universität wien

MASTERARBEIT

Titel der Masterarbeit

Molecular characterisation of marine nematodes of the genus *Robbea* and detection of their symbionts in the environment

angestrebter akademischer Grad

Master of Science (MSc)

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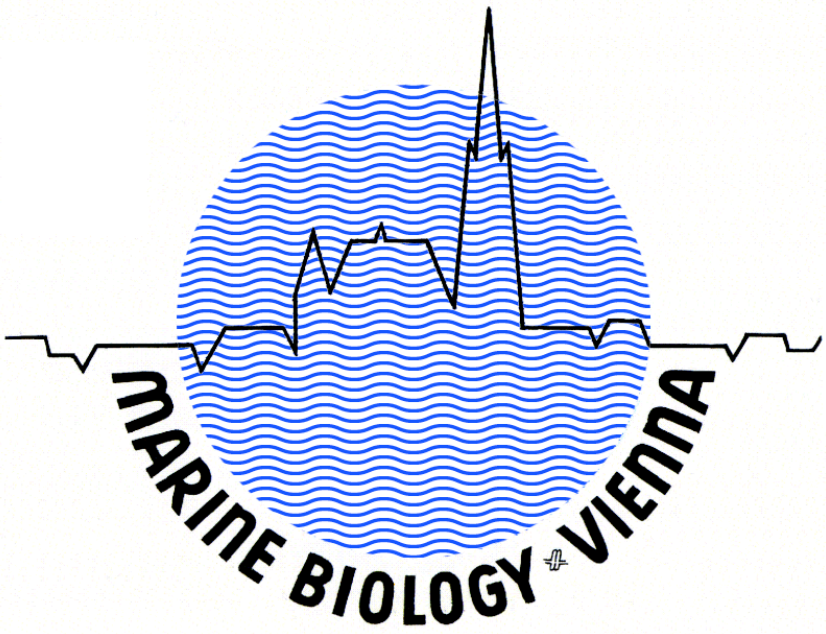
Studienrichtung (lt. Studienblatt):	Zoologie
Betreuerin / Betreuer:	Univ. Prof. Dr. Jörg A. Ott

Wien, im Mai 2010


MARINE BIOLOGY VIENNA

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Einleitung

Begriffsklärung: Symbiose und Mutualismus

Dem Begriff Symbiose liegen im deutschen und englischen Sprachraum zwei unterschiedliche Definitionen zugrunde. Letztere wurde bereits im Jahre 1878 von De Bary formuliert, und faßt unter Symbiose alle möglichen Formen des Zusammenlebens zwischen verschiedenen Arten zusammen. Darin einbezogen werden insbesondere der Kommensalismus, Parasitismus und der Mutualismus, der als eine für beide Partner profitable Beziehung verstanden wird (De Bary, 1878). Die deutsche Definition geht auf den Zoologen und Zytologen Paul Ernst Christof Buchner zurück, der bezogen auf intrazelluläre Zooxanthellen bei Coelenteraten und Protozoen im Jahre 1926 vom Verdienst seiner Vorgänger „Geddes und vornehmlich Brandts" berichtet, daß das „Resultat ihrer Bemühungen war, daß man die Algen nicht mehr als Parasiten oder auch nur als unschädliche Commensalen ansah, sondern zu der Überzeugung kam, daß Tier und Pflanze eine, wenn auch in den einzelnen Fällen engere oder lockerere Symbiose eingegangen haben, also ein Abhängigkeitsverhältnis, aus dem für die beiden Partner wechselseitig Vorteile entspringen" (Buchner, 1921). Somit wird die Symbiose im Deutschen entsprechend dem Mutualismus, folglich in einem engeren Sinne definiert, in diesem der Begriff auch in der vorliegenden Arbeit verstanden wird. Dabei muß jedoch betont werden, daß die Verwendung im Sinne De Barys oft eine größere Nähe zur natürlichen Realität aufweist, da beim engen Zusammenleben zweier Arten, die Grenzen zwischen Kommensalismus, Parasitismus und Mutualismus häufig fallen.

Bakterielle Symbiosen

Bakterien in der Wahrnehmung der Öffentlichkeit

Bakterien genießen in der Öffentlichkeit meist nur den sehr schlechten Ruf als Überbringer von Krankheit, Tod und Verderben. Ungeachtet der Schwere von Erkrankungen wie der Tuberkulose, dem Milzbrand oder der Beulenpest, besitzt aber nur ein ausgesprochen geringer Anteil aller Bakterien überhaupt ein pathogenes Potential. Robert Kochs Bestätigung der Pasteurschen Keimtheorie im Jahre 1876 (Koch, 1876) führte zwar zu einem entscheidenden Paradigmenwechsel in der Vorbeugung und Bekämp-

fung von Infektionskrankheiten, bedauerlicherweise werden Bakterien aber bis heute, trotz ihrer technologischen und industriellen Bedeutung in der Regel nicht als nutzbringend erachtet. Eine der wenigen Ausnahmen bilden seit einigen Jahren kostspielige Werbekampagnen, welche die verdauungsfördernde und immunoregulative Kraft diverser Lactobacilli in bestimmten Milchprodukten suggerieren. Außer Zweifel steht dabei aber eher die Erhöhung der Herstellerumsätze, als eine Verbesserung der Konsumentengesundheit. Meist deutlicher am eigenen Leibe spürbar wird die Bedeutung mancher Bakterien während einer Medikation mit Antibiotika. Seit deren Entdeckung durch Ernest Duchesne und Alexander Fleming (Duchesne, 1897; Fleming, 1929), hat beinahe jeder, der sich bereits einmal einer antibiotischen Therapie unterzogen hat, Erfahrungen mit Nebenwirkungen gemacht, welche von einer gestörten Balance der körpereigenen, bakteriellen Flora herrühren.

Bakterien sind in vielerlei Hinsicht unentbehrlich. Als Primärproduzenten, Remineralisierer, Stickstoffixierer, Nahrungsquelle, aber auch als Parasiten oder Pathogene spielen sie eine fundamentale Rolle in jedem Ökosystem. Weiterhin sind sie gebräuchlich in vielerlei technologischen Prozessen, wie der Fermentation von Nahrungs- und Genußmitteln, in der Textilproduktion, sowie der pharmazeutischen Industrie. Nicht zuletzt zeichnen sie sich aber auch dadurch aus, daß sie in der Lage sind, Symbiosen mit anderen Organismen einzugehen.

Bakterielle Symbiosen der Metazoa

Eine Vielzahl rezenter Forschungsergebnisse zeigt, daß symbiotische Beziehungen, insbesondere unter Beteiligung von Bakterien, keineswegs so selten und exotisch sind wie lange Zeit zuvor angenommen. Vielmehr zeichnet sich immer mehr ab, daß symbiontische Bakterien eine Schlüsselrolle in der Biologie und Evolution beinahe jedes Eukaryoten einnehmen. Assoziationen zwischen Eukarya und Eubakterien wurden bereits für eine Vielzahl von Protozoa, Metazoa und Pflanzen (reviewed in Hentschel et al., 2000), sowie Pilzen (reviewed in Artursson et al., 2006) nachgewiesen. So vielfältig wie die beteiligten Organismen sind auch die Funktionen und der gegenseitige Nutzen innerhalb

dieser Symbiosen. Wirte stellen ein Habitat, das heißt Struktur und Substrat zur Verfügung. Die Partner profitieren vom gegenseitigen Transfer energiereicher Metabolite, essentieller Verbindungen oder vom Erhalt neuer, emergenter Eigenschaften, welche sogar die gesamte Erscheinung und Lebensweise eines Holosymbionten charakteristisch bestimmen können.

Ein Beispiel für die Weitergabe essentieller Kofaktoren findet sich bei der Tsetsefliege, die von ihrem Symbionten *Wigglesworthia glossinida brevipalpis* mit Vitaminen des B-Komplexes versorgt wird (Nogge, 1982; Zientz et al., 2004). Der Symbiont der Erbsenblattlaus *Acyrthosiphon pisum*, *Buchnera aphiodicola*, nutzt hingegen Enzyme seines Wirts, um den eigenen, unvollständigen Isoleucin-Syntheseweg zu komplettieren (Thomas et al., 2009). Bakterien können selbst die Vermehrung ihrer Wirte stark beeinflussen. Eine Haltung von *Hydra viridis* in steriler Umgebung beispielsweise, ohne deren assoziierter mikrobiellen Flora, führt zu einem knospungunfähigen Phänotyp (Rahat & Dimentman, 1982). Dort, wo photosynthetische Primärproduktion aufgrund von Lichtmangel unmöglich wird, können chemoautotrophe Bakterien ganze Nahrungsnetze am Leben erhalten. Ein herausragendes Beispiel sind die Lebensgemeinschaften an den Hydrothermalquellen der Tiefsee – ein hochproduktives Ökosystem, das einzig auf bakterieller, chemolithoautotropher Primärproduktion basiert (Fisher, 1996). Der dort lebende Bartwurm *Riftia pachyptila* bildet anstelle eines Darms ein hochorganisiertes Gewebe, das *Trophosom* aus, welches symbiotische, sulfidoxidierende Bakterien beherbergt (Cavanaugh et al., 1981). Die Würmer versorgen die Symbionten über ihr Blut mit Sauerstoff, Kohlendioxid und Schwefelwasserstoff und leben im Gegenzug ausschließlich von deren organischen Produkten.

Die Wolbachia Pandemie

Beeindruckend ist der Einfluß, den *Wolbachia* spp. auf die Reproduktion ihrer Wirte ausüben. Die den Rickettsiales zugeordneten Bakterien sind obligatorische intrazelluläre Endosymbionten. Wurde erstmals eine Infektion von *Culex pipiens* mit *Wolbachia pipientis* im Jahre 1924 durch (Hertig & Wolbach, 1924) entdeckt, so nennt eine aktuelle

Schätzung, daß weltweit alleine mehr als 65 %, das heißt zumindest 10^6 aller Insektenarten, Wirte dieser α-Proteobakterien sind (Hilgenboecker et al., 2008). Zudem sind Assoziationen mit *Wolbachia* noch von Isopoda, Chelicerata, sowie filarioden Nematoden bekannt (Werren et al., 2008).

An *Drosophila* konnte gezeigt werden, daß hexapode Wirte von einer erhöhten Resistenz gegen den entomopathogenen Ascomyceten *Beauveria bassiana* (Panteleev et al., 2007) und das Flock House Virus (FHV) (Hedges et al., 2008; Teixeira et al., 2008) profitieren können. Bei weiteren RNA-Viren, dem Drosophila C Virus (DCV), Cricket Paralysing Virus und dem Nora Virus, wurde gegenüber symbiontenbefreiten Fruchtfliegen zumindest eine verlängerte postinfektiöse Lebensdauer von Holosymbionten nachgewiesen (Hedges et al., 2008; Teixeira et al., 2008).

Allerdings verleihen eine Reihe phänotypischer Effekte auf ihre Wirte, *Wolbachia* auch den Charakter reproduktiver Parasiten (reviewed in Werren et al., 2008). Die fehlende Übereinstimmung in der Phylogenie von *Wolbachia* und seiner Wirte deutet darauf hin, daß eine Übertragung von *Wolbachia* horizontal erfolgen kann, das heißt von einem Individuum auf ein anderes derselben oder gar einer anderen Spezies. Dieser Infektionsweg ist aber eher in evolutiven Zeiträumen relevant (Baldo et al., 2006; Baldo & Werren, 2007; Verne et al., 2007). Der eigentliche und wesentlich häufigere Übertragungsweg findet vom Muttertier auf die Nachkommenschaft über die Eizelle statt (Werren et al., 2008), und kann als vertikal maternal bezeichnet werden. Dabei spiegelt eine Batterie an reproduktiven Manipulationen die Evolution von *Wolbachia* als obligat intrazelluläre Bakterien wieder:

– *Cytoplasmatische Inkompatibilität* (CI): Spermien infizierter Männchen sind nicht kompatibel mit Eizellen, welche einen anderen Stamm von *Wolbachia* tragen (Werren, 1997).

– *Parthenogenetische Induktion* (PI): bei Tieren wie Milben, Wespen oder Thripsen entwickeln sich aus unbefruchteten Eiern im Normalfall haploide Männchen (Arrhenotokie), bei *Wolbachia*-Trägern jedoch diploide Weibchen (Thelytokie) (Stouthamer et al., 1990; Arakaki et al., 2001; Weeks & Breeuwer, 2001)

– *Feminisierung*: in der Embryonalentwicklung des Isopoden *Armadillidium vulgare* kann *Wolbachia* die Expression von Genen des männlichen Z Heterochromosoms inhibieren. In Folge entwickeln sich genotypische Männchen zu phänotypischen Weibchen (Vanderkerckhove et al., 2003).

– *Male killing*: mit Tetrazyklin von *Wolbachia* befreite Mütter der Lepidopterenart *Ostrinia scapulalis* produzieren ausschließlich männliche Nachkommen (Kageyama et al., 2002), *Wolbachia*-Trägerinnen hingegen ausschließlich weibliche. Dabei werden während der Larvalentwicklung männliche Nachkommen zunächst feminisiert und sterben in Folge ab (Kageyama & Traut, 2004).

– *mate discrimination*: im Vergleich zu unbehandelten *Drosophila melanogaster* Populationen, zeigen mit Tetrazyklin behandelte eine signifikant höhere Bereitschaft, sich mit Populationen zu paaren, die Träger eines anderen *Wolbachia* Stammes sind (Koukou et al., 2006)

Die genannten Reproduktionseffekte fördern die Effizienz der maternalen Übertragung und können im Falle von CI und PI Reproduktionswege ohne Infektion kategorisch ausschließen. Am Beispiel *Wolbachia* zeigt sich besonders die Sinnhaftigkeit und die Nähe zur natürlichen Realität von de Barys Definition von Symbiose (De Bary, 1878), indem hier, in ein und demselben System, die hypothetische Grenze zwischen Mutualismus und Parasitismus eindeutig verschwindet.

