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Characterization of the association between bacterial ectosymbionts and *Robbea* sp. 2 nematodes from the Caribbean

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Einleitung

Symbiose

Was ist Symbiose?

Diese Frage möchte ich an den Anfang meiner Diplomarbeit stellen. Es ist wichtig diesen Begriff näher zu erläutern, da er in der wissenschaftlichen Literatur nicht eindeutig verwendet wird. Ins Leben gerufen wurde der Ausdruck „Symbiotismus“ von dem Botaniker Albert Bernhard Frank 1877 in Deutschland. Entscheidend geprägt wurde der Begriff „Symbiose“ allerdings von dem Botaniker Anton de Bary, der durch die Erforschung von Flechten Ansehen erlangte, und in diesem Zusammenhang die Bedeutung entscheidend prägte. In seiner ursprünglichsten Form bedeutet Symbiose das enge Zusammenleben von Organismen meist unterschiedlicher Art. Das Wort leitet sich von den griechischen Wörter *syn* (zusammen) und *bios* (das Leben) ab und bedeutet „Zusammenleben“. (Wilkinson, 2001) Diese Definition enthält also das Zusammenleben zu beiderseitigem Vorteil (Mutualismus), den Nutzen der einen Seite zum Schaden der anderen (Parasitismus) und der Vorteil des Einen ohne Nutzen oder Schaden für den Anderen (Kommensalismus). Im allgemeinen Sprachgebrauch und in der wissenschaftlichen Literatur wird Symbiose allerdings oft mit Mutualismus gleichgesetzt. In eine Symbiose involvierte Organismen werden oft in Wirt und Symbiont unterteilt, wobei es üblich ist, dass meist der kleinere Partner als Symbiont bezeichnet wird (Monika Bright, persönliche Mitteilung).

Wer geht Symbiosen ein?

Die Symbiose ist ein Phänomen, das sich bei Vertretern aus allen drei Domänen des Lebens (*Archaea*, *Eubakteria* und *Eukaryota*) findet(Sapp, 2005). Bekannte und gut beschriebene Beispiele sind mutualistische Assoziationen zwischen Grünalgen und Pilzen (Flechten), zwischen Stickstoff fixierenden α-Proteobakterien (*Rhizobiaceae*) und Hülsenfrüchtlern (*Fabaceae*) oder zwischen dem gramnegativen Bakterium *Vibrio fischeri* und dem Tintenfisch *Euprymna scolopes*. Symbiontische Beziehungen

haben überdies einen entscheidenden Beitrag zur Entwicklung eukaryotischen Lebens auf der Erde schon vor über 1,5 Millarden Jahren geleistet. Die Mitochondrien, Zellorganellen, die mit wenigen Ausnahmen in allen eukaryotischen Zellen vorkommen, sind bakteriellen Ursprungs, ebenso wie die Chloroplasten, die die Grundlage für die pflanzliche Photosynthese darstellen (Dyall et al., 2004). Weiters vertritt Takemura (2001) die Hypothese, dass sich der Zellkern durch Aufnahme eines pockenvirusähnlichen Organismus in ein Archaebakterium entwickelt hat und es findet auch zwischen mikrobiellen Evolutionisten ein belebter Diskurs darüber statt, ob der Zellkern nicht aus der Fusion zwischen zwei miteinander in Symbiose lebenden Prokaryoten entstanden ist (Sapp, 2005). Für Mikroorganismen hat sich mit der Entstehung von multizellulärem Leben ein neues Habitat eröffnet, das sowohl stabile Verhältnisse bietet als auch reich an Nährstoffen ist. So reicht die Verbreitung mutualistischer Assoziationen zwischen Eukaryoten und Prokaryoten von Mikroorganismen, die sich im menschlichen Darm befinden, den sogenannten Mikrobiota (früher auch „Darmflora“), (Gill et al., 2006) bis hin zu Symbiosen zwischen marinen Nematoden und schwefeloxidierenden Bakterien (thiotrophe Symbiose) in kalkhaltigen Sanden tropischer Meere. Letztere werden in der hier vorgelegten Arbeit näher behandelt.

Thiotrophe Symbiose

Die thiotrophe Symbiose wurde erstmals 1977 bei den Riesenröhrenwürmern der Klasse *Vestimentifera* in der Tiefsee in der Nähe von Hydrothermalquellen beschrieben. Während ihrer Entwicklung werden diese Würmer von freilebenden schwefeloxidierenden Bakterien infiziert, woraufhin ihr Verdauungssystem degeneriert (Cavanaugh et al., 1981)(Nussbaumer et al., 2006). Im Zuge der Entwicklung bilden sie ein Organ (Trophosom) aus, in dem sich ihre Symbionten sammeln und vom Wirt mit reduzierten Schwefelverbindungen und CO_2 versorgt werden. Die Bakterien dienen dem Wurm als Nahrungsquelle, während der Wirt ihnen ein stabiles nährstoffreiches Habitat bietet. In Abbildung 1 wird der Metabolismus von schwefeloxidierenden Bakterien skizziert. Schwefelwasserstoff (H_2S) diffundiert in die Zelle und wird über die Enzyme APS reductase (Apr), ATP sulfurylase zu Sulfat oxidiert. Bei diesem Prozess wird ATP gewonnen das unter anderem für die Kohlenstofffixierung im Calvin-Zyklus verwendet wird.

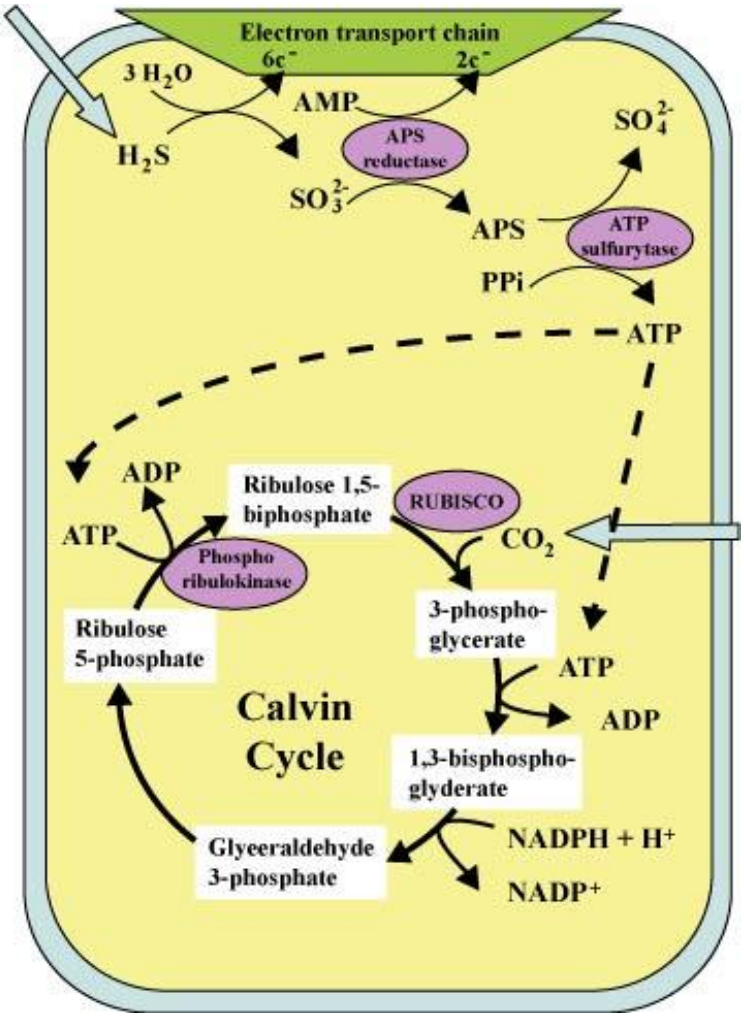


Abbildung 1 Metabolismus eines schwefeloxidierenden Bakteriums. Zusammenhang zwischen Schwefelmetabolismus zur Energiegewinnung und dem Calvin-Zyklus zur Kohlenstofffixierung (siehe Text). APS, Adenosine 5'Phosphosulfat; RUBISCO, Ribulose-1,5-bisphosphat-carboxylase. (aus Minic und Herve[T], 2004)

Thiotrophe Symbiose der *Stilbonematinae*

Die Besonderheit einer Nahrungskette, die unabhängig von photosynthetischen Primärproduzenten ist, also ohne Sonnenlicht auskommt, hat die weitere Erforschung der thiotrophen Symbiose vorangetrieben. Kurz nach ihrer Entdeckung wurde klar dass die thiotrophen Symbiosen nicht nur auf die Hydrothermalquellen beschränkt, sondern weit verbreitet sind. Eine davon ist die thiotrophe Symbiose zwischen Nematoden der Unterfamilie *Stilbonematinae* und schwefeloxidierenden Bakterien.

