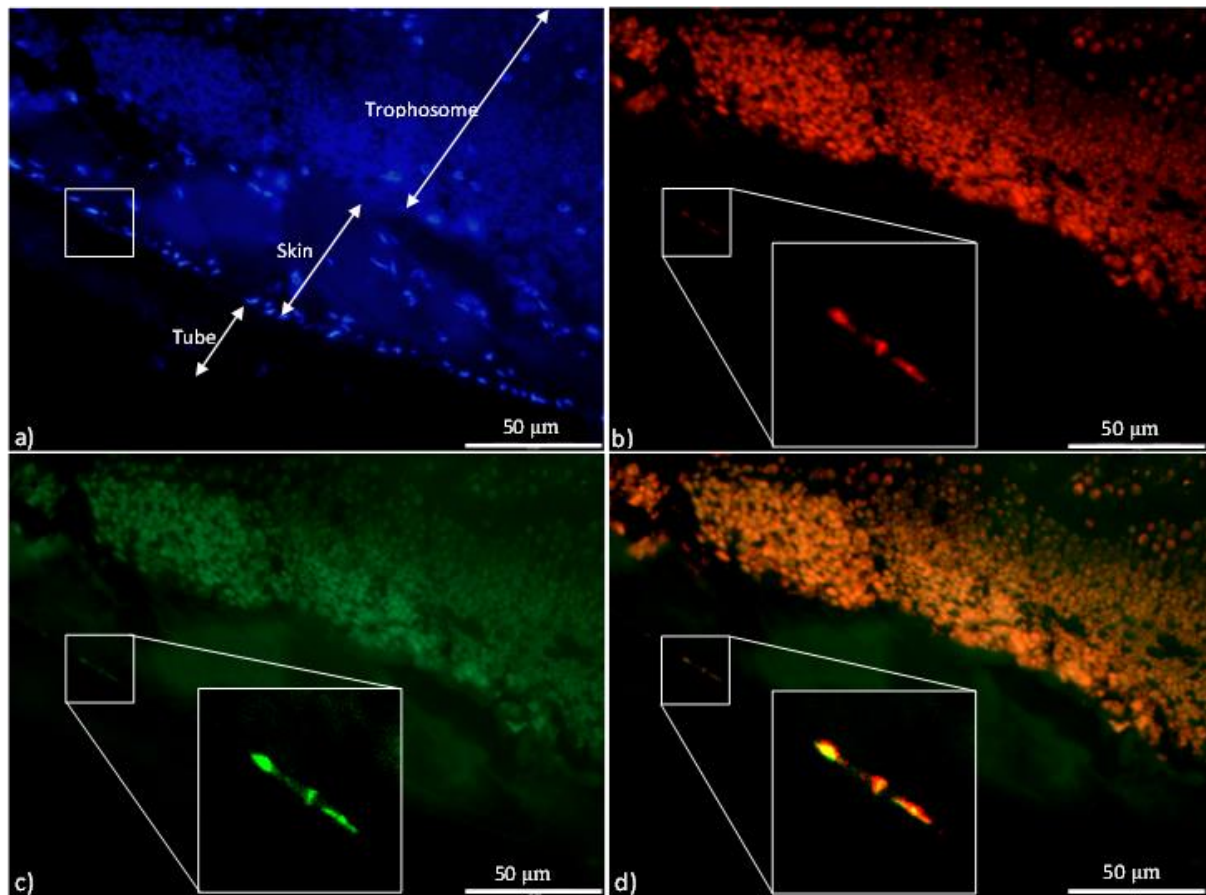


Fig.7. Epifluorescence images of sections of a juvenile *Riftia* (1467) with symbionts located in the trophosome and epidermis of the host. DAPI staining of DNA (a), EUB-mix probe in Cy3 (b), symbiont-specific probe RifTO 445 in FLUOS (c), and overlay of symbiont-specific and EUB-mix probe (d). Arrows in Fig.6a) show the size of the different layers. Endoriftia was found in the epidermis which is described here as part of the skin, the exact position is highlighted with white frames in all images.

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