Bakterielle Symbionten als treibende Kraft in der Evolution

Gemäß der Endosymbiontentheorie sind eukaryotische Zellen aus der endozytotischen Aufnahme prokaryotischer Zellen in ebenfalls prokaryotische, praeeukaryotische Zellen entstanden (Alberts et al., 2008). Die aufgenommenen Zellen erlangten in Folge in ihrer Wirtszelle Organellenstatus. Die zugehörige, von Andreas Franz Wilhelm Schimper im Jahre 1883 (Schimoer, 1883) und von Konstantin Sergejewitsch Mereschkowski im Jahre 1905 (Mereschkowsky, 1905) aufgestellte, und durch Lynn Margulis (vormals Sagan) im Jahre 1967 (Sagan, 1967) bekannt gewordene Endosymbiontenhypothese, genießt durch eine Vielzahl von Nachweisen längst den Status einer außergewöhnlich gefestigten The-

orie. Erwähnt sei hier lediglich der massive Gentransfer, der offensichtlich bei den Animalia zwischen den Vorgängern der Mitochondrien und dem Wirtsgenom, und bei den Plantae mit den Plastiden stattgefunden hat (Blanchard and Lynch, 2000). Noch heute besitzen diese Organellen ihr eigenes Genom und lassen sich anhand ihrer bakteriellen 16S rRNA phylogenetisch nächst zu den Rickettsiales, bzw. bei den Cyanobacteria einordnen (Emelyanov, 2001). Die Endosymbiontentheorie ist somit ein besonders herausragendes Beispiel dafür, welche bedeutende Kraft Symbiosen als Motor für einen evolutiven Trend zu höherer Komplexität darstellen können.

Auch bei *Wolbachia* - wohlgemerkt ebenfalls Rickettsiales und wie die Mitochondrien maternal übertragen - lassen sich aus den oben genannten, parasitären Eigenschaften evolutive Implikationen ableiten. So spielt CI eine Schlüsselrolle bei der reproduktiven Inkompatibilität der pteromaliden Wespenschwesternarten *Nasonia giraulti* und *longicornis* und somit bei deren Speziation (Bordenstein et al., 2001). Auch gibt es Hinweise, daß erst PI durch Bakterien zur Entstehung von parthenogenetischen Insekten geführt haben könnte: die ebenfalls pteromalide Wespe und *Wolbachia*-Trägerin *Muscidifurax uniraptor*, welche sich ausschließlich durch thelytoke Parthenogenese reproduziert, ist nach Behandlung mit Rifampicin vermehrungsunfähig. Scheinbar wurde in Folge der Infektion durch PI im Laufe ihrer Evolution die Selektion auf sexuelle Eigenschaften gänzlich aufgegeben (Gottlieb and Zchori-Fein, 2001). Weiterhin findet verbreitet auch Gentransfer von *Wolbachia* auf die Wirte statt, der in einem Drittel aller bis dato sequenzierten Evertebratengenome nachweisbar ist. Das Genom der tropischen Ananasfruchtfliege *Drosophila ananassae* enthält dabei sogar beinahe ein gesamtes *Wolbachia*-Genom (> 1 Mb). Daß dabei sämtliche ORFs intakt sind, und diese zu mehr als 99,9 % mit denen der Symbionten identisch sind, deutet auf einen relativ jungen Ursprung dieser Insertion hin (Hotopp et al., 2007).

Eingedenk der vielfältigen Weise, in der bakterielle Symbionten den Lebensstil, das Verhalten und die Evolution ihrer Wirte beeinflussen, sowie der Tatsache, daß der gesunde menschliche Körper circa zehnmal mehr assoziierte, mikrobielle als eigene Zellen beherbergt, sowie ein Darmmikrobiom, dessen Anzahl an Genen die des Menschen um

das mehr als 100-fache übersteigt (Backhed et al., 2005), müssen die drei elementaren Fragen des Menschseins, „Wer sind wir? Woher kommen wir? Wohin gehen wir?“, zu ihrer Beantwortung durch drei weitere Fragen, bezogen auf unsere assoziierten Mikroben ergänzt werden: Wer sind sie? Woher kommen sie? Wohin gehen sie?

Chemoautotrophe Symbiosen

Chemoautotrophie

Im Gegensatz zu photoautotrophen Organismen, die Sonnenenergie zur Assimilation des im CO_2 enthaltenen Kohlenstoffs nutzen, beziehen chemoautotrophe Lebewesen die benötigte Energie aus der Oxidation reduzierter, anorganischer Stoffe. Chemoautotrophe Bakterien gewinnen ebenfalls durch oxidative Phosphorylierung ATP. Dazu wird aus der elektrochemischen Energie des Redoxpotentials zwischen einem reduzierten Elektronendonator und einem Elektronenakzeptor mithilfe einer Elektronentransportkette ein Protonengradient über der Zellmembran erzeugt: der Motor der ATP Synthase. Ausschlaggebend für den Energiegewinn ist dabei in allen Fällen ein positives Redoxpotential zwischen dem Elektronendonator und dem Elektronenakzeptor. Um Kohlendioxid über den Calvin-Zyklus fixieren zu können, wird NAD^+ über einen umgekehrten Elektronenfluß zu NADH reduziert. Als terminaler Elektronenakzeptor der Elektronentransportkette dient bei aerober Oxidierung Sauerstoff. Fakultativ anaerobe und obligat anaerobe Chemoautotrophe, wie zum Beispiel die Desulfovibrionaceae können hierzu Nitrat, beziehungsweise Sulfat verwenden (Meyer-Reil, 2005; Madigan et al., 2009).

Ökologische Bedeutung erlangen chemoautotrophe Organismen vor allem in Habitaten, in denen photosynthetische Primärproduktion aufgrund von Lichtmangel unmöglich, und der Eintrag organischen Materials von außen gering ist. Dies trifft insbesondere auf hydrothermale Tiefseequellen und *deep subsurface*-Ökosysteme zu (McCollom and Amend, 2005). An Orten, wo durch anaeroben, mikrobiellen Abbau organischen Materials reduzierte, anorganische Stoffe entstehen, bewirkt die chemoautotrophe Oxidation, daß deren Energie im Kreislauf des System erhalten bleibt (Meyer-Reil, 2005). Die Weitergabe von chemischer, metabolischer Energie ist maßgeblich für trophische Inter-

aktionen. Dort wo sich die Primärproduktion ausschließlich auf chemoautotrophe Archeen und Bakterien stützt, spielen diese Organismen eine Schlüsselrolle für die Größe, Biomasse, Zusammensetzung und trophische Struktur dieser Lebensgemeinschaften (McCollom and Amend, 2005).

Chemoautotrophe Symbiosen mariner Evertebraten

Chemoautotroph erzeugte Biomasse kann von der beweidenden oder suspensionsfressenden Meio- und Makrofauna in einer kurzen Nahrungskette aufgenommen werden. Manche Tiere sind jedoch in der Lage, die Primärproduzenten direkt an sich zu binden oder im Körper aufzunehmen. Sowohl die Wirte, als auch die Symbionten zeichnen sich dabei durch eine hohe Diversität aus. Bislang sind entsprechende Wirte aus nicht weniger als sieben Phyla bekannt: Ciliata, Porifera, Platyhelmintes, Nematoda, Mollusca, Annelida und Arthropoda (reviewed in: Dubilier et al., 2008). Ebenso divers sind die Symbionten, welche mehreren Abstammungslinien innerhalb der α-, γ- und ε-Proteobakterien zugeordnet werden können und sowohl sulfid-, als auch methanoxidierende Bakterien beinhalten (Dubilier et al., 2008; Gruber-Vodicka et al., 2009). 16S und 18S rRNA basierte, phylogenetische Daten darauf hin, daß diese Symbiosen nicht koevolviert sind, sondern – mit wenigen Ausnahmen, wie bei den *Paracatenula* (Gruber-Vodicka et al., 2009) und vesicomyiden Muscheln (Hurtado et al., 2003) – auch innerhalb der Gruppen mehrfach unabhängig voneinander entstanden sind (Dubilier et al., 2008; Bayer et al., 2009;). Dieser Umstand findet sich auch in der morphologischen Diversität der Wirte reflektiert. So wird das Trophosom der vestiminiferen, beziehungsweise monoliferen Polychaeten *Rifta pachyptila* und *Sclerolinum* aus dem viszeralen Mesoderm gebildet (Bright & Sorgo, 2003; Nussbaumer et al., 2006; Eichinger, pers. Komm.), bei dem frenulaten Polychaeten *Oligobrachia* höchstwahrscheinlich aber aus dem Entoderm (Dubilier et al., 2008).

Thiotrophe Symbiosen

Sulfidische Habitate

Sulfidvorkommen sind vielfältig im Meer und reichen von der Tiefsee bis in seichte Küstengebiete. An den hydrothermalen Quellen der mittelozeanischen Rücken dringt Wasser mehrere Kilometer tief unter die neu entstehende Erdkruste. Durch die von der darunterliegenden Magmakammer erzeugten Hitze entsteht ein reduziertes, mit Sulfid, Methan und Metallen angereichertes, anoxisches Gemisch, welches durch Spalten im Grabenbruch mit bis zu 400 °C ins Meer zurückschießt oder langsam durch das Sediment hinaufsickert (Von Damm, 1990). Eine ähnliche Art des Sulfideintrags findet sich auch in seichteren Gebieten an vulkanischen Magmakammern (Dando et al., 1995). In anderen Habitaten ensteht Sulfid durch die Zersetzung organischen Materials durch chemoorganothrophe, sulfatreduzierende Bakterien. Dazu gehören die sogenannten cold seeps (Suess et al., 1987), herabgesunkene Walskelette und Holzstücke (Rouse et al., 2004), makrophytischer Debris (Fenchel and Riedl, 1970; Mann, 1976), sowie Flachwassersedimente. Letztere erstrecken sich über alle organisch angereicherten Sedimente, das heißt im Wesentlichen alle Schelfsedimente, über dysoxische Becken bis zu Sedimenten am oberen Kontinentalabhang (Ott et al., 2004b).

Küstennahe Sedimentböden

Mit Ausnahme stark bebrandeter Sandstrände erstreckt sich sauerstoffhaltiges Porenwasser nur wenige Millimeter bis Zentimeter in die Flachwassersedimente. Darunter liegt eine tiefere, anoxische und sulfidische Schicht, welche von der Oberflächenschicht durch eine Chemokline, die Redox Potential Diskontinuität (RPD) getrennt wird (Fenchel and Riedl, 1970). Die RPD weist eine charakteristische Abfolge der Elektronenakzeptoren von Sauerstoff, über Nitrat, Eisenoxid und Mangan, zu Sulfat auf. Dort gewinnen chemoorganotrophe Bakterien aus der anaeroben Reduzierung von Sulfat Ener-

gie zur Oxidation von organischem Material oder Wasserstoff. Das dabei enstehende Sulfid diffundiert nach oben und wird durch Sauerstoff oxidiert. Die bei dieser Oxidation freiwerdende Energie wird von chemoautotrophen Bakterien genutzt, um den im Kohlendioxid enthaltenen Kohlenstoff zu fixieren (Jøregensen, 1989).

Mutualistische Effekte thiotropher Symbiosen

Sulfidreiche Sedimentschichten können einige Millimeter bis Zentimeter von Sauerstoff enthaltenden Schichten entfernt liegen. Besonders für den Fall schwacher Sulfid- und Sauerstoffgradienten, haben sulfidoxidierende Bakterien Wege gefunden, ihre relativ geringe Größe mit ihren energetischen Bedürfnissen in Einklang zu bringen. Manche Vertreter, wie die *Beggiatoa* oder das Spaghetti-Bakterium *Thioploca,* besitzen eine fädige Wuchsform und eine für Bakterien außergewöhnlich hohe Mobilität, was ihnen ermöglicht, die Kluft zwischen sulfidischem und oxischem Porenwasser zu überbrükken (Ott et al., 2004b). Sulfidoxidierende Symbionten hingegen nutzen ihre Wirte, um sich entweder auf und ab tragen, oder in einem spezialisierten Organ, dem *Trophosom*, sowohl mit Sauerstoff, als auch mit Sulfid versorgen zu lassen (Ott et al., 1991; Giere, 1992; Pflugfelder et al., 2005). Zwar ist Sulfid als Hemmer der Cytochrom-c-Oxidase toxisch für die meisten Metazoa, thiotrophe Wirte hingegen besitzen eine erhöhte Resistenz gegenüber sulfidischen und anoxischen Bedingungen (Vismann, 1991). Zusätzlich können die Symbionten ihre Wirte durch den Oxidationsprozess vor erhöhten Konzentrationen schützen (Hentschel et al., 1999). Im Trophosom von *Riftia pachyptila* werden die Symbionten über das Kreislaufsystem des Wirts mit Sulfid versorgt. Anders als die meisten anderen Metazoa verfügt *Riftia* über ein extrazelluläres Hämoglobin, das sowohl Sauerstoff, als auch davon unabhängig, Sulfid gleichzeitig und reversibel zu binden (Zal et al., 1996; Zal et al., 1998; Flores et al., 2005).

Die Symbiosen der Stilbonematinae

Die Unterfamilie der Stilbonematinae

Freilebende Nematoden der interstitiellen Fauna mariner Flachwassersedimente können, wie *Astomonema* oder *Parastomonema*, sulfidoxidierende Bakterien im Körperinneren tragen (Ott et al., 1982; Vidakovic and Boucher, 1987; Giere et al., 1995; Kito, 1989; Musat et al., 2007), oder außen auf der Kutikula. Letztere gehören zu der Unterfamilie der Stilbonematinae (Desmodoridae, Chromadoria, Adenophorea), bestehend aus den Gattungen *Adelphus* Ott, 1997, *Catanema* Cobb, 1920, *Eubostrichus* Greef ,1869, *Laxus* Cobb, 1894, *Leptonemella* Cobb, 1920, *Robbea* Gerlach, 1956, *Squanema* Gerlach, 1963 und *Stilbonema* Cobb, 1920 (Ott et al., 2004a; Ott et al., 2004b). Die Würmer wandern an der Chemokline zwischen sauerstoffreichen, oberen Sandschichten und tieferen, anoxischen und sulphidischen Schichten auf und ab. Dabei versorgen sie ihre Symbionten sowohl mit dem benötigten Elektronenakzeptor Sauerstoff, als auch mit dem Elektronendonator Sulfid (Ott et al., 1991; Polz et al., 1992; Hentschel et al., 1999). Im Gegenzug sind die Ektosymbionten der Hauptbestandteil der Wirtsnahrung, wie anhand von Messungen stabiler Kohlenstoffisotopen in den Wirten, den Symbionten und organischem Sedimentmaterial gezeigt werden konnte (Ott et al., 1991). Weiterhin sind die Bakterien auch in der Lage, ihren Wirt vor einer Vergiftung mit Sulfid zu schützen (Hentschel et al., 1999).