Die *Stilbonematinae* sind eine im Flachwasserbereich weit verbreitete Gruppe mariner Nematoden (Fadenwürmer). Beschrieben sind derzeit (anno 2008) Vertreter der Gattungen *Adelphos*, *Catanema*, *Eubostrichus*, *Laxus*, *Leptonemella*, *Robbea*, *Squanema* und *Stilbonema*. Mit Sicherheit ist dies nur die Spitze des Eisberges, und es existieren noch mehr unbeschriebene Genera. (Ott, J.A. persönliche Mitteilung) Die *Stilbonematinae* sind eine Unterfamilie der *Desmodoridae* und sind charakterisiert durch die Anordnung und Anzahl von Zellen in speziellen Organen, den glandulären sensorischen Organen (GSO), und einem artspezifischem Überwuchs mit symbiontischen, schwefeloxidierenden Bakterien. Letztere bilden den wesentlichen Bestandteil der Nahrung der *Stilbonematinae.* Diese symbiontischen thiotrophen Bakterien speichern elementaren Schwefel und verleihen damit dem jeweiligen Wirt ein weißes Erscheinungsbild. Durch die Bewegung des Nematoden im Sand zwischen einem oberflächennäheren O_2- und einem tiefer gelegenen H_2S-Gradienten werden die Bakterien mit Nährstoffen versorgt(Ott et al., 1991).

Die Auslässe der drüsenartigen GSOs sind gekennzeichnet durch auffällige Borsten (*setae*), die regelmäßig über den ganzen Nematoden verteilt sind. Im Inneren der Borsten befinden sich die Fortsätze von einer sensorischen und einer glandulären Zelle.

Die genaue Funktion der GSOs ist nicht bekannt. Allerdings gilt es als sehr wahrscheinlich, dass sie den Mucus produzieren, der die Oberfläche der Nematoden (Kutikula) bedeckt. Neben der glandulären Funktion wird den GSOs noch eine chemosensorische Funktion zugeschrieben, die eine Rolle bei der Absonderung und der Steuerung der Zusammensetzung des Mucus bestehen könnte (Bauer-Nebelsick et al., 1995). Im Fall der Stilbonematiden *Laxus oneistus* und *Stilbonema majum* konnte gezeigt werden dass der vom Nematoden sezernierte Mucus „Mermaid“ enthält, ein zuckerbindendes Protein (C-Typ-Lektin, CTL). Dieses Protein erkennt bestimmte Zuckerreste an Bakterienoberflächen und trägt damit zur Anheftung des Symbionten an der Oberfläche des Wirtes bei. Wie die Spezifität der Rekrutierung der symbiontischen Bakterien zustande kommt ist unklar, da „Mermaid“ auch andere Bakterien wie zum Beispiel *Escherichia coli* erkennen und binden kann (Bulgheresi et al., 2006). Um die Interaktion zwischen Wirt und Symbiont von Seite der Nematoden weiter zu ergründen wurde aus den exprimierten Genen von *L. oneistus* eine c-DNA Daten-

bank erstellt, die auch im Internet zur Verfügung steht. (http://www.nematodes.org/NeglectedGenomes/NEMATODA/Laxus_oneistus/index.html). Ebenfalls ungeklärt ist wann es zum ersten Kontakts zwischen den thiotrophen Symbionten und den juvenilen Würmern kommt. Bisher konnte noch kein Symbiont der *Stilbonematinae* freilebend, ohne Wirt, nachgewiesen werden.

Abstract

Thiotrophic symbiosis is of fundamental importance for life, not only in deep sea habitats, where no sunlight can be harnessed to generate energy, but also in shallow water environments.

Free-living marine nematodes carrying sulfur-oxidizing bacterial symbionts on their cuticle (*Stilbonematinae, Desmodoridae*) occur in shallow water at the interface between O_2-rich and hydrogen sulfid-rich sand layers. Migration of the nematodes through the chemocline favours the growth of the symbionts, which represent the nematodes' major food source. Due to the relative simplicity of this association, stilbonematids have already proven to be useful for the understanding of symbiosis establishment. Nevertheless, only the *Laxus oneistus* association was analyzed so far and the bacterial symbiont found to belong to one single phylotype of *Gammaproteobacteria*. Here, we characterized *Robbea* sp.2, a yet undescribed stilbonematid inhabiting a back-reef sand bar off Little Cayman (Cayman Islands). 18S rDNA-based phylogenetic analysis showed that the nematode belongs to the *Stilbonematinae*. Although morphological analysis assigned it to the genus *Robbea,* this genus was not supported at the molecular level in our phylogenetic reconstruction. The surface of *Robbea* sp.2 is covered by a monolayer of morphologically undistinguishable white cocci. We performed PCR and FISH-based analysis and could show that only one phylotype is present on the worms' cuticle. In a 16S rDNA-based tree, the symbionts of *Robbea* sp.2 could be placed in one cluster comprising the sulfur-oxidizing symbionts of *L. oneistus* and those of marine gutless oligochaetes. The presence and phylogeny of the *aprA* gene indicated that the symbionts can use reduced sulfur compounds as an energy source. Finally, we detected the 16S rDNA of the *Robbea* sp.2 symbionts not only in the vicinity of the worm´s habitat, but also in seawater collected far off the reef. This is the first report of the presence of a thiotrophic symbiont in off-shore surface seawater.

Introduction

Symbiosis between chemoautotrophic sulfur bacteria and marine organisms inhabiting sulfur-rich environments was first described in the giant tube worm *Riftia pachyptila* that occurs at hydrothermal vents on the bottom of the Pacific ocean (Cavanaugh et al., 1981). In the following years it became clear that symbioses between marine invertebrates and thiotrophic bacteria are not restricted to hydrothermal vents. Apart from *R. pachyptila*, the variety of marine thiotrophic symbioses spans over diverse organisms such as ciliates (eg. *Zoothamnium niveum*; Bauer-Nebelsick et al., 1996), crustacaeans (eg. *Rimicaris exoculata*; Van Dover, 1994), mollusks (eg. *Codakia orbicularis*;Felbeck et al., 1981), annelids (eg. *Inanidrilus leukodermatus*; Giere, 1981), platyhelminthes (eg. *Paracatenula* sp. J. A. Ott, personal communication) and stilbonematid nematodes. (e.g. *Laxus oneistus*; Ott et al., 1995).
The geographical distribution of these symbioses is as diverse as the phyla of their hosts: *Z. niveum* is found on mangrove peat walls and sea grass debris in the Caribbean and Mediterranean Sea (Ott et al., 1998), *R. exoculata* is the main occurring genus of the macrofauna near hydrothermal vents at the Mid-Atlantic Ridge (Polz and Cavanaugh, 1995) and symbiotic nematodes and oligochaetes occur patchily distributed in shallow-water sand all over the globe (Ott et al., 1995) (Dubilier,1986).

The prokaryotic symbionts can either be inside their host, intracellular or extracellular, (endosymbiosis) as in the nematodes *Astomonema* spp. (*Siphonolaimidae*) or on the outer surface of the host (ectosymbiosis) as in the small nematode subfamily *Stilbonematinae* (*Desmodoridae*).