Morphologie

Der Mantel aus Ektosymbionten bedeckt die Würmer und verleiht ihnen eine hellweiße Farbe, welche auf Einschlüße elementaren Schwefels in den Bakterien zurückzuführen ist (Himmel et al., 2009). Die Vorderregion mancher Arten ist symbiontenfrei, wie auch die äußerste Schwanzspitze. Bei diesen nimmt beim Übergang von der Vorder- in die Hinterregion der Durchmesser des Wurmes entsprechend der Dicke des Bakterienmantels ab, so daß der Gesamtdurchmesser des Holosymbionten durchwegs gleich groß erscheint. Neben der Ektosymbiose besitzen die Stilbonematinae als autapomorphes Merkmal noch besonders ausgebildete *glandular sense organs* (GSOs). Über die ges-

amte Fläche des Wurms verteilt, sondern sie einen Schleim ab, der zumindest bei *Laxus oneistus* ausschließlich im posterioren Teil des Wurms die Symbionten zu binden vermag. Das GSO ist eine hypodermale Struktur bestehend aus einer Epidermiszelle, einer sensorischen Nervenzelle und zwei Drüsenzellen, A und B. Bei den nicht-symbiotischen Desmodorida ist nur eine vorhanden (Nebelsick et al., 1992; Nebelsick et al., 1995; Bulgheresi et al., 2006). Weitere Charakteristika stellen die reduzierte, unbewaffnete Buccalhöhle, der schwach ausgebildete Pharynx und der zumeist drüsige Terminalbulbus dar. Bei den Gattungen *Catanema*, *Laxus* und besonders *Robbea* ist der vordere Abschnitt des dreigeteilten Pharynx reich an Muskulatur und klar abgesetzt. Bei mehreren *Robbea* Arten besitzen die Männchen eine Reihe postpharyngealer Saugnäpfchen. Die Anzahl dieser vermutlich der Kopulation dienenden Kutikularstrukturen ist dabei artspezifisch (Ott, 1993; Ott et al., 2004a).

Übertragung und Aufrechterhaltung des Symbiontenmantels

Die Übertragung der Symbionten ist essentiell für die Aufrechterhaltung von entsprechenden Assoziationen über mehrere Wirtsgenerationen hinweg. Generell werden zwei Arten der Übertragung unterschieden: horizontal von der Umwelt und vertikal mit maternalem, seltener biparentalem Symbiontentransfer (Bright and Bulgheresi, 2010). Eine horizontale Übertragung impliziert das Vorhandensein freilebender Symbionten im Wirtshabitat. Gestützt auf die Analyse von 16S rRNA-Gen Fragmenten konnten bisher freilebende Phylotopen der Symbionten folgender Wirte nachgewiesen werden: die lucinide Muschel *Codakia orbicularis* im Sediment von Seegräsern (Gros et al., 2003), der siboglinoide Polychaet *Oligobrachia mashikoi* im Sediment von Tsukomo Bay, Japan (Aida et al., 2008), sowie *Riftia pachyptila* an hydrothermalen Quellen in der Tiefsee (Harmer et al., 2008).

Eine horizontale Übertragung wird auch für die Stilbonematinae vermutet (Ott et al., 2004a; Ott et al., 2004b). Diese häuten sich während ihrer Entwicklung bis zu viermal (Jörg A. Ott, pers. Komm.). Eine horizontale Übertragung würde ihnen ermöglichen, den Bakterienmantel jedesmal neu zu etablieren. Desweiteren konnten auf der Eihülle

früher, ungeschlüpfter Embryos von *Laxus oneistus* bisher keine Bakterien festgestellt werden (Silvia Bulgheresi, unpublizierte Beobachtung). Die Kolonisierung frisch geschlüpfter oder gehäuteter Würmer verläuft vermutlich ausgesprochen schnell, da bei Sammlungen im Feld bisher nur sehr selten aposymbiotische Jungwürmer gefunden wurden (Jörg A. Ott, pers. Komm.).

Bei der Anheftung der Symbionten spielen höchstwahrscheinlich Mannose-bindende, Ca^{2+}-abhängige Lektine, die *Mermaids*, eine Rolle. Es konnte gezeigt werden, daß diese von den GSOs der hinteren Körperregion von *Laxus oneistus* und *Stilbonema majum* sezerniert werden und an der Anheftung zwischen Symbionten, sowie Wirt und Symbionten beteiligt sind (Bulgheresi et al., 2006). Weiter wurde indirekt nachgewiesen, daß sich die entsprechenden Mannosereste auf der Oberfläche der Bakterien befinden (Nussbaumer et al., 2004).

Ziele dieser Arbeit

In einer kürzlich erschienen molekularen Beschreibung des neu entdeckten Stilbonematinen *Robbea* sp.2 und seiner Symbionten wurde anhand der Gensequenzen für die 16S rRNA und der Adenosinphosphosulfatreduktase A (aprA) nachgewiesen, daß der Symbiontenmantel von *Robbea* sp.2 aus γ-Proteobakterien eines phylogenetischen Clusters von sulfidoxidierenden Oligochaeten- und Nematodensymbionten besteht und dabei monospezifisch ist (Bayer et al., 2009). Weiterhin konnte anhand des Nachweises von Fragmenten des symbiontischen 16S rRNA Gens von 1kb Länge gezeigt werden, daß diese in 100-prozentiger Sequenzübereinstimmung sowohl im Sediment und Porenwasser des Wirtshabitats, als auch im darüberliegenden Oberflächenwasser vorkommen (Bayer und Bulgheresi, unpublizierte Daten).

Ziel dieser Arbeit ist die Komplettierung der molekularen Charakterisierung zweier weiterer neu entdeckten Arten, *Robbea* sp.1 und *Robbea* sp.3, sowie ihrer Symbionten. Dies beinhaltet den bislang fehlenden Nachweis der 16S rRNA-Gene der Symbionten von *Robbea* sp.1 und *Robbea* sp.3, sowie des 18S rRNA Gens von *Robbea* sp.1. Weiterhin soll die Charakterisierung der *internal transcribed spacer region* (ITS) der Symbionten eine

höhere Auflösung der Verwandtschaftsverhältnisse der Bakterien auf den einzelnen Wirten erlauben, um Rückschlüsse auf den Infektionsmodus dieser Symbiose zu erhalten. Um die Hypothese einer horizontalen Übertragung der stilbonematinen Bakterien aus der Umwelt weiter zu stützen, sowie die globale Verbreitung eng verwandter Phylotypen einzuschätzen, wurde mithilfe der Polymerasekettenreaktion (PCR) und Fluoreszenz-in-situ-Hybridisierung (FISH) versucht, Fragmente der 16S rRNA-gene sowohl der Symbionten von *Robbea* sp.1 und *Robbea* sp.3, als auch von *Robbea* sp.2 und *Laxus oneistus* weit außerhalb der Wirtshabitate, im küstenfernen Oberflächenwasser des Mittelmeers und der Karibik nachzuweisen.

Abstract

Marine nematodes that bind sulphur-oxidising bacteria on their cuticle (Chromadorea: Stilbonematinae) are found worldwide in shallow seawater sediments. Migrating between an oxidised upper sand layer and a reduced one underneath, they allow the bacteria to obtain both the oxygen they need as electron acceptor and reduced sulphur compounds as electron donors. In turn, the symbionts constitute their hosts‘ major food source. For decades it has been assumed that the symbionts are acquired from the environment at each generation anew and after each time the cuticle is shed to be replaced by a newly synthesised one. Among these highly specific and stable associations the symbiont of *Laxus oneistus* was the first to be analysed and found to belong to a single phylotype of γ-proteobacteria. This study completes the molecular characterisation of three recently discovered species of the genus *Robbea* and their associated bacteria and supports environmental acquisition of nematode symbionts. The small rRNA-gene subunits of the Mediterranean *Robbea* sp.1 host as well as the symbionts of *Robbea* sp.1 and the Caribbean *Robbea* sp.3 have been sequenced, their symbiotic origin confirmed by fluorescence in situ hybridisation (FISH) and phylogenetically analysed. Both *Robbea* hosts and symbionts do not form a distinct cluster within the Stilbonematinae or the γ-proteobacterial sulphur-oxidising nematode and marine gutless oligochaete symbionts, respectively. The analysis of the internal transcribed spacer region (ITS) of the *Robbea* sp.1 and sp.3 symbionts suggests that one or two founder cells from an environmental pool might be sufficient to establish the bacterial coat. Furthermore, Mediterranean and Caribbean offshore surface seawater samples were tested for presence of 16S rRNA-gene sequence types of all three *Robbea* symbionts, by means of polymerase chain reaction (PCR) and FISH. Strikingly, the symbiont phylotypes associated with the Mediterranean nematode *Robbea* sp.1 and the Caribbean nematode *Robbea* sp.3 were detected in both Mediterranean and Caribbean offshore seawater. FISH experiments also revealed that the planktonic counterparts are metabolically active, as they divide by binary fission. The conspicuous presence in a habitat, which is chemically, physically and geographically completely different from their hosts‘, is unprecedented for marine sulphur-oxidising symbionts.

Introduction

The Thiobios

Ecological significance

Biocenoses under aphotic conditions and with low influx of extraneous organic matter may solely be dependent on chemoautotrophic primary production. This particularly applies to deep-sea hydrothermal vent communities and deep subsurface ecosystems (McCollom and Amend, 2005). Furthermore, wherever reduced inorganic compounds emerge from anaerobic decomposition of organic material, chemoautotrophic oxidation secures the conservation of energy within ecosystematic redox cycles (Meyer-Reil, 2005). Trophic interactions are based on the interspecific transfer of metabolic chemical energy. Where primary production predominantly relies on chemoautotrophic archaea and bacteria, these organisms possess a key role for the size, biomass, composition and trophic structure of their dependent communities (McCollom and Amend, 2005). In the sulphate-rich marine environment, reduced sulphur compounds, such as sulphide and thiosulphide, widely arise from biogenic and geochemical processes. They provide the source of energy for a highly diverse form of life: the thiobios (Reise and Ax, 1980; Powell and Bright, 2007).

Sulphidic habitats

Chemoautotrophic sulphur-oxidisers are widespread among habitats where sulphide or thiosulphate occur. At hydrothermal vents of mid-oceanic ridges, sea water penetrates nascent oceanic crust several kilometres deep. Underneath the crust, overheated by the underlying magma chamber, sulphate from the intruding water is reduced to sulphide. Mingled with metals and other reduced compounds, the heated anoxic fluid wells up through cracks in the rift valley or seeps through sediments at temperatures up to 400 °C (Von Damm, 1990). In contrast, sulphide found in other habitats results from the degradation of organic matter by chemoorganotrophic, sulphate-reducing bacteria. These habitats mainly include cold seeps (Suess et al., 1987), whale and wood falls (Rouse et al., 2004), macrophyte debris (Fenchel and Riedl, 1970; Mann, 1976) and

sheltered shallow water sediments. The latter constitute the largest thiotrophic habitat and extend to all sediments with sufficient influx of organic matter, which means substantially all shelf sediments to dysoxic basins and upperslope sediments (Ott et al., 2004b).

The redox potential discontinuity

In sheltered shallow water sediments, the anoxic, sulphidic layer lies under an oxygenated surface layer of variable thickness in the millimetre to centimetre-range. The chemocline in between these layers is named the redox potential discontinuity (RPD) (Fenchel and Riedl, 1970). Chemically, the RPD is characterised by a downward sequence from oxygen to nitrate, ferric iron, manganese and sulphate. Chemoorganotrophic bacteria gain energy for the decomposition of organic matter from reducing sulphate. Upward diffusion of the resulting sulphide leads to its oxidation, where chemoautotrophic bacteria use this energy to fix carbon from sulphide oxidation (Jøregensen, 1989).

Molecular basis of sulphide oxidation

Chemolithoautotrophic oxidation of reduced sulphur compounds to sulphate occurs stepwise. The overall electron yield is eight electrons per molecule. *Paracoccus pantotrophus* and many other predominantly α-proteobacteria use a distinct sulphide and thiosulphate oxidation system, the so called Sox system. It comprises 15 genes encoding various cytochromes and other proteins, that directly oxidise sulphide or thiosulphate to sulphate. Other bacteria oxidise sulphide to elemental sulphur or to sulphite in a first major step, yielding two electrons per molecule or six, respectively (Madigan et al., 2009). Enzymes involved may include the reverse dissimilatory sulphite reductase (rDsr) cluster or a truncated Sox- system (Beller et al., 2006). In a second step, four electrons are released, when - if present - elemental sulphur is oxidised to sulphite. Then, the third step occurs in either of two ways: the final oxidation of sulphite to sulphate is most commonly accomplished by a sulphite oxidase. Instead, some bacteria use adeno-

sine phosphosulfate (APS) reductase, a reversible key enzyme in the metabolism of both sulphate-reducing and many sulphide-oxidising bacteria (Madigan et al., 2009).

Electrons from sulphide, thiosulphate and elemental sulphur are fed into the electron transport chain at different levels and generate a proton motive force used for ATP generation. Electrons from enzymatic sulphide oxidation are passed to cytochrome bc_1 via quinone. Then, via cytochrome *c*, they are carried to a terminal oxidase. Electrons enzymatically released from thiosulphate, elemental sulphur and sulphite are used to directly reduce cytochrome *c*. Under oxic conditions, sulphur oxidisers preferably use oxygen as the electron acceptor, but some species such as *Thiobacillus denitrificans* may also reduce nitrate, using a nitrate reductase (NarG) when conditions are anoxic. The advantage in the use of oxygen stems from its higher redox potential versus nitrate, hence yielding more energy. In a reverse electron flow NAD^+ is reduced to NADH by the NADH-quinone oxidoreductase. In this case, the electron donors possess a more electropositive redox potential than NAD^+/NADH does, thus making NADH generating reactions energy-consuming. NADH enters the CO_2-fixing Calvin cycle as a reducing agent in the reduction of 1,3-bisphosphoglycerate to glyceralaldehyde 3-phosphate (Madigan et al., 2009).

Thiotrophic symbioses

The mutual benefits in thiotrophic symbioses

Sulphide-enriched sediment layers may be separated from those containing the optimum electron acceptor oxygen by several millimetres to centimetres. In cases of weak oxygen and sulphide gradients, sulphur-oxidising bacteria have to cope with this incongruity between their size and their energetic demands (Ott et al., 2004b). Some bacteria, like the *Beggiatoa* and *Thioploca* grow large in size and are mobile enough to bridge the gap (Huettel et al., 1996; Ott et al., 2004b). Instead, symbiotic sulphur-oxidisers use their hosts to either carry them to-and-fro, or to house them in a particular organ, the trophosome, in which they are provided with both agents. The hosts have also repeatedly adapted to this situation. Motile hosts can migrate between oxygenated and anoxic sand

layers, to provide their bacteria with both sulphide and oxygen (Ott et al., 2004a). As sulphide inhibits cytochrome c oxidase, it is toxic to most metazoans. Thiotrophic hosts possess an increased tolerance towards sulphidic and anoxic conditions (Vismann, 1991). In addition, symbionts may protect their hosts by the oxidation process (Hentschel et al., 1999). The symbionts in the trophosome of *Riftia pachyptila* are simultaneously supplied with sulphide and oxygen by the worm's circulatory system. Unlike most other metazoans, *Riftia* synthesises extracellular haemoglobins that are able to reversibly bind sulphide, independent from oxygen transport (Zal et al., 1996; Zal et al., 1998; Flores et al., 2005).

Diversity of thiothrophic symbioses

Both hosts and symbionts are remarkably diverse and most probably their association evolved multiple times independently (Dubilier et al., 2008). To date, several lineages of the α-, γ- and ε-proteobacteria are known to contain sulphur-oxidising symbionts (Gruber-Vodicka et al., 2009; Dubilier et al., 2008). These bacteria repeatedly engaged in symbiotic associations with hosts belonging at least to seven different phyla: Ciliata, Porifera, Platyhelmintes, Nematoda, Mollusca, Annelida and Arthropoda (Dubilier et al., 2008). Even on lower taxonomic levels, the incongruity between 16S and 18S rRNA-gene based phylogenetic data reflects the lack of co-evolution among sulphur-oxidising symbionts and their hosts (Dubilier et al., 2008; Bayer et al., 2009). So far the only exceptions are found in vesicomyd clams (Hurtado et al., 2003) and in the recent finding of a perfectly matching 16S/23S and 18S/28S rRNA-gene phylogeny of catenulid flatworms, *Paracatenula* spp., and their intracellular α-proteobacterial symbionts (Gruber-Vodicka et al., 2009).

In view of the high diversity, many hosts share convergent adaptions. Polychaete and nematode hosts show simple gut anatomy or even complete reduction of intestinal structures and up and down migration behaviour. Another example can be found among the Polychaeta, the Vestiminiferan *Rifta pachyptila*, as well as the Monoliferan Sclerolinum and the Frenulate Oligobrachia developed a trophosome to harbour their

chemoautotrophic symbionts. Although functionally similar, these trophosomes differ in their organogenesis, as the Vestiminferan and Monoliferan trophosome is of mesodermal origin (Nussbaumer et al., 2006; Eichinger, pers. comm.) whereas the Frenulate very likely are entodermal (Dubilier et al., 2008).

Stilbonematid symbioses

The subfamily of the Stilbonematinae

Nematodes of the interstitial fauna of marine sandy bottoms may, as *Astomonema* or *Parastmonema*, carry sulphur-oxidising bacteria in their body interior (Ott et al., 1982; Vidakovic and Boucher, 1987; Giere et al., 1995; Kito, 1989; Musat et al., 2007) or on their surface. The latter belong to the subfamily Stilbonematinae (Desmodoridae, Chromadoria, Adenophorea), which consists of the genera *Adelphus* Ott, 1997, *Catanema* Cobb, 1920, *Eubostrichus* Greef, 1869, *Laxus* Cobb, 1894, *Leptonemella* Cobb, 1920, *Robbea* Gerlach, 1956, *Squanema* Gerlach, 1963 and *Stilbonema* Cobb, 1920 (Ott et al., 2004a; Ott et al., 2004b).

The worms migrate along the chemocline between oxygenated upper sand layers and anoxic sulphidic deeper ones, to supply their bacteria with both the oxygen they need as e^--acceptor and reduced sulphur compounds as e^--donor (Ott et al., 1991; Polz et al., 1992; Hentschel et al., 1999). In turn, the ectosymbionts constitute the major part of their hosts‘ diet, as shown by stable carbon isotope composition measurements (Ott et al., 1991), and protect their hosts from sulphide poisoning (Hentschel et al., 1999).

In contrast to non-symbiotic Desmodoridae, Stilbonematinae are characterised by a reduced, unarmed buccal cavity, a weak pharynx and a mostly glandular terminal bulb. In the genera *Catanema*, *Laxus* and especially *Robbea*, the anterior part (corpus) of the tripartite pharynx is set off and muscle-rich. The males of most *Robbea* species are provided with a row of postpharyngeal ventromedian suckers, supposed to be copulation-helping organs. The number of these cuticular structures is constant and species-specific. The stilbonematid body is divided into an anterior region, which may be free of symbionts, and the posterior region carrying the symbionts. In *Laxus*, the diameter does

not increase by the thickness of the bacterial monolayer, as the worm's diameter in the posterior region is fittingly decreased. Elemental sulphur inclusions in the ectosymbiotic bacteria are responsible for the stilbonematids' bright white appearance (Himmel et al., 2009). In all species, the very tip of the tail is symbiont-free (Ott, 1993; Ott et al., 2004a).

Besides their ectosymbiosis, the stilbonematids possess another autapomorphic character, the glandular sense organs (GSOs). Distributed all over their body, they secrete a mucus, that only in the posterior region is able to attach the symbionts. The stilbonematid GSO is a hypodermal structure consisting of one epidermal cell, one sensory cell and two glandular cells, A and B, instead of only one, like in subcuticular glands of non-symbiotic desmodorid nematodes (Nebelsick et al., 1992; Nebelsick et al., 1995).

Transmission and maintenance of stilbonematid symbionts

Symbiont transmission is vital for the maintenance of beneficial associations over host generations. Basically, two different modes of transmission can be distinguished: horizontal from the environment and vertical with maternal, and more rarely biparental, symbiont transfer (Bright and Bulgheresi, 2010). The first implies the existence of free-living symbionts in the host habitat. Based on 16S rRNA-gene fragments, free-living symbiont phylotypes of the following hosts have already been described: the lucinid clam *Codakia orbicularis* in seagrass sediment (Gros et al., 2003), the siboglonid polychaete *Oligobrachia mashikoi* in the sediment of Tsukomo Bay, Japan (Aida et al., 2008), and *Riftia pachyptila* at deep-sea hydrothermal vents (Harmer et al., 2008).

Horizontal transmission has also been hypothesised for the Stilbonematinae (Ott et al., 2004a; Ott et al., 2004b), since no bacteria were detected on the eggshells of early unhatched *Laxus oneistus* embryos so far. Colonisation on newly hatched or molted stilbonematids must happen very rapidly, since aposymbiotic juveniles are rarely found in field collections (Jörg A. Ott, pers. comm.).

Recruitment of symbionts from the environment most probably involves mannose-binding, Ca^{2+}-dependent lectins, the Mermaids. They were shown to be secreted by the GSOs in the posterior region of *Laxus oneistus* and mediate symbiont-symbiont, as well as worm-symbiont attachment (Bulgheresi et al., 2006). Indirect evidence suggests that corresponding mannose residues are present on the bacterial surface (Nussbaumer et al., 2004).

Aims of this study

The recent characterisation of the 16S rRNA and the APS reductase A (AprA) gene of the symbionts of the newly found stilbonematid *Robbea* sp.2 has shown that the symbiont coat of this nematode consists of a monospecific single layer of sulphur-oxidising γ-proteobacteria belonging to the oligochate-nematode symbiont cluster (Bayer et al., 2009). Furthermore, 100 % identical fragments of 1 kb in length could be detected in the sediment, overlaying water and surface water of the host habitat (Bayer et al., 2009).

To complete the molecular characterisation of two other yet undescribed species, *Robbea* sp.1 and *Robbea* sp.3, the 16S rRNA-genes of their symbionts and the 18S rRNA-gene of *Robbea* sp.1 were sequenced and phylogenetically analysed. In order to gain deeper insights into the mode of symbiont transmission and to assess the degree of kinship within the bacteria on single host individuals, the internal transcribed spacer region (ITS) of symbionts on single hosts was sequenced. To further support the hypothesis of a horizontal transmission in stilbonematid symbioses, as well as to assess the distribution of symbiont-related phylotypes, fragments of the 16S rRNA-gene from the symbionts of *Robbea* sp.1, *Robbea* sp.2, *Robbea* sp.3 and *Laxus oneistus* were searched for in offcoast surface seawater of the Mediterranean and Caribbean Sea, by means of polymerase chain reaction (PCR) and fluorescence in situ hybridisation (FISH).

Material and Methods

Sample collection

Specimen collection

Specimen of *Robbea* sp.1 were collected in July 2007 from a subtidal sand patch in close vicinity to a *Posidonia oceanica* meadow in around 2 m depth in the boat harbour of the Station de Recherches Sous-Marines et Océanographiques (STARESO), Calvi, France (42° 34'49 N, 8° 43'27 W). *Robbea* sp.2 was collected in October 2006 in around 1 m depth from a shallow water back-reef sand bar off Point of Sand Beach on Little Cayman, Cayman Islands (19° 42'08 N, 79° 57'47 W). *Robbea* sp.3 was collected in November 2007 in approximately 1 m depth from a shallow water back-reef sand bar off Carrie Bow Cay, Belize (16° 48' 11 N, 88° 04'55 W). The worms were extracted from the sand by shaking it in seawater and pouring the supernatant through a 63 µm-pore-size mesh screen. Single individuals were then isolated under a dissecting microscope using fine needles. *Robbea* sp.1 and *Robbea* sp.3 were either fixed in 80 % ethanol for DNA extractions or in methanol and partially postfixed one hour at room temperature in 1% osmiumtetroxide for fluorescence in situ hybridisation (FISH). Worms were stored in 80 % ethanol at 4 °C .

Environmental samples

For the detection of free living symbionts, open water samples were taken in a depth of an armlength, approximately one kilometre offshore from the STARESO, Calvi, France (CALVI W); La Spezia, Italy (LASP W); Little Cayman, Cayman Islands (CAY W) and Carrie Bow Cay, Belize (CBC W).

For DNA extractions, 1l of each sample was filtered through 0.22 µm pore size 47 mm GTTP MILLIPORE filters (MILLIPORE, Billerica, Massachusetts), subsequently stored in bead tubes of the UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, California).

Morphological analysis of *Robbea* sp. 1 symbionts

Scanning electron microscopy (SEM)

Specimens of *Robbea* sp.1 stored in 70 % ethanol were postfixed in a 2.5 % glutaraldehyde, 0.1 M sodium cacodylate, 2 % sucrose solution for 2 hours, rinsed with 0.1 M sodium cacodylate buffer 3 times 10 minutes, postfixed in an 1 % osmiumtetroxide, 0.1 M sodium cacodylate, 2 % sucrose solution for 1 hour and rinsed again. Samples were subsequently dehydrated in an ascending alcohol series of 30 %, 50 %, 70 %, 80 %, 90 % and 100 % v/v ethanol and transferred into 100 % acetone. After critical point dehydration, worms were gold sputter coated and examined with a Philips XL 20 scanning electron microscope.

18S rRNA-gene library of *Robbea* sp. 1 single worms

DNA extraction

For extraction of genomic DNA, single specimen of *Robbea* sp.1 stored in 70 % ethanol were isolated in 0.2 ml reaction tubes using fine needles and washed two times in 1x PBS. According to Schizas et al., 1997, they were transferred into 10 µl PCR reaction buffer (invitrogen, Paisley, UK), spun down briefly and supplemented with 1 µl of a 1 mg/ ml proteinase K solution (Sigma-Aldrich, St. Louis, Missouri). During incubation at 55 °C for three hours, samples were hourly shaken and spun down, briefly and carefully. Proteinase K was inactivated by incubation at 99 °C for 5 minutes and samples were subjected to three cycles of freezing in liquid nitrogen and thawing at 65 °C, both for 3 min per cycle. According to Musat et al., 2007, 10 µl of GeneReleaser (Eurogentec, Seraing, Belgium) were added and tubes were transferred into a thermal cycler and subjected to the GeneReleaser manufacturer's protocol: 65 °C for 30 s, 8 °C for 30 s, 65 °C for 90 s, 97 °C for 3 min, 8 °C for 1 min, 65 °C for 3 min, 97 °C for 1 min, 65 °C for 1 min, and finally 80 °C for at least 1 min. Tubes were then centrifuged at 13000 g for 1 min, the clear supernatants transferred into fresh ones and stored at -20 °C.

PCR conditions

The 18S rRNA-gene of the *Robbea* sp.1 host was amplified from three worm individuals in parallel 50 µl PCRs with each 2 µl genomic DNA used as template. Additionally, a fourth reaction with sterilised double distilled water was performed as a negative control. All reactions contained final concentrations of 1x PCR buffer - Mg (invitrogen, Paisley, UK), 1.5 mM $MgCl_2$, and 0.2 mM of each dNTP.. The primers in these reactions were 1F_short and 2023R (MWG Biotech AG, Ebersberg, Germany), each in a final concentration of 0.5 µM. The cycling conditions were: 94 °C initial denaturation for 4 min, 35 loops with each 45 s denaturation at 94 °C, 30 s annealing at 49 °C and 1 min 45 s elongation time at 72 °C, followed by a final elongation of 10 min at 72 °C. To allow hot start polymerisation, 2.5 U Taq polymerase (invitrogen, Paisley, UK) were added to each reaction after the initial denaturation. After cycling, reactions were checked by loading 5 µl plus 1 µl of 6x loading buffer on an 1 % agarose gel containing 1 µl/mg ethidium bromide and subsequent electrophoretic separation. The gel was then photographed in a UV gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Cloning procedure

PCR reactions containing a product of the expected size of approximately 1.8 kb with additional 5 µl of loading buffer were loaded on a 0.8 % agarose gel containing 1 µl/mg ethidium bromide and electrophoretically separated. The respective bands were sharply cut out of the gel and purified using the Gel Elution Gold Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) following the manufacturer's protocol. Purified products were ligated into the pCR®2.1-TOPO® cloning vector and transformed into TOP 10 cells, according to the manufacturer's protocol (invitrogen, Paisley, UK). Cells were spread on LB agarose plates containing X-gal and incubated overnight at 37 °C. The next day, white colonies were picked randomly and transferred onto 96-well plates with 50 µl sterilised LB broth (Sigma-Aldrich, St. Louis, Missouri) per well. 2 µl of the resulting suspensions were used to screen the colonies for the right insert by PCR. All reactions contained 1x PCR buffer with KCl (Fermentas, St.Leon-Rot, Germany), 1.0 mM $MgCl_2$, 0.125 mM

of each dNTP and 1.25 U Taq polymerase (Fermentas, St.Leon-Rot, Germany). Primers in these reactions were M13F and M13R (MWG Biotech AG, Ebersberg, Germany), each in a final concentration of 0.375 µM. Cycling conditions were: 94 °C initial denaturation for 3 min, 35 loops with each 15 s denaturation at 94 °C, 20 s annealing at 49 °C and 30 s elongation time at 72 °C, followed by a final elongation of 10 min at 72 °C. After cycling, reactions were checked by loading 9 µl plus 1 µl of loading buffer on an 1 % agarose gel containing 1 µl/mg ethidium bromide and electrophoretically separated. The gel was then photographed in a UV gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Plasmid multiplication and isolation

Overnight cultures from each colony with an insert of the expected size were inoculated by adding 10 µl to 5 ml of sterile LB broth (Sigma-Aldrich, St. Louis, Missouri) in culture tubes and growing overnight at 37 °C and a horizontal shaking speed of 145 times per minute. When reached an OD_{600} of approximately 1.0, cultures were pelleted at 2100 g for 10 min and the supernatants discarded. Plasmids were isolated from the bacterial pellets using the peqGOLD Plasmid Miniprep Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany), following the manufacturer‘s protocol.