The *Stilbonematinae* consist of the genera *Adelphos* Ott 1997, *Catanema* Cobb 1920 *Eubostrichus* Greef 1869, *Laxus* Cobb 1894, *Leptonemella* Cobb 1920, *Robbea* Gerlach 1956, *Stilbonema* Cobb 1920 and *Squanema* Gerlach 1963 and they form a monophyletic group according to morphological and molecular data. (Kampfer et al., 1998). The habitat is predominantly sufide-rich shallow-water sandy bottoms, where the worms live a few centimeters below the sediment surface. There, between the oxygenated upper and the anoxic sulfidic deeper layers lies the Redox Potential Discontinuity (RPD). In many types of sediment the RPD is devoid of both O_2 and reduced sulfur compounds. At these sites the worms move between the deeper layers and the surface allowing the bacteria to obtain the oxygen they need as e^- acceptor and reduced sulfur compounds (e.g. hydrogen sulfide, thiosulfate) as e^- donor, thus creating ideal conditions for the bacterial symbionts (Ott et al., 1991). Although sulfide is toxic to all organisms by inhibiting the enzyme Cytochrome c Oxidase, even at nanomolar concentrations, it reaches up to mM concentrations in the nematodes habitat. Here, the ectosymbionts act like a shield and thus prevent the worm from taking up toxic amounts of sulfide (Ott, 1995; Hentschel et al., 1999). Symbionts present in the worm's gut revealed by TEM together with stable carbon isotope ($\delta^{13}C$) analysis indicate that the symbionts are the worms' major food source (Ott et al., 1991). The only known autapomorphic character of the subfamily *Stilbonematinae* is the complex structure of special mucus secreting glandular sensory organs (GSO). These organs, located right under the cuticle, are formed by three to four cells. In contrast, the GSOs found in closely related but non-symbiotic taxa *Spiriniinae* and *Desmodorinae* are composed of only two cells (Nebelsick et al., 1992; Bauer-Nebelsick et al., 1995). The second important uniting characteristic of the *Stilbonematinae* is their genus and even species-specific obligate association with sulfur-oxidizing bacteria attached to the worms' cuticle (Ott and Novak, 1989). These ectosymbiotic bacteria give the nematodes a white appearance in incident light, probably due to inclusions of elemental sulfur.

Regarding the bacterial coat, in most *Leptonemella* and *Stilbonema* individuals it consists of unordered multiple layers of coccoid-shaped bacteria. On the other hand representatives of the genera *Robbea*, *Laxus* and *Catanema* show a highly ordered monolayer of rod-shaped bacteria on the cuticle. The bacteria can either be attached parallel to the nematode's body or are aligned perpendicularly to the cuticle. Some of the symbionts have particular features: Scanning electron microscopic pictures sug-

gest that *Robbea* sp. 3 (J.A. Ott, unpublished) and *L. oneistus* symbionts perform an unusual longitudinal fission that was also observed in thiotrophic symbionts of marine gutless oligochaetes and ciliates (Fenchel and Finlay, 1989; Polz et al., 1994; Olav Giere, 2001). *Eubostrichus* spp. symbionts reach up to 35 µm in *E. topiarius* or *E. parasitiferus* and up to 100 µm in *E. dianae* where they form a fur-like coat on the worm's cuticle and contain several nucleoids (Polz et al., 1992; Polz et al., 1994; Berger et al., 1996). The bacterial volume share of the consortium varies strongly among the stilbonematids: in *L. oneistus* it is about 12% of the total consortium whereas it comprises 22% in *S. maium*, 7% in *E. parasitiferus* and up to 44% in *E. dianae* (Ott et al., 2004a).

The symbionts are probably acquired from the environment as female worms do not appear to transmit them to early embryos (S. B., unpublished data). Moreover, nematodes molt four times during their life cycle (Ott et al., 2004b). Nevertheless, no evidence of a free-living form of the stilbonematid symbionts in the marine environment is available yet. As for the mechanisms of symbiont recruitment by *L. oneistus*, it appears to be mediated by mannose and by the Ca^{2+}-dependent lectin Mermaid secreted from the GSO onto the bacteria associated region of the cuticle (Bulgheresi et al., 2006).

In this study we focused on a marine, yet undescribed, nematode that was collected on a back-reef sandbar at Little Cayman Island in the Caribbean Sea. A monolayer of regularly arranged bacteria covers the whole body except for its anteriormost part and a small region at the tip of the tail. According to the presence of bacterial epigrowth and distinctive morphological traits it was classified as a species of the subfamily *Stilbonematinae* and placed in the genus *Robbea*. It was provisionally named *Robbea* sp. 2 (J.A. Ott, personal communication). In the course of this study we wanted to

(1) confirm whether the worm is truly a member of the *Stilbonematinae* also on a molecular basis,

(2) elucidate the nature of the symbiotic bacteria,
(3) find out whether the worm's bacterial coat constitutes a monospecific biofilm as in *L. oneistus* and
(4) assess the presence of the symbionts in the environment.

Thus, we first analyzed the phylogenetic position of *Robbea* sp. 2 and its associated bacterial symbionts by establishing a clone library of 18S rRNA and 16S rRNA genes, respectively, and calculated phylogenetic trees. To gain further evidence that the sequenced 16S rDNA clones were derived from *Robbea* sp. 2 symbionts we applied Fluorescence In Situ Hybridisation (FISH) on the symbiotic worm. We confirmed the sulfur-oxidizing nature of the symbionts by cloning a part of the *adenosine 5'-phosphosulfate reductase* gene (*aprA*) of *Robbea* sp. 1, 2 and 3 symbionts that is involved in sulfur metabolism for energy generation purposes. Finally, we searched for the *Robbea* sp. 2 symbionts in the environment by using symbiont-specific 16S rDNA PCR primers on sand and seawater samples.

Throughout the present work, the *Robbea* sp. 2 symbiosis was compared, both at the morphological and molecular level, with two other yet undescribed symbioses: the one involving *Robbea* sp.1, from the Belize Barrier Reef and the one involving *Robbea* sp. 3 from the Mediterranean Sea.

Experimental procedures

Specimen collection

Robbea sp. 2 was collected in October 2006 at a 0.5 m depth from a back- reef sand bar off Point of Sand Beach on Little Cayman, Cayman Islands (19° 42'08.3"N, 79° 57'46.7" W). The worms were extracted by shaking the sand and pouring the supernatant through a 63-µm-pore-size mesh screen. They were then picked by hand under a dissecting microscope, fixed in alcohol and flash frozen in liquid N_2 to -196°C.

Scanning Electron microscopy

Robbea sp. 2 worms were prefixed in a 2.5% glutaraldehyde, 0.1 M sodium cacodylate, 2% sucrose solution, rinsed with 0.1 M sodium cacodylate buffer, and post-fixed in a 1% osmium tetroxide, 0.1 M sodium cacodylate, 2% sucrose solution.dehydrated and sputter coated (sputter time: 50 sec). Specimens were analyzed through a Philips XL 20 scanning electron microscope.

DNA Extraction and polymerase chain reaction (PCR) amplification

16S rDNA

Symbionts were washed off a deep frozen pellet of 500 *Robbea* sp. 2 individuals with 50 µl of ddH_2O, transferred to a fresh 1.5 ml tube and incubated at 94°C for 10 min. 5 µl of this solution was directly used as template for PCR. PCR was performed using the eubacterial primers 616V (5´-AGA GTT TGA TYM TGG CTC-3´) and 1492R (5´-GGY TAC CTT GTT ACG ACT T-3´) with the following thermal cylcing program: 95°C for 5 min 1X; 95°C for 45 sec, 47°C for 45 sec, 72°C for 3 min 35X; 72°C for 10 min 1X. The PCR product was 1,499 nucleotides.

We extracted and purified the DNA from single *Robbea* spp. worms exactly as described in Schizas et al. (1997). 18S rDNA was amplified by PCR with the general eukaryotic primers 1f (5´-CTG GTT GAT YCT GCC AGT-3´) and NS8 (5´-ATG ATC CTT CCG CAG GTT CAC-3´). Cycling conditions were: 95°C for 5 min 1X; 95°C for 45 sec, 48°C for 45 sec, 72°C for 2 min 35X; 72°C for 10 min 1X. The PCR product was 1,755 nt. To amplify a ~400 nt adenosine *phosphosulfate reductase alpha subunit* (*aprA*) gene fragment we used the primer pair aps1F (5´-TGGCAGATCATGATYMAYGG-3´), aps4R (5´-GCGCCAACYGGRCCRTA-3´) as described in Blazejak et al. (2006, Primer designed by J. Kuever; Meyer and Kuever, 2007a) . Cycling conditions were: 95°C for 3 min 1X; 95°C for 1 min, 54 °C for 1 min, 72°C for 3 min 35X; 72°C for 10 min 1X.