Sequencing

Sequencing reactions with a total volume of 10 µl contained approximately between 100 and 400 ng of plasmid as template, 50 ng of each M13F, M13R and 600F primers (MWG Biotech AG, Ebersberg, Germany) and 1 µl of BigDye (Applied Biosystems, Foster City, California). Cycling conditions were: 26 loops with 20 s denaturation at 96 °C, 10 s annealing at 48 °C and 4 min elongation time at 60 °C. Finished reactions were analysed in an ABI PRISM® 310 Genetic Analyzer (Applied Biosystems, Foster City, California).

Sequence data analysis

Chromatograms with .ab1 file name extensions were vector trimmed and pre-edited using the 4Peaks software, version 1.7.2 (4Peaks by A. Griekspoor and Tom Groothuis, mekentosj.com), aligned and further edited in the CodonCode Aligner V 2.0.4 software (CodonCode Corporation, Dedham, Massachusetts). A majority weighted consensus was compared to the GenBank non redundant sequence collection using n-blast (Altschul et al., 1990).

16S rRNA-gene libraries of *Robbea* sp.1 and sp.3 symbionts

For the 16S rRNA-gene amplification of the symbiont of *Robbea* sp.1, exactly the same template as for the 18S rRNA-gene amplification was used. Concerning *Robbea* sp.3, another DNA extraction was performed analogously to the one described above for the 18S rRNA-gene library of *Robbea* sp.1 single worms.

The 16S rRNA-gene of the symbionts of *Robbea* sp.1 and *Robbea* sp.3 were each amplified in three parallel 50 µl PCR reactions with 2 µl of the respective different single worm genomic DNA as template. In addition, a fourth reaction with sterilised double distilled water was performed as a negative control. All reactions contained final concentrations of 1x PCR buffer - Mg (invitrogen, Paisley, UK), 1.5 mM $MgCl_2$ and 0.2 mM of each dNTP. The primers in these reactions were 616V and 1492R (MWG Biotech AG, Ebersberg, Germany), each in a final concentration of 0.5 µM. The cycling conditions were: 94 °C initial denaturation for 4 min, 35 loops with each 45 s denaturation at 94 °C, 30 s annealing at 49 °C and 1 min 30 s elongation time at 72 °C, followed by a final elongation of 10min at 72 °C. To allow hot start polymerisation, 2.5 U Taq polymerase (invitrogen, Paisley, UK) were added to each reaction after the initial denaturation. After cyc-

ling, reactions were checked by loading 5 µl plus 1 µl of 6x loading buffer on an 1 % agarose gel containing 1 µl/mg ethidium bromide and subsequent electrophoretic separation. The gel was then photographed in a UV gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany)

Ligation, transformation, cloning, blue/white screen, PCR screen, overnight cultures, plasmid isolation and sequencing and were performed exactly as described above for the 18S rRNA-gene library of *Robbea* sp.1 single worms.

Sequence data analysis

Chromatogram editing and alignments were performed exactly as described above for the 18S rRNA-gene. A majority weighted consensus was chimera checked with the Mallard software (Ashelford et al., 2006) and compared to the GenBank non redundant sequence collection (nr/nt) using n-blast (Altschul et al., 1990).

Phylogenetic sequence analyses

In order to resolve the phylogenetic relationship of the *Robbea* sp.1 host among other Stilbonematids, as well as the symbionts of *Robbea* sp.1 and sp.3, phylogenetic trees were calculated. The majority weighted consensus sequences from the 18S and 16S rRNA-gene library, respectively, were aligned to the Silva ssu_jan04_corrected database (Pruesse et al., 2007) using the NT4 alignment tool of the ARB software package (Ludwig et al., 2004) and added to the default tree. Related sequences plus outgroup species were exported in fasta format and aligned using the online MAFFT version 6 tool (http://mafft.cbrc.jp/alignment/server/; Katoh et al., 2005). Bayesian inference was performed using Parallel MrBayes @ BioHPC (http://cbsuapps.tc.cornell.edu/mrbayes.aspx; Huelsenbeck and Ronquist, 2001; Ronquist and Huelsenbeck 2003; Altekar et al.; 2004) and Maximum Likelihood using RAxML at the CIPRES cluster at the San Diego Super-

computing Center (http://www.phylo.org/news/RAxML; Stamatakis et al.; 2005; Stamatakis et al.; 2008), both with 1000 bootstraps. GenBank accession numbers for outgroup sequences in 18S rRNA based trees are Y16923, Y16913 and Y16911, as well as in 16S rRNA based trees AF452609, AF507818 and L35510.

ITS libraries of *Robbea* sp.1 and sp.3 symbionts

For the 16S rRNA-gene amplification of the symbiont of *Robbea* sp.1, exactly the same template as for the 18S rRNA-gene amplification was used. Concerning *Robbea* sp.3, another DNA extraction was performed analogously to the one described above for the 18S rRNA-gene library of *Robbea* sp.1 single worms.

The 16S to 23S rRNA internal transcribed spacer (ITS) region of the *Robbea* sp.1 and *Robbea* sp.3 symbionts was amplified in two times three parallel PCR reactions with each 1 µl of a different single worm genomic DNA as template. Additionally, two negative controls were performed by using sterilised double distilled water as template. All reactions contained final concentrations of 1x PCR buffer with KCl, including 2.0 mM $MgCl_2$ (Fermentas, St.Leon-Rot, Germany) and 0.2 mM of each dNTP. Forward primers used in these reactions were Rcas635F or Rhs438F, specifically targeting the 16S rRNA-gene of *Robbea* sp.1 or *Robbea* sp.3, respectively. A 23S rDNA reverse primer, 1035R+ (5´-TTCGCTCGCCRCTACT- 3´), was modified from the 1035R primer described in Ludwig et al., 1992. Both, forward and reverse primers were used in a final concentration of 0.5 µM per reaction. The cycling conditions were: 94 °C initial denaturation for 4 min, 35 loops with each 45 s denaturation at 94 °C, 30 s annealing at 46 °C and 1 min 45 s elongation time at 72 °C, followed by a final elongation of 10 min at 72 °C. To allow hot start polymerisation, 2.5 U Taq polymerase (Fermentas, St.Leon-Rot, Germany) were added to each reaction after the initial denaturation. Finished Reactions were checked by loading 5 µl plus 1 µl of 6x loading buffer on an 1 % agarose gel containing 1 µl/mg ethidium bromide and subsequent electrophoretic separation. The gel was then photographed in a UV gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Ligation, transformation, cloning, blue/white screen, PCR screen, overnight cultures, plasmid isolation and sequencing and sequence data analysis were performed as described above for the 18S rRNA-gene library of *Robbea* sp.1 single worms.

Fluorescence in situ hybridisation (FISH) on single worms

FISH was performed according to Manz et al., 1992. For a detailed overview of all probes used, formamide concentrations and hybridisation times refer to Table2.

Probe design

FISH probes specifically targeting the 16S rRNA-gene of *Robbea* sp.1 and *Robbea* sp.3, respectively, were designed using the probe design tool of the ARB software package (Ludwig et al., 2004) and the SILVA ssu_jan04_corrected database (Pruesse et al., 2007), with the respective sequences added. Best hits were chosen and additionally checked for specificity by comparing them to the GenBank non redundant nucleotide collection (nr/nt) via blastn (Altschul et al., 1990) and the Greengenes (DeSantis et al., 2006) and SILVA 16S/18S databases (Pruesse et al., 2007) via probeCheck (Loy et al., 2008). Specific probes were manufactured and fluorescently 5´ labelled with Cy3 by Thermo Scientific, Germany.

CloneFISH

Due to a scarcity of *Robbea* sp.1 specimens, a CloneFISH procedure was performed, in order to determine optimum hybridisation conditions. Therefore, approximately 100 ng of a plasmid carrying a representative sequence of the *Robbea* sp.1 16S rRNA-gene were electroporated into 65 µl of JM109(DE3) cells. Cells were regenerated in 250 µl S.O.C. medium at 37 °C for one hour, plated on 0.1 mg/ml kanamycin LB agar and incubated overnight at 37 °C. The next day, colonies were picked and PCR screened with M13F and M13R primers. Positive clones were sequenced and grown overnight in 5 ml LB broth containing 0.1 mg/ml kanamycin. The next day, 1 ml of each overnight culture was

transferred into 100 ml LB broth containing 0.1 mg/ml kanamycin and grown to an OD_{600} of 0.4 at 37 °C. Expression of the cloned 16S rRNA-genes was immediately induced by adding 100 µl of 1 M IPTG. After one hour at 37 °C, 100 µl of 170 mg/ml chloramphenicol were added and cells were left at 37 °C for another four hours. Afterwards, cultures were pelleted carefully, washed twice in 1x PBS, fixed in 4 % PFA for one hour at 4 °C, washed again and stored in 1 vol 1x PBS plus 1 vol 96 % ethanol at -20 °C.

Of each clone suspension 2 µl were applied onto a teflon coated microscopy slide, air dried and coated with 10 µl of 10 % hybridisation buffer. 30 ng of Cy3 labelled Rca470 probe, specific to Robbea sp.1 and 1 µl of 50 ng of FLUOS labelled eubacterial EUB338 probe were added and the slide was put into a 50 ml tube stuffed with hybridisation buffer soaked paper and incubated at 46 °C for 1.5 hours. The slide was then transferred into the correspondent washing buffer, incubated at 48 °C for 10 min, transferred into ice-cold water for a few seconds and dried quickly under a weak stream of compressed air. Prior to examination on a Zeiss Axio Imager AX10 epifluorescence microscope, stained cells were mounted in DAPI Vectashield (Vector Labs) under a cover slip. By eye, the clone with the strongest Cy3 signal was chosen for the consequent formamide series.

Next, a formamide series including the corresponding washing buffers was performed with the following concentrations: 10 %, 20 %, 25 %, 30 %, 35 %, 40 %, 45 % and 55 %, following the same hybridisation procedure. Evaluation was done on a Leica TCS-NT confocal laser scanning microscope. Gain, offset and zoom values were adjusted to the brightest fluorescent sample. Once set for all consequent samples, ten random images were made per each formamide concentration. Editing and analysis of the images were done in the daime 1.1 software (Daims et al., 2006).

Single worm FISH conditions

Specimens of symbiont coated, ethanol fixed *Robbea* sp.1 were put on a glass slide, air dried briefly, coated with 46 °C warm 0.1 % agarose, air dried again and coated with 10 µl of 35 % formamide hybridisation buffer. To sample worms, 50 ng EUB338 (FLUOS) and 30 ng GAM42a (Cy5) were added, to control worms 30 ng NON338 (Cy5), 30 ng Gam42a

(Cy5) and 50 ng BET42a (FLUOS). Both sample and control slides were put into 50 ml tubes stuffed with hybridisation buffer soaked paper, tightly screwed and incubated at 46 °C for 1.5 hours. Transferred into washing buffer, slides were incubated at 48 °C for 10 min, transferred into ice-cold water for a few seconds and dried quickly under a weak stream of compressed air. Control worms were stored at 4 °C, whereas sample worms were coated with 25 % formamide hybridisation buffer plus 1 µl of 30 ng/µl Rca470 (Cy3) and subjected to exactly the same hybridisation and washing procedure. Prior to examination on a Leica TCS-NT confocal laser scanning microscope, worms were mounted in DAPI Vectashield™ (Vector Labs Peterborough, UK) under a cover slip.

Specimens of *Robbea* sp.3 fixed in methanol were hybridised in 35 % formamide hybridisation buffer. To sample worms, 30 ng Rhs465 (Cy3), 30 ng GAM42a (Cy5) and 50 ng EUB338 (FLUOS) were added, to control worms 30 ng NON338 (Cy5), 30 ng Gam42a (Cy5) and 50 ng BET42a (FLUOS). Hybridisation was performed at 46 °C for 3 hours. Taken off the slides with a fine needle and transferred into small glass bowls filled with washing buffer, worms were then incubated at 48 °C for 10 min and afterwards again transferred onto a new glass slide. Worms were coated with ice-cold water for a few seconds, by once pipetting down and up, and quickly dried under a weak stream of compressed air. Prior to examination on a Leica TCS-NT confocal laser scanning microscope, worms were mounted in DAPI Vectashield™ (Vector Labs, Peterborough, UK) under a cover slip.

16S rRNA-gene libraries of environmental samples

DNA extraction and random amplification

Before extracting environmental DNA with the UltraClean™ Soil DNA Isolation Kit according to the manufacturer's protocol, filters were torn using sterile sharp tweezers. The extracted DNA was randomly amplified using the standard protocol of the GenomiPhi™ V2 Kit (GE Health Care Europe, Vienna, Austria). In parallel, PCR grade water routinely used in the lab was also randomly amplified to serve as a template in negative control PCR reactions.

Specific primer design

Primer sets each specifically targeting the symbionts' 16S rRNA-genes of *Robbea* sp.1, Robbea sp.2, *Robbea* sp.3 and *Laxus oneistus* were designed using the PROBE_DESIGN tool of the ARB software package (Ludwig et al., 2004) and an individually enlarged version of the SILVA ssu_jan04_corrected database (Pruesse et al., 2007). By using ARB's integrated PROBE_MATCH tool, most specific hits were chosen and modified to the following criteria: mismatches to the next similar target sequences had to be shifted to the 3' end, thereby resulting in a 20 to 22-mer with a GC content from 40 to 60 % and, as possible, a melting temperature between 60 and 64 °C. To further check the specificity of the resulting primers, their sequences were tested against the GenBank non redundant nucleotide collection (nr/nt) via blastn (Altschul et al., 1990), as well as the greengenes (DeSantis et al., 2006) and SILVA 16S/18S databases (Pruesse et al., 2007) via probeCheck (Loy et al., 2008). Primers were manufactured by MWG Biotech AG, Ebersberg, Germany, and are listed in Table 1.

PCR conditions

A nested PCR was performed by first amplifying a 16S rRNA-gene pool of each environment's amplified total genomic DNA. These pools served as template for reactions with specific primers. The 50 µl 16S Pool reactions contained 1 µl amplified total genomic DNA as template, and final concentrations of 1x PCR buffer - Mg (invitrogen, Paisley, UK,), 1.5 mM MgCl2 and 0.2 mM of each dNTP. Primers in these reactions were 616V and 1492R (MWG Biotech AG, Ebersberg, Germany), each in a final concentration of 0.5 µM. Cycling conditions were: 94 °C initial denaturation for 4 min, 25 loops with each 45 s denaturation at 94 °C, 30 s annealing at 49 °C and 1 min 30 s elongation time at 72 °C, followed by 10 loops with each 45 s denaturation at 94 °C, 30 s annealing at 49 °C and 2 min 30 s elongation time at 72 °C, and a final elongation of 10min at 72 °C. To allow hot start polymerisation, 2.5 U Taq polymerase (invitrogen, Paisley, UK) were added to each reaction after the initial denaturation.

The unpurified 16S rRNA-gene pools were then used as templates for the consequent specific reactions. Primers sets and environments tested are listed in Table 1 and 4. All 50 µl reactions with specific primers contained 1 µl template, and final concentrations of 1x PCR buffer - Mg (invitrogen, Paisley, UK), 1.5 mM MgCl2, 0.2 mM of each dNTP and 0.5 µM for each specific primer. The cycling conditions were: 94 °C initial denaturation for 4 min, 35 loops with each 45 s denaturation at 94 °C, 30 s annealing at the respective temperature described in Table 5 and 1 min elongation at 72 °C and a final elongation of 10 min at 72 °C. For hot start polymerisation, 2.5 U Taq polymerase (invitrogen, Paisley, UK) were added to each reaction after the initial denaturation. For each primer set a parallel negative control reaction was performed with 1 µl double distilled water as template. In the case of Laxus oneistus, a nested PCR was performed. First, the Los69F and Los1012R primers (MWG Biotech AG, Ebersberg, Germany) were used in a first reaction. The resulting fragment then was gel purified with the Gel Elution Gold Kit (PEQLAB Biotechnologie GmbH, Erlangen, Germany) and then used as template in a second reaction with Los224F and Los646R (MWG Biotech AG, Ebersberg, Germany). Reactions were checked by loading 5 µl plus 1 µl of 6x loading buffer on an 1 % agarose gel containing 1 µl/mg ethidium bromide and subsequent electrophoretic separation. The gel was then photographed in a UV gel documentation system (PEQLAB Biotechnologie GmbH, Erlangen, Germany).

Ligation, transformation, cloning, blue/white screen, PCR screen, overnight cultures, plasmid isolation and sequencing were performed exactly as described above for the 18S rRNA-gene library of *Robbea* sp.1 single worms.

Sequence analysis

In the CodonCode Aligner V 2.0.4 software (CodonCode Corporation, Dedham, Massachusetts), sequences of the clones obtained from the environmental samples were aligned to the respective 16S rRNA-gene sequences obtained from *Robbea* sp.1 and *Robbea* sp.3 single worms' symbionts, as well as from *Robbea* sp.2 symbionts, described in Bayer et al., 2009. Any character differing from the original single worm sequence

isoforms in one position was declared a mismatch. The relative number of mismatches compared to the total number of positions in the respective environmental fragment was taken as measure for the sequence similarity between original and environmental sequences. The environmental sequence most similar to the original data was then chosen the environmental equivalent.

Fluorescence in situ hybridisation (FISH) on environmental samples

Filter preparation

To 22.5 vol of seawater, 1 vol of 37 % formaldehyde was added and incubated overnight at 4 °C. In aliquots of 94 ml, the fixed samples were then vacuum filtered through 0.22 µm pore size, 47 mm GTTP filters (MILLIPORE, Billerica, Massachusetts) and washed with 1x PBS immediately afterwards. Thereby, it was taken care the vacuum did not exceed 130 mbar (Hicks et al., 1992). Filters were air dried on a sheet of cellulose paper and stored airtight at 4 °C in a 6 cm petri dish.

Environmental FISH conditions

Fixed bacteria were transferred from the filters onto the wells of epoxy resin masked slides (Marienfeld, Germany), after these were moistened with 0.1 % agarose. 1 cm^2 pieces were cut from the filters and placed face down onto the wells. Slides were then vacuum-dried for approximately 10 min in an exsiccator before peeling off the pieces again (Hicks et al., 1992). Transferred bacteria were hybridised with the fluorescein-labelled Gam42a (Manz et al., 1992) and the Cy3-labelled probes Rca470, Rss456 or Rhs465, specifically targeting the 16S rDNAs of the symbionts of *Robbea* sp.1, *Robbea* sp.2 and *Robbea* sp.3, respectively.

All environmental FISH experiments were performed with Gam42a (FLUOS) and the respective specific probe Rca470 (Cy3), Rss456 (Cy3) or Rhs 465 (Cy3) for the symbionts of *Robbea* sp.1, *Robbea* sp.2 and *Robbea* sp.3. However, it should be noted, that the Rss456 probe (Bayer et al., 2009) also matches the 16S rRNA sequence of Inanidrilus

leukodermatus (AJ890100). Each well was coated with 10 µl hybridisation buffer (see Table 6) plus 1 µl of the respective probes, concentrated 30 ng/µl and 50 ng/µl in case of Cy3- or FLUOS-labelled probes, respectively. All hybridisations were performed with 35 % formamide overnight, except Rcas470 (Cy5), which was hybridised overnight with 25 % formamide, after the samples have been prehybridised with Gam42a (FLUOS) alone. Subsequently, samples have been transferred to the corresponding washing buffer (see Manz et al., 1992) for 10 min at 48 °C, dipped in ice-cold water for a few seconds and dried quickly under a weak stream of compressed air. Prior to examination on a Zeiss Axio Imager AX10 epifluorescence microscope, stained cells were mounted in DAPI Vectashield™ (Vector Labs, Peterborough, UK) under a cover slip.

Results

Morphological analysis of *Robbea* sp.1 symbionts

Scanning electron micrographs of *Robbea* sp.1 show that the posterior region of the worm is covered by a monolayer of tightly packed morphologically similar coccoid bacteria, of about 2 µm in diameter (Fig. 1). The white colour of the coating bacteria on the rather dark cuticle generates a contrast visible to the naked eye.

Molecular characterisation of the *Robbea* sp.1 host

18S rRNA-gene based phylogenetic analysis

In order to assess the phylogenetic position of *Robbea* sp. 1, the 18S rRNA-gene of three single specimen was amplified. All three reactions yielded a product with the expected size of approximately 1.8 kb (Fig. 4). Two of the obtained products were cloned, and from each, the inserts of two colonies were sequenced. All four sequences were 100 % identical and their consensus was added to GenBank (accession number EU768870). The three most similar BLAST hits corresponded to 18S rRNA-gene sequences of the stilbonematid nematodes *Laxus cosmopolitus* (Y16918), *Laxus oneistus* (Y16919) and *Stilbonema majum* (Y16922).

18S rRNA-gene based phylogenetic trees of stilbonematid hosts including *Robbea* sp.1 have been calculated and a consensus tree is shown in Figure 5. It confirms the phylogenetic position of stilbonematid nematodes within other non-symbiotic chromadorid species, but the three *Robbea* hosts do not form a distinct cluster. Furthermore, geographic proximity also does not necessarily imply phylogenetic relatedness, since the Caribbean (Cayman Islands) *Robbea* sp.2 (EU768871) 18S rRNA is mostly similar to that of the geographically distant, Mediterranean species *Catanema* sp. (Y16912).

Molecular characterisation of the *Robbea* sp.1 and sp.3 symbionts

16S rRNA-gene based phylogenetic analysis

The amplification of the *Robbea* sp. 1 symbionts' 16S rRNA-gene yielded a product of the expected size (approximately 1.5 kb) from genomic DNA obtained from three individuals (Fig. 6). A total of 13 clones resulting from two different host specimens was sequenced. The alignment of these sequences showed similarities of ≥ 99.8 %. Their consensus sequence was added to GenBank (accession number EU711427). The three most similar BLAST hits correspond to 16S rRNA-gene sequences belonging to the ectosymbiont of *Laxus oneistus* (FM955323), the gamma 1 endosymbiont of *Olavius ilvae* (AJ620498) and the ectosymbiont of *Robbea* sp.2 (EU711426).

Genomic DNA derived from three *Robbea* sp.3 individuals each yielded a 16S rRNA-gene PCR product (Fig. 7). The sequence similarities within the resulting clone library were ≥ 99.8 % and a consensus sequence was added to GenBank (accession number EU711428). The three most similar BLAST hits consisted of 16S rRNA-genes belonging to the sulphur oxidising endosymbiont of *Olavius algarvensis* (AF328856), the ectosymbiont of *Robbea* sp.2 (EU711426) and the endosymbiont 1 of *Inanidrilus leukodermatus* (AJ890100).

16S rRNA-gene based phylogenetic relationships of stilbonematid and gutless oligochaete symbionts including the *Robbea* sp.1 and sp.3 ectosymbionts have been calculated and a consensus tree is shown in Figure 8. The symbionts of the three newly discovered *Robbea* species all belong to the γ-proteobacterial nematode and marine gutless oligochaete symbionts. However, just like their hosts, they do not form a distinct cluster within this group. Comparing the host and symbiont trees (Fig. 5 and 8) does not indicate co-evolution. Furthermore, the discrepancy between symbiont phylogeny and host geography suggests a host independent environmental distribution of these symbionts.

Fluorescence in situ hybridisations (FISH) on single worms

In order to verify that the γ-proteobacterial 16S rRNA-gene fragments obtained by PCR originated from the bacteria associated with *Robbea* sp.1 and sp.3, fluorescence in situ hybridisations were performed with 16S rRNA-specific probes. For a detailed overview of all probes used, formamide concentrations and hybridisation times refer to Table2.

• ***Robbea sp.1***

Due to a scarcity of *Robbea* sp.1 specimen, the optimum hybridisation conditions with the specific probe Rca470 (Cy3) were determined by CloneFISH. Transformed cells expressing the respective 16S rRNA-gene were hybridised with the Rca470 (Cy3) probe under different formamide concentrations. According to the daime 1.0 (Daims et al., 2006) analysis , a formamide concentration of 25 % was chosen for further hybridisation experiments, which is close to the maximum of the polynomial regression line of fluorescence intensities of the formamide series (Fig. 9).

All bacteria were triple stained with the eubacterial probe EUB338 (FLUOS), the γ-proteobacterial probe GAM42a (Cy5) and Rca470 (Cy3), the probe specific to the 16S rRNA-gene of the *Robbea* sp.1 symbiont. In the control experiment, all bacteria stained by the γ-proteobacterial probe GAM42a (Cy5) were neither stained by the nonsense probe NON338 (Cy3), nor by the β-proteobacterial probe BET42a (Cy5), which equals GAM42a in position, length and sequence except for one nucleotide (Fig. 10).

• ***Robbea sp.3***

All bacteria present were triple stained with EUB338 (FLUOS), GAM42a (Cy5) and Rhs465 (Cy3), the probe specific to the 16S rRNA-gene of the *Robbea* sp.3 symbiont. In the parallel control experiment, all bacteria stained by the γ-proteobacterial probe GAM42a (Cy5) were neither stained by the nonsense probe NON338 (Cy3), nor by the β-proteobacterial probe BET42a (Cy5) (Fig. 11).

High clonal homogeneity among symbionts associated with *Robbea* sp.1 and sp.3

To gain information on the number of founder bacteria necessary to make up the coat on *Robbea* sp.1 and *Robbea* sp.3, the 16 to 23S rRNA-gene internal transcribed spacer region (ITS) of three individuals' genomic DNA per species was amplified (Fig. 12 and 13), cloned and sequenced. This approach is based on the fact that the ITS is subjected to a weaker selection pressure than the 16S rRNA-gene and has already been extensively used to assess symbiont diversity (Won et al., 2003; Harmer et al., 2008).

In the case of *Robbea* sp.1 symbionts, fragments of 1619 (variant A) and 1568 nt (variant B) were obtained. The ratios between the two variants differ between the surveyed worms. 83 % of variant A and 17 % of variant B are assigned to both specimen #1 (18 clones) and #2 (12 clones), 39 % of variant A and 61 % of variant B to specimen #3 (13 clones). Concerning *Robbea* sp.3 symbionts, fragments of 1803 nt were obtained. Each *Robbea* sp.3 specimen harboured symbionts with a single distinct ITS, with sequence similarities of 99.8 % between specimen #1 and #2, as well as #2 and #3 , and 99.1 % between specimen #1 to #3 (Table 3 and 4).

High similarities between 16S rRNA sequences of symbionts and offshore environmental samples

Previous experiments indicated that the *Robbea* sp.2 16S rRNA-gene is present not only in the host habitat at the Cayman Islands, but also in surface seawater collected outside the reef (Christoph Bayer and Silvia Bulgheresi, unpublished data). Therefore the search for the 16S rRNA-genes in offshore surface seawaters was extended to all three newly discovered *Robbea* symbionts plus *Laxus oneistus*. After having assessed the presence of the 16S rRNA-genes in surface seawater sampled offshore from the respective host habitats, i.e. *Robbea* sp.1 in the Mediterranean Sea from Calvi (Corsica, France)

as well as in the Caribbean Sea, *Robbea* sp.2 from Little Cayman (Cayman Islands), *Laxus oneistus* and *Robbea* sp.3 from Carrie Bow Cay (Belize), the search was extended to seawater samples from geographic regions where the respective worms have not yet been found.