Sequences

Sequences were deposited in Genbank under the following accession Numbers: *Robbea* sp. 2 18S rDNA (EU768871); *Robbea* sp. 3 18S rDNA (EU784735); *Robbea* sp. 2 symbiont 16S rDNA (EU711426); *Robbea* sp. 1 symbiont aprA (clone 1-3, EU864035) (clone 1-16, EU864036), *Robbea* sp. 2 symbiont aprA (clone 1-3, EU864037) (clone 1-83, EU864038); *Robbea* sp. 3 symbiont aprA (clone 1-13, EU864039 (clone 1-10, EU864040) (clone 1-14, EU864041).

Cloning

The PCR products were gel purified and cloned into pCR2.1-TOPO using the TOPO TA Cloning Kit (Invitrogen Life Technologies, Germany). 19 clones of the *Robbea* sp. 2 symbiont 16S rDNA fragment and 7 and 6 clones of the 18S rDNA fragment of *Robbea* sp. 2 and *Robbea* sp. 3, respectively were randomly picked and fully sequenced. For the *aprA* fragment, 15 clones from *Robbea* sp.1 symbiont (11 clones encoding for EU864035, 4 encoding for EU864036), 23 clones from *Robbea* sp. 2 symbiont (21 encoding for EU864037, 2 encoding for EU864038) and 24 clones from

Robbea sp. 3 symbiont (13 encoding for EU864039, 7 encoding for EU864040, 4 encoding for EU864041) were randomly picked and fully sequenced as above. The sequences were aligned and compared with CodonCode Aligner 1.6.3 software (CodonCode Corporation, USA)

Phylogenetic analysis

The *Robbea* sp. 2 symbiont 16S rDNA sequence, *Robbea* sp. 2 and *Robbea* sp. 3 18S rDNA sequences were compared with sequences in GenBank by using BLASTN (Altschul et al., 1990) and the *Robbea* spp. symbiont aprA sequences by using BLASTX (Altschul et al., 1997). Phylogenetic analysis was carried out using the ARB program package (Ludwig et al., 2004).

To evaluate the phylogenetic position of *Robbea* and of *Robbea* sp. 2 symbiont various tree-building methods were used: maximum likelihood (AxML), PhylipDNAPARS 3.573 (calculated with 100 resamplings), PhylipDistance, neighbor joining (with the Jukes Cantor correction) and TreePuzzle 5.0 with various filter sets were tested. In the final consensus tree PhylipDNAPARS 3.573 and TreePuzzle 5.0 were used. Similarity matrices were calculated using the similarity matrix option in the neighbor joining field of the ARB software package.

We applied a filter that considered only those alignment positions that were conserved in at least 50% of the species in our tree and only sequences with length above 1,325 bp were used for symbiont phylogeny and above 1450 bp for host phylogeny. Sequences of Thiotrix spp. were used as out-group in the symbionts 16S rDNA tree and sequences of *Priapulus* spp. (X80234) and *Halycriptus* spp. (AF342790) (Phylum *Priapula*) were used as out-groups in the host 18S rDNA tree.

For the AprA protein tree an alignment with selected members of sulfur-oxidizing bacteria (SOB), sulfate-reducing prokaryotes (SRP) and the *Robbea* spp. symbiont sequences was done with T-coffee (Notredame et al., 2000). The resulting multiple alignment was imported into ARB (with the fasta with gap import-filter) and first a TreePuzzle tree without the new *Robbea* spp. symbionts sequences was calculated. To increase phylogenetic resolution the short *Robbea* spp. symbiont AprA sequences

were added using the ARB Parsimony interactive tool. Two filters were used: one insertion deletion (indel) filter with at least 50% similarity for the initial tree and a second indel filter with at least 50% similarity for adding the *Robbea* spp. symbionts with the ARB interactive tool. The filters considered 406 and 103 columns in the alignment, respectively. As outgroup, members of the Apr-lineage I (Meyer and Kuever, 2007a, 2007b) were used.

FISH (Fluorescence In Situ Hybridization)

By using the ARB PROBE_DESIGN tool, we designed two FISH probes for *Robbea* sp. 2 symbionts, Rss456 and Rss1439, binding at *E. coli* position 456 and 1439, respectively. Rss456 was also specific for *Inanidrilus leukodermatus* endosymbiont 1 (GenBank accesion number AJ890100.1), while Rss1439 was *Robbea* sp. 2 symbiont-specific (Table 1). Probes were fluorescently labeled on their 5' end (Thermo, Germany). FISH was done according to Manz et al. (1992). Briefly, MeOH fixed worms with attached symbionts were placed on a glass slide and covered with hybridization buffer (0.9 M NaCl, 20 mM TrisHCl (pH 8.0), 0.001 % SDS, 35% formamide). Subsequently, probes were added at the final concentration of 3 ng/µl for EUB338, 6 ng/µl for Rss456, and 3 ng/µl for Gam42R. Each slide was put into a 50 ml tube stuffed with paper soaked in hybridization buffer and incubated in this moist chamber at 46°C overnight. To remove unspecific bound probe, worms were transferred to small glass bowls filled with washing buffer (70 mM NaCl, 20 mM Tris·HCl (pH 8.0), 0.125 M EDTA, 35% formamide). Worms were then incubated at 48°C for 7 min, re-transferred to the slides and dried under a weak stream of compressed air. Finally, a layer of DAPI Vectashield (Vector Labs) was applied. Hybridized samples were examined using a Leica TCS-NT confocal laser scanning microscope.

Environmental samples

Environmental samples were collected from four different sites: 0.5 g worm-free sand samples (SS) and 1 liter surface seawater (SW) were collected at the *Robbea* sp. 2 collection site described above; 0.5 g sand samples (NSS) were collected from the

Little Cayman Research Station beach (19° 41´43. 23 ´´ N; 80° 03´41. 67´´ W) and 1 liter from superficial seawater (NSW) was collected far off this beach (19° 42´04. 86´´ N; 80° 03´47. 57´´ W; water depth of 35 m). Seawater samples were filtered through 0.2 µm filters. Both sand samples and filters were stored frozen in the extraction buffer-containing vials provided by the Ultra Clean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc.). DNA was extracted from the environmental samples according to the manufacturer's instructions and 1 µl of the extracted DNA was randomly amplified with the GenomiPhi V2 DNA Amplification Kit (GE Healthcare), also according to the manufacturer's instructions. 1 µl of the amplified environmental DNA was subjected to PCR with eubacterial primers 616V and 1492R, resulting in a 1,499 nt PCR band representing an eubacterial pool of 16S rDNA fragments. We then used the gel purified 1,499 nt PCR band as a template to amplify a 1,000 nt 16S rDNA fragment from the *Robbea* sp. 2 symbiont with unlabeled FISH probe 456Rss (see above) and 1439Rssr primer (5´CCCTCCCGAAAGTTAAGCTAC3´). 16S rDNA fragments from each environmental sample were cloned in the pCR2.1-TOPO vector with the TOPO TA Cloning Kit (Invitrogen Life Technologies, Karlsruhe, Germany) and four clones per environment were picked, fully sequenced, and aligned with CodonCode Aligner 1.6.3 software. Cycling conditions for both PCRs were: 95°C for 5 min 1X; 95°C for 45 sec, 55°C for 45 sec, 72°C for 2 min 35X; 72°C for 10 min 1X.