Since the sequence of the PCR fragment obtained by using *Laxus oneistus* 16S-rRNA-specific primers on Carrie Bow Cay offshore samples (CBC W) only had a maximum similarity of ≤ 97 % compared to the original 16S rRNA-gene, this search was aborted. *Robbea* sp.2 symbiont sequence types with up to 98.4 % similarity could be detected in Carrie Bow Cay (CBC W), as well as in Calvi offshore water samples (CAL W) (Table 5, Fig. 16). The most striking result was obtained with *Robbea* sp.3 symbiont 16S rRNA: two widely overlapping fragments covering 1.5 kb of the 16S rRNA-gene fragment with 100 % sequence similarity to the same gene retrieved from worm associated bacteria. Surprisingly, the same fragments were not only obtained from Carrie Bow Cay (CBC W), but also from Calvi offshore water samples (CAL W; Table 5, Fig. 17 and 18). Last but not least, also a sequence type of approximately 400 nt with 100 % sequence identity of the *Robbea* sp.1 symbiont 16S rRNA could be detected in La Spezia (LASP W) and Calvi offshore water samples (CAL W), as well as in Carrie Bow Cay (CBC W) with 99.5 %, and Little Cayman (CAY W) with 99.8 % (Table 5, Fig. 14 and 15). In this case, however, the trial to cover the full length 16S rRNA fragment by using semispecific primer sets only resulted in sequences with similarities of ≤ 97 %, compared to the original.

It should be noted, that all respective environmental clone libraries were highly heterogeneous. Negative controls with PCR grade water, as well as randomly amplified PCR grade water did not yield any products. Hence, the possibility of plasmid contaminations can be widely excluded. For an overview of sample origins, primer sets, number of clones, exact sequence lengths, hypervariable region coverages and sequence similarities, refer to Table 5.

Visualisation of free-living phylotypes of the *Robbea* symbionts

In order to verify the wide distribution of *Robbea* symbiont phylotypes, fluorescence in situ hybridisations (FISH) were performed on filtrates of surface seawater sampled 1km offshore from Portovenere, La Spezia, Italy. Three experiments were conducted, each with the γ-proteobacterial probe GAM42a (FLUOS) and one of the Cy3-labelled probes Rca470, Rss456 or Rhs465, specifically targeting the 16S rDNAs of the symbionts of *Robbea* sp.1, sp.2 and sp.3, respectively (Fig. 20-23). All specific stainings were co-stained with the GAM42a probe. For a detailed overview of all probes used, formamide concentrations and hybridisation times refer to Table 6. The results confirmed the presence of symbiont 16S rRNA-genes in a habitat, which is chemically, physically and geographically completely different from the one of their hosts'. Moreover, they yielded another striking insight: the planktonic counterparts of the *Robbea* symbionts could be found undergoing cell division, hence suggesting that they are alive and active. Finally, the phylotype of the *Robbea* sp.3 symbiont does not divide longitudinally, like its cognate attached to the cuticle, but rather seems to undergo a conventional transversal binary fission. In addition, hybridisations with the Rss456 probe, specific not only to the 16S rRNA of the *Robbea* sp.2 symbiont, but also to the oligochaete *Inanidrilus leukodermatus* endosymbiont 1, yielded another interesting result: oftentimes, bacteria stained specifically were found in consortia with other, γ- or non-γ-proteobacteria (Fig. 22). This finding corresponds to the subcuticular situation in oligochaete hosts, where γ-proteobacterial sulphur-oxidisers are associated with δ-proteobacterial sulphate-reducers and other γ-proteobacterial sulphur-oxidisers (Dubilier et al., 2008).

Discussion

Morphological and molecular characterisation of *Robbea* sp.1

The genus *Robbea* was established by (Gerlach, 1956) and is characterised by a tripartite pharynx with a clearly set off and muscle-rich distal part (corpus). All males are provided with a row of postpharyngeal ventromedian suckers, supposed to be copulation-helping organs. The number of these cuticular structures is constant and species-specific. By light microscopy observations claiming conformity with these characteristics, the newly discovered and yet undescribed nematode collected in Calvi, Corsica was assigned to the genus *Robbea* (Fig. 1), as well as *Robbea* sp.2 (Fig. 2) and sp.3 (Fig. 3) from the Caribbean Sea. However, in the 18S rRNA-gene based phylogenetic reconstruction (Fig. 5), *Robbea* sp.1 and two the other recently discovered species, *Robbea* sp.2 and sp.3, do not form a monophyletic lineage within the Stilbonematinae (see also Bayer et al., 2009). *Robbea* sp.1 (EU768870) shows highest 18S rRNA-gene sequence similarity with the sympatric Mediterranean stilbonematid *Laxus cosmopolitus* (Y16918), as *Robbea* sp.3 does with the co-occuring Caribbean species *Stilbonema majum* (Y16922). In contrast, *Robbea* sp.2 (EU768871) 18S rRNA-gene sequence is mostly similar to those of the geographical distant, Mediterranean species *Catanema* sp. (Y16912). Although further sequence information, such as the 28S rRNA-gene, the internal transcribed spacer (ITS) region, as well as other genes might be needed to support the genus *Robbea* on a molecular level, the data obtained in this study suggest an evolutionary scenario in which the distinct morphological traits have evolved several times independently. From this point of view, the genus *Robbea* should probably be regarded as a polyphyletic taxon.

Morphological and molecular characterisation of the *Robbea* sp.1 and sp.3 symbionts

The symbionts of the *Robbea* sp.1 and sp.3 hosts significantly differ in size, shape and alignment on the cuticle, but both form highly ordered monolayers (Fig. 1 and 3). It should be noted that *Robbea* sp.1 and *Robbea* sp.2 symbionts obviously divide transversally (see arrows in Fig. 1 and 2), whereas *Robbea* sp.3 symbionts seem to divide longitudinally (see arrows in Fig. 3). This rare cytoengeneering feat has also been observed in

Laxus oneistus symbionts (Polz et al., 1994) and may ensure that in the monolayer of upright rod-shaped bacteria, daughter cells stay in contact with the host cuticle.

The high morphological homogeneity within *Robbea* sp.1 and sp.3 symbiont monolayers is mirrored by the high sequence homogeneity of the 16S rRNA-gene libraries, i.e. sequence similarities ≥ 99.8 %. Fluorescence in situ hybridisation (FISH) inequivocably proved the monospecificity of the *Robbea* symbioses, as each bacterium was stained by three probes of increasing specificity: the eubacterial, the γ-proteobacterial and the specific ones targeting the 16S rRNA of the *Robbea* sp.1 and sp.3 symbionts, respectively. Both symbionts can be assigned to two distinct clone groups within the γ-proteobacterial nematode and marine gutless oligochaete symbiont cluster (Fig. 5). Moreover, stilbonematid symbionts do not necessarily cluster with geographically close nematode and oligochaete symbionts. Since the stilbonematid symbionts depicted in Figure 5 are not consistently grouped according to their geographical origin, their phylogenetic reconstruction suggests that these bacteria might be abundant at least along the whole Atlantic Ocean and its marginal Mediterranean Sea, both connected by bidirectional currents in the Strait of Gibraltar. In this hypothetical scenario, speciation and engagement in symbiotic associations could have evolved several times independently at many geographical sites. The resulting phylogenetic incongruence between host and symbiont phylogenies is typical for horizontally transmitted symbioses (Moran and Wernegreen, 2000; Bright and Bulgheresi, 2010) and also observed for marine gutless oligochaetes and their sulphur-oxidising symbionts (Dubilier et al., 2001; Blazejak et al., 2006; Dubilier et al., 2008). Regarding the close relatedness of the nematode-oligochaete symbiont cluster to the Chromatiaceae, it is intriguing that some of the latter – although in general free-living – engage in symbiotic phototrophic consortia with other unrelated bacteria (Tonolla et al., 2000; Overman, 2002).

Symbiont transmission in stilbonematid symbioses

The establishment of stilbonematid symbioses has never been observed. To gain better insight in this process, the cultivation of aposymbiotic stilbonematid symbionts has been attempted several times, but to date failed (J.A. Ott, pers. comm.). In general, hosts acquire their symbionts by two different strategies. In vertical transmission, symbionts are passed directly from the parental generation to their offspring - in most cases maternally. Horizontally transmitted symbionts are taken up from the environment at each generation anew. The term environment in this case may of course also include other holobiont conspecifics. Although rare, a strict vertical transmission implies that the symbiont population is cut off from the environmental population, leading to speciation via genetic drift. In contrast, horizontally transmitted symbionts are periodically released to the environment, where they can mingle with the free-living population (Dubilier et al., 2008; Bright and Bulgheresi, 2010). In reverse, the presence of symbionts and closely related phylotypes is an essential prerequisite for environmentally, i.e. horizontally transmitted symbioses.

The internal transcribed spacer region (ITS) libraries obtained in this study show that for *Robbea* sp.1 and sp.3, the acquisition of at least one or two symbionts as founder cells could be sufficient to establish the symbiont coat (Table 3). This is consistent with findings in the intestines of insect killing nematodes (Martens and Goodrich-Blair, 2005; Ciche et al., 2008) and the crypts of the *Euprymna scolopes* light organ (Wollenberg and Ruby, 2009), both colonised by a maximum of two founder cells. Also the microbial population inhabiting the trophosome of the giant tubeworm *Riftia pachyptila* is understood as a clonal expansion of only few founder cells (Nussbaumer et al., 2006; Harmer et al., 2008). Frank, 1996, hypothesises that natural selection favours associations with limited clonal diversity of microbial symbionts, as thus the host would profit from reduced competition among different symbiont clones. The high clonal homogeneity within stilbonematid symbiont coats, as it is suggested by the 16S rRNA and ITS analyses, is indeed surprising, regarding the fact that in these ectosymbioses the attached symbionts are constantly exposed to a definitely non-sterile environment. In fact, the coat seems to

be maintained at least nearly monoclonal even against other competent environmental symbiont strains.

Microscopic observations of newly hatched stilbonematid embryos showed no signs of maternally transmitted symbionts (S. Bulgheresi and J.A. Ott, unpublished observation), supporting environmental transmission. However, embryo release was induced artificially and field-derived evidences are needed. But as stilbonematids may molt up to four times during their life cycle, they also have to regain their symbiont coat after each molting, regardless of the initial mode of symbiont acquisition. The symbionts may therefore again be acquired from the environment or, hypothetically, by reinfection with bacteria from the shedded cuticle. The latter case could be supported by the fact that for every ITS variant, libraries were extremely homogenous with no less than 100 % sequence congruence. Moreover, some molting stilbonematids occasionally found in the field collections exhibit an old cuticle on top of the new one, both symbiont coated (S. Bulgheresi and J.A. Ott, unpublished observation). Even though hypothetical, in this case it cannot be excluded that a higher number of founder cells may colonise the juvenile host, but some become lost during each molting. To address this question, an alternative experimental set up would be needed, considering a size classification of the surveyed specimen.

Unexpectedly wide distribution of free-living symbiont phylotypes

As mentioned before, the existence of a free-living environmental pool of competent symbiont bacteria is a prerequisite for horizontal transmission. For lucinid clams, a previous study shows that the sulphur-oxidising symbionts of *Codakia orbicularis* are present in its habitat, the *Thalassia testudinum* seagrass sediment. Inoculation experiments with this sediment led to successful infections of aposymbiotic *Codakia orbicularis* mussels (Gros et al., 2003). However, to date no evidence of the presence of sulphur-oxidising lucinid symbionts in water samples could be published. Similarly, 16S rRNA-gene fragments of the beard worm's *Oligobrachia mashikoi* thiotrophic symbionts were only

detected in sediment samples collected in a bay where the host resides (Aida et al., 2008). Also the γ-proteobacteria that invade the larval throphosome of the deep-sea tubeworm *Riftia pachyptila* (Rinke et al., 2006) can be found only in sediment surrounding the tubes and in biofilms covering microscopy slides that were deployed up to 100 m away to where the host thrives (Harmer et al., 2008). However, PCR amplification of the symbionts' 16S rRNA-gene from surrounding water samples failed to yield positive results.

Previous work points to the presence of free-living *Robbea* sp.2 symbiont phylotypes around the worm's habitat at the coast of the Cayman Islands (Christoph Bayer and Silvia Bulgheresi, unpublished data). To further support a horizontal transmission in stilbonematid symbioses, as well as to test the hypothesis of an atlantic-wide radiation of symbiont phylotypes, 16S rRNA-gene fragments of *Robbea* sp.1, sp.2 and sp.3 symbionts were searched in off-shore surface seawaters from the Caribbean and the Mediterranean Sea (Table 4). The most striking results were achieved with two widely overlapping fragments covering the whole known sequence of the *Robbea* sp.3 symbiont's 16S rRNA-gene. The sequence similarity of 100 % compared to the original sequence in both environments strongly supports an ubiquitous presence of these phylotypes at least in the Atlantic Ocean and its marginal Mediterranean Sea. In addition, a shorter fragment of the Mediterranean *Robbea* sp.1 symbiont's 16S rRNA-gene could be amplified from the Mediterranean off-shore environment with 100 % sequence similarity, as well as from the Caribbean environment with 99.8 %. This wide distribution of symbiont 16S rRNA sequence types is an unprecedented result and strongly supports horizontal transmission of stilbonematid symbionts. However, the degree of 16S rRNA similarity may not reflect that of a whole genome This implies that very similar bacterial 16S rRNA phylotypes might carry very different genetic signatures affecting their competence to engage in symbiotic associations. Vice versa, bacteria carrying different 16S rRNA-genes might share the same degree of competence to establish symbioses. Thus, comparative whole genomic or at least multi locus sequence typing (MLST) data are needed.

At first glance, the relatively low percentages of ≤ 98.4 % and ≤ 97 % in sequence similarity of the environmental clone libraries of *Robbea* sp.2 and *Laxus oneistus* symbionts compared to their original 16S rRNA-genes might contradict the conclusions drawn from the results obtained with *Robbea* sp.1 and sp.3 symbionts. But at least two considerations should be taken into account. On the one hand, the number of gene homologues in all databases is extremely low compared to the vast quantity of gene homologues that actually do exist in the environment. Any oligonucleotide designed to specifically target a certain sequence may undoubtedly also match any other yet unrecorded sequence. This could be a possible explanation why fragments of the 16S rRNA-gene of the *Robbea* sp.1 symbiont obtained with semispecific primer sets failed to achieve similarities as high as those obtained with two specific primers. In the case of *Robbea* sp.2 and *Laxus oneistus* symbionts, the amplified environmental 16S rRNA fragments were only distantly related to those of the symbionts. One possible explanation is that the seawater samples contain yet undiscovered bacterial phylotypes bearing highly similar 16S rRNA. On the other hand, it is also possible that *Robbea* sp.2 and *Laxus oneistus* symbionts are absent from the water column or under the detection limit. Notably, the attachment of the symbionts between the four different host species highly varies in its force. There actually is a downward sequence in the strength of symbiont attachment as follows: *Laxus oneistus*, *Robbea* sp.2, *Robbea* sp.1 and *Robbea* sp.3. *Laxus oneistus* hardly looses its symbiont coat even under vigourous mechanical treatment, whereas the symbionts of *Robbea* sp.3 tend to fall off their hosts easily, already at very low mechanical stresses, such as the surface tension when changing the medium. Surprisingly, the downward sequence in the force of attachment perfectly matches the sequence similarity percentages in reverse. Thus, any deviation in environmental symbiont abundance between the four surveyed species could be mirrored in a gradation of their adaption to a symbiotic lifestyle. Bacteria like the *Laxus oneistus* symbiont may be more dependent on host attachment as is the *Robbea* sp.3 symbiont. Although hypothetical, this could possibly lead to similar effects usually observed among vertically transmitted symbioses, such as lowered environmental abundance and speciation via genetic drift (Wernegreen and Moran, 1999; Bright and Bulgheresi, 2010).