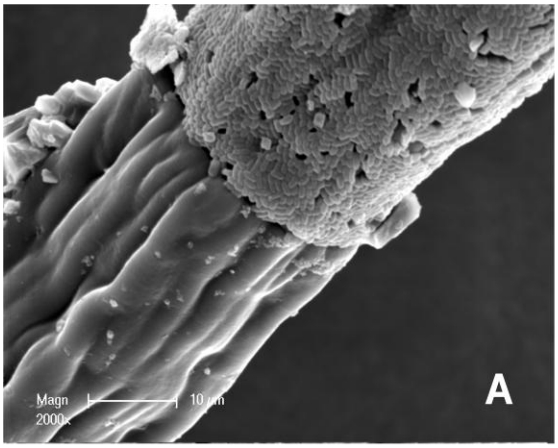
Table 1. Probes used for FISH

Probe	Standard probe name[1]	Specificity	Sequence	Target RNA	Position[2]	Reference
B338	S-*-BactV-0338-a-A-18	Most Bacteria	5´-GCT GCC TCC CGT AGG AGT-3´	16S	338-355	(Amann et al., 1990)
GAM42a	L-C-gProt-1027-a-A-17	Gammaproteobacteria	5´-GCC TTC CCA CAT CGT TT-3´	23S	1027-1043	(Werner Manz et al., 1992)
456Rss	S-*-Robss-0456-a-A-21	*Robbea* sp. ectosymbiont	5´-CTT GGG TTA ATA GCT CAG GGT-3´	16S	457-477	This paper
1439Rss	S-*-Robss-1439-a-A-21	*Robbea* sp. ectosymbionts	5´-CTT GGG TTA ATA GCT CAG GGT-3´	16S	1439-1459	This paper
NON338	Not named	Negative control	5´-ACT CCT ACG GGA GGC AGC-3´	16S	338-355	(Wallner et al., 1993)

[1]according to (Alm et al., 1996)

[2]16SrRNA position, E.coli numbering (Brosius et al., 1978)

[3]23SrRNA position, E.coli numbering (Brosius et al., 1981)



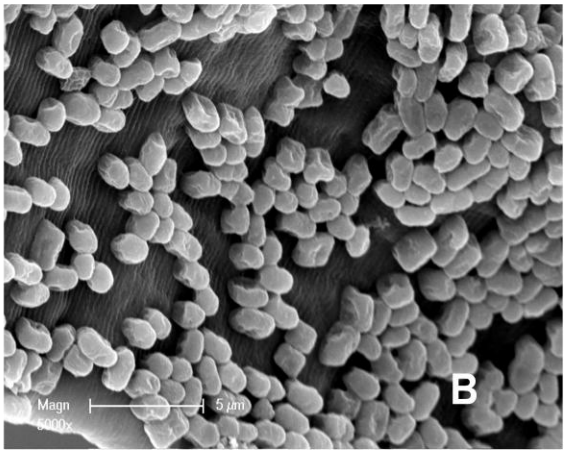
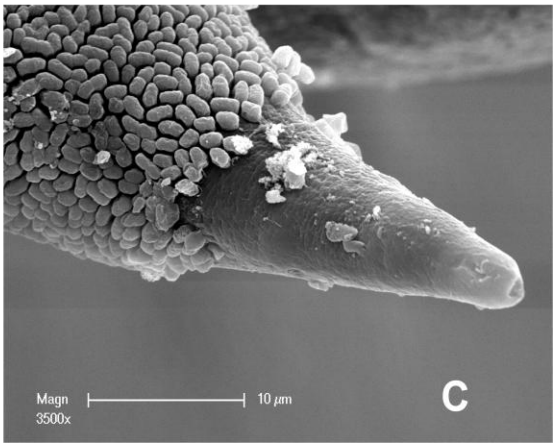


Fig.1 .: Scanning electron microscope pictures of the *Robbea* sp. 2 . A) The beginning of the bacterial coat, some distance behind the anterior end. (right). The furrows on the worm´s bacteria free region (left) is an artifact of the ethanol fixation. B) a deranged region in the bacterial coat where the coccoid shape of the bacteria is more evident. C) bacteria free region at the tip of the tail.

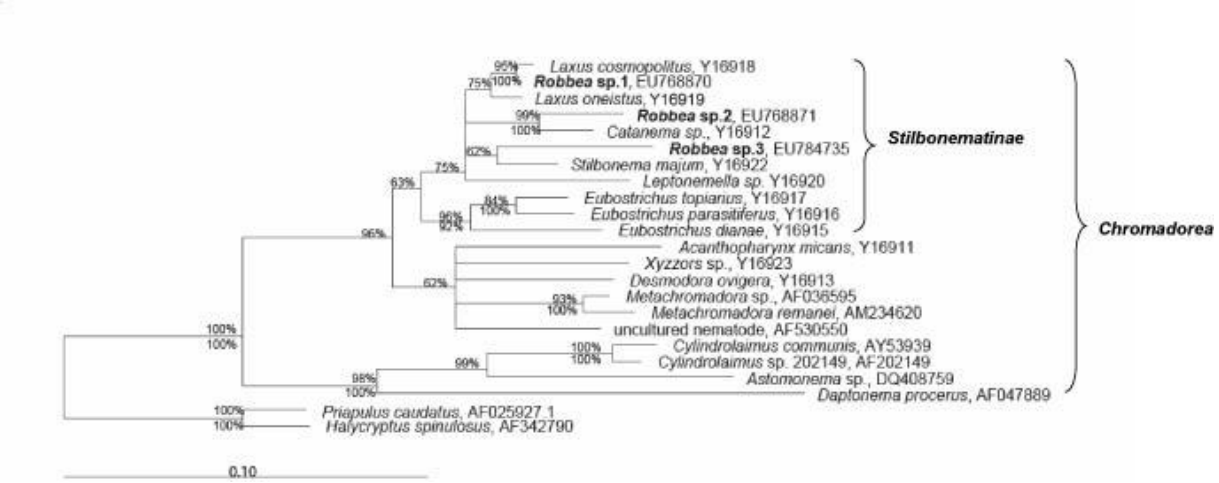


Results

Morphology of *Robbea* sp. 2 and its symbionts

Robbea sp. 1 and *Robbea* sp. 2 are very similar being long and slender, with a length/width ratio (a) >100, rather small papillae and amphids with a pore-like fovea. In comparison, *Robbea* sp. 3 is shorter and stouter, with a < 100, large papillae and spiral to loop-shaped amphids (J.A. Ott, personal communication). Nearly the entire cuticle of *Robbea* sp. 2 is covered with a morphologically uniform monolayer of “corn kernel”-shaped cocci (Fig. 1). Only the first 400-500 µm, in males, and 260-310 µm, in females, of the anterior end, plus a small region at the tip of the tail are free of symbionts. The sharp transition between the overgrown and the symbiont-free part of the worm at the anterior is accompanied by a decrease in the worm’s diameter. Whether the bacteria are arranged parallel or perpendicular to the

worm´s surface cannot be determined due to the symbionts' unusual morphology. The bacteria form a densely packed monolayer and give the worm a white appearance in incident light, probably due to inclusions of elemental sulfur inside the bacterial vacuoles.



Treepuzzle analysis showing the relationship of the *Robbea* worms (in bold) with other Stilbonematinae and other Chromadorea. Treepuzzle supported values are depicted above the respective branches and maximum parsimony bootstrap values below the branches. Only Treepuzzle support values above 75% and parsimony bootstrap values higher than 70% are displayed. The scale bar represents 10% estimated sequence divergence.

Molecular Phylogenetic analysis of *Robbea* sp. 2 and its symbionts

In all applied phylogenetic computation models the 18S rDNA sequences of *Robbea* sp. 2 fell in the subfamily Stilbonematinae, class Chromadorea of the phylum Nematoda (Fig. 2). *Robbea* sp. 2 18S rDNA showed 96.2% sequence identity with that of Catanema sp., and ≥ 91.3% sequence similarity with that of the rest of the stilbonematids in our tree, with *Robbea* sp. 3 showing the lowest sequence similarity. In our consensus tree *Robbea* spp. nematodes do not form a monophyletic distinct cluster within the stilbonematids. *Robbea* sp. 2 symbiont 16S rDNA clones were randomly picked and comparison of their complete sequences showed that they all could be grouped into one single

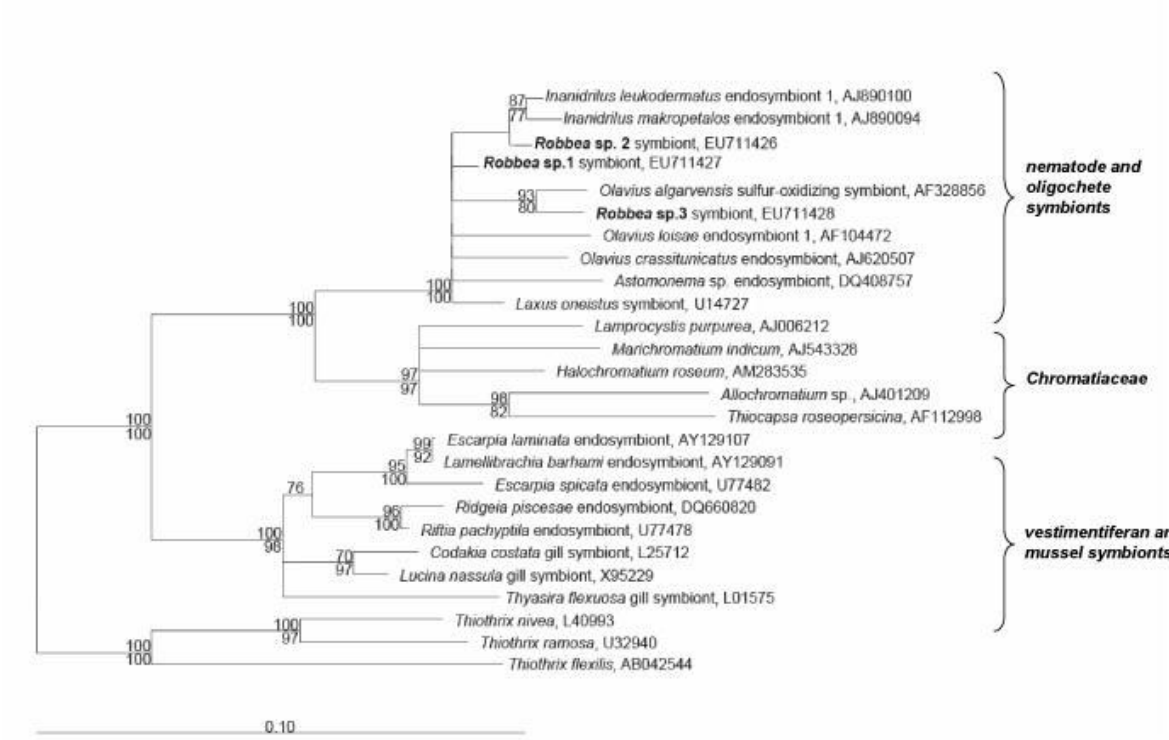


Fig. 3. 16S rDNA-based consensus phylogenetic tree based on maximum parsimony and Treepuzzle analysis showing the relationship of the *Robbea* symbionts (in bold) with other stilbonematid and oligochaete symbionts, as well as other bacteria belonging to the *Chromatiaceae* and other vestimentiferan and mussel symbionts. Treepuzzle supported values are depicted above the respective branches and maximum parsimony bootstrap values below the branches. Only Treepuzzle support values above 75% and parsimony bootstrap values higher than 70% are displayed. The scale bar represents 10% estimated sequence divergence.

clone family belonging to the Gammaproteobacteria, with a sequence similarity within this clone family ≥ 99.8 %. In all the applied methods this gammaproteobacterial 16S rDNA was consistently grouped together in a cluster with the symbionts of the stilbonematid *Robbea* sp. 3 and of the oligochaetes *I. leukodermatus*, *I. makropetalos*, and *Olavius algarvensis* (Fig.3).

Analysis of the gammaproteobacterial 16S rDNA by a similarity matrix showed that the highest sequence similarity was found with *I. leukodermatus* Gamma endosymbiont 1 (99%), while the sequence similarity inside the nematode-oligochaete symbionts cluster was ≥ 96.3% , with *O. crassitunicatus* displaying the lowest sequence similarity.

To prove that the gammaproteobacterial 16S rDNA sequence from *Robbea* sp. 2 derived from its ectosymbionts, we carried out FISH with Rss456, an oligonucleotide probe targeting a central, 21 nt-long fragment of this sequence. For the second probe

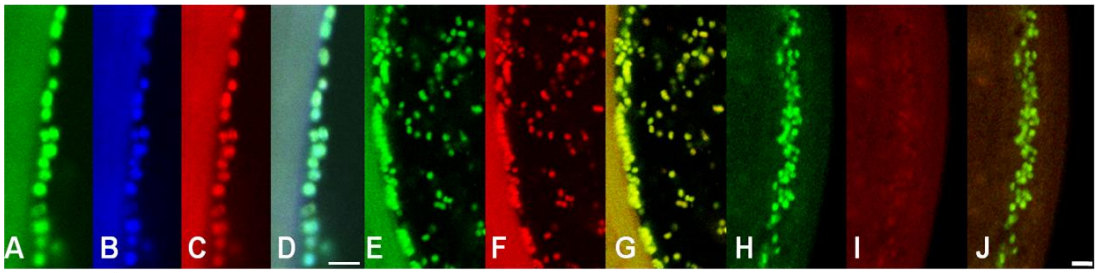


Fig. 4 FISH confocal microscope pictures of *Robbea.* sp.2 attached to the worm's surface. The symbionts are triple stained with a eubacteria-specific probe (green), a *Gammaproteobacteria*-specific probe (blue), and a symbiont-specific probe (red). Overlay pictures: D (of A,B and C) G (of E and F), J (of H and I). Control reaction: all symbionts show staining with the eubacterial probe (H) but no staining with the nonsense probe (I) Scale bars D) and J) are 2µm

we designed, Rss1439, we could not detect any signal. All the bacteria that were attached to the *Robbea* sp. 2 cuticle were stained by the eubacterial probe EUB338, by the *Gammaproteobacteria*-specific probe GAM42a, and by the symbiont-specific probe Rss456 (Fig. 4). Furthermore, all the dissociated bacteria in its proximity which were stained with the eubacterial probe were also stained by the Rss456 probe (Fig. 4). This indicates that the bacteria covering the worm belong to a single phylotype i.e. no additional bacteria are present. This is consistent with the electron microscopy analysis displaying only one bacterial morphotype on the worm´s surface. No FISH signal, instead, was detectable with the negative control probe NON338 (Fig. 4 I).

Sulfur metabolism

To gain further evidence that *Robbea* sp. 2 symbionts are indeed sulfur-oxidizing bacteria, we cloned a fragment of the *adenosine 5'-phosphosulfate reductase* gene, which encodes for an enzyme involved in sulfur metabolism, and calculated a phylogenetic tree with selected sulfur-oxidizing and sulfate-reducing bacteria (Fig.5). Because no sequences of aprA genes of stilbonematid symbionts were known we also oned *Robbea* sp.1 and *Robbea* sp. 3 symbiont aprA gene.

Fig. 5. Phylogenetic reconstruction based on Treepuzzle analysis of AprA sequences from the *Robbea* symbionts (in bold). Only support values above 75% are displayed. The scale bar represents 10% estimated sequence divergence.

In our phylogenetic tree, we got a similar picture for all investigated symbionts: each *Robbea* spp. symbiont possesses one variant that falls into the AprA lineage I (AL I) and one variant that falls into the sulfate-reducing prokaryotes related AprA lineage II (AL II). Both lineages are described in Meyer and Kuever (2007b) and contain several sulfur-oxidizing bacteria, whereas aprA genes in AL II are more closely related to sulfate-reducing prokaryotes (SRP) *aprA* genes.

Robbea sp. 3 clone 1-14 will not be discussed, since it probably derived from a contaminating *Allochromatium*-related bacterium, as it clusters with relatives of this genus (Fig.5). The sequences of *Robbea* spp. symbionts that fall into the AL I share ≥ 97.7% sequence identity among each other and the sequences that fall into the AL II share ≥ 90.1%.

Fig.6: Little Cayman Island, Carribean Sea. Sampling sites of Environmental samples. Spots marked SS+SW refer to the location where *Robbea* sp. 2 worms where found and NSS+NSW refer to the sites where no *Robbea spp.* nematodes where found. Abbreviations.: SS-"Symbiotic Sand", SW-"Symbiotic Water", NSS-"Nonsymbiotic Sand", NSW-"Nonsymbiotic Water". (aus Google Earth, 22.October.08)

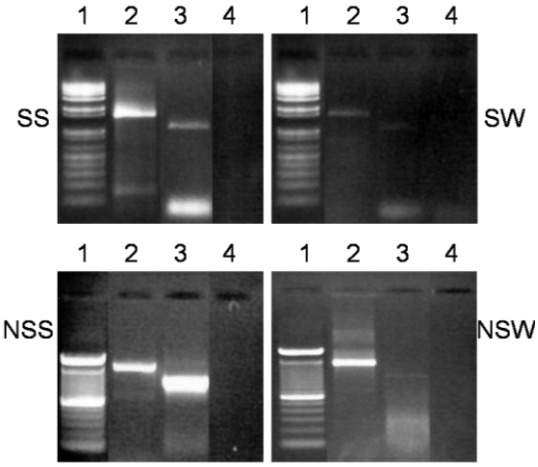


Fig. 7: PCR on environmental samples. Lane 1: Marker. Lane 2 product of eubacterial primer 616V and 1492R. Lane 3: PCR with *Robbea* sp.2 symbiont-specific primer Rss456 and Rss1439. Lane 4: negative control.

Free-living symbionts

To elucidate the mechanism of symbionts acquisition by the nematodes, we looked for the *Robbea* sp. 2 symbionts in sand and in seawater samples from the Point of Sand collection site at Little Cayman. (Fig. 6+7 SS, SW) Moreover, we also searched for the symbionts in a shallow water sand bar at a site where we could never find any *Robbea* sp. individuals, as well as in surface seawater collected off this very sand bar, where the sea reaches a depth of 35 m (Fig. 6+7 NSS, NSW). By using 16S rDNA-specific primers on DNA extracted from all 4 environmental samples, we could detect the symbionts not only where *Robbea* sp. 2 occurs, but also where the worms were never found (Fig.6+7).

Discussion

In this work we characterized *Robbea* sp. 2 and its symbiont, a newly identified symbiotic marine nematodes from the Caribbean. The genus *Robbea* was established by Gerlach (1956) for the type species *R. caelestis* from the coast of Brazil. The diagnostic feature which led to the erection of the new genus was the peculiar morphology of the pharynx: the anteriormost part (corpus) of the tripartite pharynx is conspicuously muscular and distinctly set off from the next part (isthmus), which has a much smaller diameter. In a second species described by Gerlach (1963) from the Maldive Islands, *R. tenax*, the males bear a row of conspicuous sucker-like ventral papillae which extends from the posterior pharyngeal region for some distance posteriad and are involved in the mating process.

Confusion arose, when Platt and Zhang (1982) described two new species of *Stilbonematinae* with distinctly set-off corpus, which they assigned to the genus *Catanema* Cobb 1920, *C. smo* and *C. macintyri*, respectively, and subsequently synonimized the genus *Robbea* with *Catanema*. The detailed drawing by Cobb (1920), however, shows a slightly dilated corpus for the type species, *Catanema exile*, but no sign of this part being set off from the isthmus.

Robbea spp. 1, 2, and 3 bear organ-like suckers in the anterior region and show a pharyngeal muscular corpus clearly set off the isthmus.
The molecular data shows a contradictory, unexpected picture: although they cluster together with other stilbonematids in all phylogenetic trees derived from 18S rDNA sequence data, each species appears to be more closely related to a member of a different genus, than to each other *Robbea* sp.1 clusters with the two *Laxus* species (*L. cosmopolitus* and *L. oneistus*), *Robbea* sp. 2 with a yet undescribed *Catanema* species, and *Robbea* sp. 3 with *Stilbonema majum*. It is possible that the phylogenetic resolution of the *18S rRNA* gene alone is not sufficient to resolve the morphological highly similar nematodes of the genus *Robbea*. Nevertheless, a combined approach with an added mitochondrial gene like *cytochrome oxidase 1* or mitochondrial *16S rRNA*, may be required to obtain a clearer picture of the phylogenetic positions inside the subfamiliy *Stilbonematinae*.

The symbionts of *Robbea* sp. 2 form a morphological uniform bacterial layer on top of the nematodes cuticle. The FISH experiment shows that all *Robbea* sp.2 associated bacteria are stained with the *Robbea* sp. 2 symbiont-specific probe (Rss456) indicating the monophyly of the symbionts. It has to be mentioned that probe Rss456 is also specific for *I. leukodermatus Gamma 1* symbiont (acc.No. AJ890100). However, obtained PCR amplification products of 16SrDNA and *aprA* genes from *Robbea* sp. 2 worm extracts clearly show a difference to available *I. leukodermatus* sequences ruling out an association of *Robbea* sp. 2 with *I. leukodermatus* symbionts. All *Robbea* spp. symbionts cluster with oligochaetes and stilbonematid symbionts in our consensus tree. *Robbea* sp. 2 symbiont branches off as a sister species to the oligochaetes *I. leukodermatus* and *I. makropetalos* endosymbionts. Both *Inanidrilus* spp. endosymbionts were already shown to possess the gene for the alpha subunit of *adenosine phosphosulfate reductase* (*aprA*), indicating their ability to use sulfurous compounds for energy generation purposes.

There were earlier, yet unsuccessful attempts to detect APS reductase by enzymatic assays in *Stilbonematinae* by Polz et al. (1992). We chose to follow a DNA-based approach and were able to obtain a fragment of the *aprA* gene from all three *Robbea* spp. stilbonematid symbionts. The presence of two *aprA* copies and their clustering in different AprA SOB lineages suggest that one copy originated from lateral gene transfer (Meyer and Kuever, 2007b). The ectosymbiotic life-style of stilbonematid symbionts supports interaction with bacteria from the environment and thus makes horizontal gene transfer likely. Accordingly, the endosymbionts of *Inanidrilus*, of the vestimentiferan tubeworm *Riftia pachyptila* and those of *Astomonema* sp. seem to possess only one form of the *aprA* gene (Blazejak et al., 2006; Musat et al., 2007; Meyer and Kuever, 2007b). The AprA enzymes of AL I and AL II have been reported to differ in their cytoplasmic location: bacteria that harbor an *aprA* gene of the AL I seem to express a membrane bound AprA enzyme, whereas for AL II, free cytoplasmic AprA proteins have been identified. It has been shown that in *Thiocapsa roseopersicina* and *Thiobacillus denitrificans* (SOB), that possess copies from both lineages, the SRP related *aprA* is preferentially expressed compared to the SOB related one (Beller et al., 2006). In this context, we hypothesize that the symbionts of all three *Robbea* sp. have acquired a second SRP-related *aprA* gene by horizontal gene transfer. This two gene-system, that probably also occurs in other stilbonematid

symbionts, could give an advantage in their habitat where fluctuations of the available reduced and oxidized nutrients occurs. These findings, together with the assumed presence of sulfur inclusion bodies, are good indications for a sulfur-oxidizing nature of the symbionts.

Absence of symbionts on unhatched embryos (S.B., personal communication) and the need to molt four times during the nematode life cycle, excludes vertical transmission of the symbionts. Nevertheless, no evidences of stilbonematid symbionts in the environment were available. The fact that we were able to detect the symbionts in environmental samples is an indication that a free-living form of the symbionts exists. It is not clear if this free-living form is metabolically active or if it occurs in the environment in a transient, inactive state only.

Future Perspectives

From the approximately 15,000 so far described marine nematodes only the members of closely related families *Epsilonematidae*, *Draconematidae* and *Desmodoridae* (comprising less than 300 species) show occasional or even frequent microbial fouling of their body surface. (J.A. Ott, personal communication) Likewise, stilbonematids often show specific regions that are free of epigrowth as in *L. oneistus* or *Robbea* sp.2. Comparison of these different areas on the worms, especially in gene expression patterns, could present an answer to how the worms developed molecular means to recruit the symbionts and on the other hand prevent unwanted epigrowth.

Robbea spp. associations with thiotrophic bacteria seem to provide a good basis for investigations of thiotrophic symbiosis. It is easier to collect material as compared to hydrothermal vents species and although attempts to cultivate any stilbonematid species have not succeeded yet, some representatives have a fairly high life expectancy in captivity of up to three weeks which renders them useful for molecular techniques like RNAi (e.g. L. oneistus, *Robbea* sp. 2). Future studies could aim to find out if all stilbonematid associations are monospecific and, if true, how specific symbionts are selected out of the vast pool of microbes present in the marine environment.

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Conclusio

In dieser Arbeit haben wir *Robbea* sp. 2 und deren Symbionten, einen unbeschriebenen symbiotischen marinen Nematoden aus der Karibik charakterisiert. Die Gattung *Robbea* wurde von Gerlach (1956) für die Typusart *R. caelestis* von der Küste Brasiliens eingeführt. Das bestimmende Merkmal das zur Errichtung der neuen Gattung *Robbea* führte war die besondere Morphologie des Schlundes (Pharynx). Der vordere Teil (Corpus) des dreigeteilten Schlundes ist muskulös ausgeprägt und eindeutig von dem nächsten Abschnitt (Isthmus) abgesetzt. Bei den Männchen von *R.* tenax, einer Spezies von den Malediven, erstreckt sich noch ventral, von der hinteren Region des Schlundes ein Stück Richtung Hinterende, eine Reihe auffälliger saugnapfartiger Papillen, die eine Rolle bei dem Begattungsvorgang spielen.
Robbea sp.2 besitzt ebenfalls diese erwähnten Merkmale und dennoch gibt es beim Vergleich mit anderen Vertretern dieses Genus auf Ebene der 18S rDNA keine Monophylie. Ein kombinierter Ansatz mit einem zusätzlichen mitochondrialen Gen wie *cytochrome c oxidase 1* oder *16S rRNA* könnten möglicherweise helfen eine bessere Auflösung der phylogenetischen Positionen in der Unterfamilie *Stilbonematinae* zu erzielen.
Die Symbionten von *Robbea* sp. 2 bilden eine morphologisch einheitliche bakterielle Schicht auf der Kutikula der Nematoden. 16S rRNA Fluoreszenz In situ Hybridisierungen zeigten dass alle mit *Robbea* sp. 2 assoziierten Bakterien mit der *Robbea* sp. 2 Symbionten spezifischen Sonde (Rss456) gefärbt werden konnten und bestätigt damit das Bild auf dass die einheitliche Morphologie der Bakterien hinweisst. Es wird bis jetzt angenommen, dass der weisse Farbton der Bakterien auf Einschlüsse elementaren Schwefels zurückzuführen ist. Durch die erstmalige Klonierung eines Gens aus dem Schwefelstoffwechsel (Adenosine 5'-Phosphosulfat Reduktase A, *aprA*) eines stilbonematinen Symbionten ist diese Annahme bestärkt worden. Die schwefeloxiderende Natur der Symbionten von *Robbea* sp. 2 und von zwei weiteren Vertretern dieser Gattung wird vor allem durch die Phylogenie ihrer *aprA* Gene bestätigt, die als Nächstverwandte auschließlich schwefeloxidierende Bakterien zeigt.
Nematoden häuten sich vier Mal in ihrem Lebenszyklus. Dieser Umstand macht eine

Übertragung der Ectosymbionten, von der Eltergeneration auf die Nachkommen unwahrscheinlich. Folglich müssen die Symbionten aus der Umwelt aufgenommen werden. Dennoch wurden bisher noch keine thiotrophen Symbionten ausserhalb der Umgebung des Habitats ihres Wirts gefunden. Der Umstand dass wir erstmals einen thiotrophen Symbionten im offenen Ozean, abseits der Küste detektieren konnten deutet auf eine freilebende Form hin.

Zusammenfassend lässt sich sagen dass diese Arbeit versucht hat, das Wissen über die Assoziationen von Stilbonematinen und ihren Symbionten zu erweitern. Vor allem die phylogenetische Seite der *Stilbonematinae* mag zwar morphologisch klarer erscheinen ist allerdings molekularbiologisch noch ungenügend untersucht und bietet noch einige Widersprüchlichkeiten und Herausforderungen. Hier wurde versucht, ausgehend von einem Vertreter der Gattung *Robbea,* etwas mehr Licht in diese Phylogenie, zu bringen. Obwohl die Morphologie eindeutig zu sein scheint spricht die DNA eine andere Sprache und zeigt größere Verwandtschaften der Vertreter der Gattung *Robbea* zu Vertretern anderer Gattungen auf als untereinander.
Mit den Untersuchungen an *Robbea* sp. 2 wurde ein weiteres Beispiel eines spezifischen Biofilms, der aus einem einzigen Phylotyp von Bakterien besteht gefunden. Dies scheint zumindest für die einschichtigen Überwüchse der *Stilbonematinae* typisch zu sein.
Allerdings wurde ein Nachweis auf genetischer Ebene über die schwefeloxidierende Natur der stilbonematinen Symbionten vor dieser Arbeit noch nicht publiziert. Ebenso neu ist die erstmalige Detektion eines thiotrophen Symbionten im offenen Ozean, weit entfernt von dem Habitat seines Wirts.

Zusammenfassung (engl., see Abstract p. 9)

Marine thiotrophe Symbiosen sind nicht nur in Lebensräumen wie der Tiefsee, in denen kein Sonnenlicht zur Energiegewinnung genutzt werden kann, von fundamentaler Bedeutung, sondern finden sich auch in seichten Gewässern. Marine Nematoden, die schwefeloxidierende bakterielle Symbionten auf ihrer Kutikula tragen (*Stilbonematinae, Desmodoridae*) kommen im Sand seichter Gewässer vor, in denen oxidierte O_2 -reiche Schichten reduzierte H_2S-reiche Schichten überlagern. Die Wanderung der Nematoden durch die Chemokline schafft gute Bedingungen für das Wachstum der angehefteten Symbionten, die den Nematoden als wichtigste Nahrungsquelle dienen. Aufgrund der Einfachheit dieser Assoziation haben sich *Stilbonematinae* für das Verständnis der Etablierung von Symbiose bereits als nützlich erwiesen. Trotzdem wurde bisher nur die Assoziation von *Laxus oneistus* beschrieben. Dort konnte gezeigt werden, dass der Symbiont einem einzigen Phylotyp der Klasse *Gammaproteobakteria* zugeordnet werden kann. In dieser Arbeit haben wir einen noch unbeschriebenen Stilbonematiden und dessen bakteriellen Symbionten von den Kaiman Inseln charakterisiert. Die Oberfläche des Wurms ist bedeckt von einer weissen, einschichtigen Lage von Kokken, die morphologisch einheitlich sind. 18S rDNA phylogenetische Analysen zeigten, dass der Nematode zu den *Stilbonematinae* gehört. Da er aus morphologischer Sicht ein Mitglied der Gattung *Robbea* ist, haben wir die 18S rDNA mit zwei Vertretern dieses Genus verglichen. Wir konnten zeigen, dass sie-obwohl morphologisch eng verwandt-in der 18S rDNA Analyse nicht in eine gemeinsame Gruppe fallen. Um die Diversität der Symbionten des Bakterienmantels zu bestimmen, haben wir auf PCR und FISH basierende Analysen durchgeführt und konnten zeigen dass nur ein Phylotyp an der Kutikula des Wurms existiert. In 16S rDNA Analysen gruppierten die Symbionten aller drei *Robbea*-Arten mit den schwefeloxidierenden Symbionten von *Laxus oneistus* und marinen darmlosen Oligochaeten. Das Vorhandensein und die Phylogenie des *aprA* Gens bei den Symbionten aller drei *Robbea*-Arten ist ein Hinweis, dass sie reduzierte Schwefelverbindungen als Energiequelle nutzen können. Schließlich konnten wir zum ersten Mal einen frei lebenden stilbonematinen Symbionten der *Stilbonematinae* in der Umwelt nachweisen.

Curriculum Vitae

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06/2006-08/2006	internship at the Center for Brain Research, Prof. Johannes Berger, Dr. Markus Kunze
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