Unexpected ecological flexibility of stilbonematid symbionts

Data obtained by Fluorescence in situ hybridisation of offshore seawater samples not only confirm the presence of stilbonematid symbiont phylotypes in an unexpected environment (Fig. 20 -22), but also suggest that they are active, since they can also be found in a dividing state (Fig. 22). The sulphur-oxidising endosymbiont of the marine gutless oligochaete *Olavius algarviensis* is phylogenetically very close to the stilbonematid symbionts (Fig. 5). Metagenomic data indicate that its wide genetic arsenal includes genes necessary for both sulphide-oxidation and heterotrophy (Woyke et al., 2006). This was unexpected because the oligochaete symbionts are assumed to be transmitted maternally (Giere and Langheld, 1987) and there are no reports on their presence in the marine environment. Given their phylogenetic closeness, it is likely that also the *Robbea* symbionts are not obligate sulphide-oxidisers and that heterotrophy enables them to survive in non-reducing habitats such as surface offshore seawater. In addition, also other metabolic adaptations might help stilbonematid symbionts to survive in very different habitats. When spread throughout oxic environments, they may use stored elemental sulphur as an energy source. Elemental sulphur globules are responsible for the bright white appearance of these bacteria and their presence has recently been determined in the sulphur-oxidising symbionts of the Stilbonematid *Eubostrichus dianae* by RAMAN spectroscopy (Himmel et al., 2009). Marine snow flocs may provide anoxic microniches containing sulphide from bacterial sulphate-reducing activity (Ploug et al., 1997). Both sulphate-reducing and sulphide-oxidising bacteria have already been shown to be present within these pelagic aggregates (Rath et al., 1998). Another possibility might be an ability to uptake and oxidise dimethylsulphide (DMS) from the sea and use it as an electron donor. The source of DMS in the sea is dimethylsulphoniopropionate (DMSP) or dimethylpropiothetin (DMPT), two molecules that are abundantly produced by marine algae worldwide. When released, DMSP and DMPT can be microbially catabolised into DMS (reviewed in Johnston et al., 2008). Free-living symbiont phylotypes may also be able to use nitrate instead of oxygen as an electron acceptor as Hentschel et al., 1999, suggested for the *Laxus oneistus* and *Stilbonema majum* symbionts. Genetic versatility certainly confers huge evolutionary advantages to ectosymbionts, as they live at the

host-environment interface instead of occupying sheltered structures inside their hosts.

Conclusions

A new nematode and a new nematode–bacteria association with very different geographical origins – the island of Corsica and the Belize Barrier Reef – were molecularly characterised. Although it cannot be excluded that the *Robbea* sp.1 and sp.3 symbionts could stably engage in associations with other marine hosts, the data show that *Robbea* sp.1 and sp.3 are always coated by one characteristic symbiont phylotype. The basis of this conclusion is the high homogeneity of the 16S rDNA library and the reproducible staining of the symbionts by a 16S rRNA-specific FISH probe. Accordingly, electron microscopy analysis revealed that individuals of *Robbea* sp.1 are always coated by one characteristic bacterial morphotype. ITS analyses of *Robbea* sp.1 and sp.3 symbiont coats suggest that a single host may be colonised by only one or two symbiont strains. However, an initial colonisation of juvenile hosts by more than two strains and a subsequent reduction up-on each molting cannot be excluded, since this experiment was conducted on adult worms only.

18S rRNA-based phylogenetic analysis shows that *Robbea* sp.1 is a Stilbonematid, albeit the genus *Robbea* can yet not be confirmed at the molecular level. Additional nuclear or mitochondrial DNA sequence information might be needed. Intriguingly, the 16S rRNA-genes of the *Robbea* sp.1 and sp.3 symbionts are tightly grouped with those of mouth- and gutless oligochaetes. One explanation is the co-occurence of nematodes and oligochaetes in shallow-water sandy bottoms and their exposure to a similar pool of environmental bacteria. In the course of the evolution, this habitat potentially promoted the establishment of these associations several times and at many different geographical locations. In this scenario, nematodes and oligochaetes recruited similar bacteria from this shared habitat as their prospective symbionts. Sequencing of stilbonematid symbiont genomes might unveil molecular adaptations shared by the oligochaete and nematode sulfur-oxidising symbionts.

The existence of free-living forms of bacterial symbionts is regarded as a prerequisite for an environmental transmission strategy, as it is supposed for the stilbonematid symbioses. Based on 16S rRNA-gene sequencing the study provides unprecedented evidence for the presence of at least two stilbonematid symbiont phylotypes in offshore surface

seawater samples – not only in proximity to their hosts‘ habitats, but also in far distant geographical regions: the Mediterranean and the Caribbean Sea. Furthermore, FISH stainings with 16S rRNA-specific probes show that these phylotypes are in a metabolic active state, as they were dividing. This is an unexpected result, as offshore surface seawaters chemically, physically and geographically completely differ from the Stilbonematids‘ shallow-water sediment habitats. These findings suggest an unexpected ecological flexibility and sulphide-independent metabolism of stilbonematid symbionts. However, more genetic information is needed to assess the competence of these free-living phylotypes to engage in symbiotic associations.

TABLES

Table 1: PCR primers used in this study

Target Gene	Primer set	T_A (°C)	Reference
Eukaryotic 18S rRNA	1f: 5′-CTGGTTGATYCTGCCAGT-3′ + 2023R: 5′-GGTTCACCTACGGAAACC-3′	49	Winnepenninckx et al., 1995 Pradillon et al., 2007
Eubacterial 16S rDNA	616V: 5′-AGAGTTTGATYMTGGGCTC -3′ + 1492R: 5′-GGYTACCTTGTTACGACTT-3′	47	Juretschko et al., 1998 Kane et al., 1993
Robbea sp.1 symbiont ITS	Rcas626F: 5′-GGAATGGCATTTGATACTGTTC-3′ + 1035R+: 5′- TTCGCTCGCCRCTACT- 3′	46	Heindl et al., submitted Heindl et al., submitted*
Robbea sp.3 symbiont ITS	Rhs438F: 5′-TCAGTCGGGAAGAAAGGTCTC-3′ + 1035R+: 5′- TTCGCTCGCCRCTACT- 3′	46	Heindl et al., submitted Heindl et al., submitted*
Robbea sp.1 symbiont 16S rRNA	Rcas626F: 5′-GGAATGGCATTTGATACTGTTC-3′ + Rcas1012R: 5′-GGCACCTGCCTATCTCTAGAC-3′	53	Heindl et al., submitted Heindl et al., submitted
	Rcas626F: 5′-GGAATGGCATTTGATACTGTTC-3′ + 1492R: 5′-GGYTACCTTGTTACGACTT-3	48	Heindl et al., submitted Kane et al., 1993
	616V: 5′-AGAGTTTGATYMTGGGCTC -3′ + Rcas1012R: 5′-GGCACCTGCCTATCTCTAGAC-3′	48	Juretschko et al., 1998 Heindl et al., submitted
Robbea sp.2 symbiont 16S rRNA	Rcs458F: 5′- TGGGTTAATAGCTCAGGGTCC-3′ + Rcs1017R: 5′- GCTCCCGAAGGCACCTATCT-3′	60	this study this study
	Rcs458F: 5′- TGGGTTAATAGCTCAGGGTCC-3′ + Rcs1449R: 5′- TGGTAAGCGCCCTCCCGAAA-3′	60	this study this study
Robbea sp.3 symbiont 16S rRNA	Rhs458F: 5′-TCAGTCGGGAAGAAAGGTCTC-3′ + Rhs998R: 5′-CCATCTCTGGAAAGTTCACAG-3′	50	Heindl et al., submitted Heindl et al., submitted
	Rhs458F: 5′-TCAGTCGGGAAGAAAGGTCTC-3′ + 1492R: 5′-GGYTACCTTGTTACGACTT-3′	50	Heindl et al., submitted Kane et al., 1993
	616V: 5′-AGAGTTTGATYMTGGGCTC -3′ + Rhs998R: 5′-CCATCTCTGGAAAGTTCACAG-3′	53	Juretschko et al., 1998 Heindl et al., submitted
Laxus oneistus symbiont 16S rRNA	Los69F: 5′-AGTCGAACGCGAAAGCCCCTT-3′ + Los1012R: 5′-GGCACCCGTCTATCTCTAGAC-3′		this study this study
	Los224F: 5′-TAGCTGGTTGGTAGGGTAAGA-3′ + Los646R: 5′-CCTCTACCATACTCTAGTCGG-3′		this study this study

* derived from 1035R (Ludwig et al., 1992)

Table 2: Probes and conditions used for FISH on single worms

Probe	Standard probe name[a]	Specificity	Sequence/ 5´-modification	target rRNA	Position[b,c]	Formamide percentage/ incubation time (h)/ probe concentration ng/µl^{-1}	Reference
EUB338	S-*-BactV-0338-a-A-18	most eubacteria	5´-GCT GCC TCC CGT AGG AGT-3´/fluorescein	16S	338-355	35%-40%/1.5-o.n./3.9	Amann et al., 1990
NON338	not named	negative control	5´-ACT CCT ACG GGA GGC AGC-3´/ Cy3	16S	338-355	35%-40%/1.5-o.n./2.3	Wallner et al, 1993
BET42a	L-C-bProt-1027-a-A-17	β-proteobacteria	5´-GCC TTC CCA CTT CGT TT -3´/ fluorescein	23S	1027-1043	35%-40%/1.5-o.n./3.9	Manz et al., 1992
GAM42a	L-C-gProt-1027-a-A-17	γ-proteobacteria	5´-GCC TTC CCA CAT CGT TT-3´/ Cy5	23S	1027-1043	35%-40%/1.5-o.n./2.3	Manz et al., 1992
Rcas470	S-Rob1s-0471-a-A-21	*Robbea* sp.1 symbiont	5´-TGC GTA ACG TCA AGA CCC TGG -3´/ Cy3	16S	471-491	35%/1.53./4.6	Bayer et al., 2009
Rhs465	S-Rob3s-a-A-21	*Robbea* sp.3 symbiont	5´-AAC GTC AGG ATC CCG AGC TAT -3´/ Cy3	16S	466-486	40%/32./2.3	Bayer, et al. 2009

[a]according to Alm et al., 1996

[b]16SrRNA position, *E. coli* numbering according to Brosius et al., 1978

[c]23SrRNA position, *E. coli* numbering according to Brosius et al., 1981

o.n.: overnight

Table 3: detailed information about *Robbea* sp.1 and sp.2 symbiont ITS clone libraries

Target symbiont ITS	specimen #	primer set	ITS sequence types	Fragment length (nt)	number of clones
Robbea sp.1 symbiont	1	Rcas626F + 1035R+	2 (A.B)	Type A: 1,619 Type B: 1,568	18 (83%A;17%B)
	2		2 (A.B)		12 (83%A;17%B)
	3		2 (A.B)		13 (39%A;61%B)
Robbea sp.3 symbiont	1	Rcas438F + 1035R+	1	1,803	15
	2		1		14
	3		1		11

Table 4: matrix displaying sequence similarities in the *Robbea* sp.3 symbiont ITS clone libraries

	worm #1	worm #2	worm #3
worm #1	100 %	99.8 %	99.1 %
worm #2	99.8 %	100 %	99.8 %
worm #3	99.1 %	99.8 %	100 %

Table 5: detailed information about clone libraries obtained from environmental water samples.**CBC W:** Carrie Bow Cay water **CAY W:** Little Cayman water **CAL W:** Calvi water **LASP OW:** La Spezia water **max.seq. identity:** maximum sequence identity; nucleotide sequence identity was calculated by counting the number of consistently identical nucleotides between each environmental 16S rDNA clone and the corresponding nematode-associated 16S rRNA gene. The sequence identity of the most similar clone is shown in %.

target	primer set	fragment length	e. coli position	hypervariable regions included	CBC OW		CAY OW		CAL OW		LASP OW	
					# of clones	max. seq. identity	# of clones	max. seq. identity	# of clones	max. seq. identity	# of clones	max. seq. identity
Robbea sp.1 symbiont 16S	Rcas626F + Rcas1012R	402 bp	627 - 1028	V 4-6	21	99.5%	5	99.8%	4	100%	4	100%
	Rcas626F + 1492R	871	627 - 1499	V 4-9	–	–	8	≤ 97%	9	≤ 97%	–	–
	616V + Rcas1012R	1019	8 - 1028	V 1-6	–	–	7	≤ 97%	5	≤ 97%	–	–
Robbea sp.2 symbiont 16S	Rcs458F + Rcs1017R	569	457 - 1026	V 3-6	6	98.4%	–	–	8	98.2%	–	–
	Rcs458F + Rcs1449R	1000	457 - 1457	V 3-9	6	98.4%	–	–	7	98.4%	–	–
Robbea sp.3 symbiont 16S	Rhs438F + Rhs998R	560 bp	439-1499	V 4-5	4	100%	–	–	8	100%	–	–
	Rhs438F + 1492R	1073 bp	439 - 1499	V 4-9	6	100%	–	–	22	100%	–	–
	616V + Rhs998R	1008 bp	8 - 1019	V 1-5	8	100%	–	–	9	100%	–	–
Laxus oneistus symbiont 16S 16S	Los224F + Los646R	424 bp	245 - 668	V 3-4	7	≤ 97%	–	–	–	–	–	–

Table 6: Probes and conditions used for FISH on environmental samples

Probe	Standard probe name[a]	Specificity	Sequence/ 5´-modification	target rRNA	Position[b,c]	Formamide percentage/ incubation time (h)/ probe concentration ng/µl^{-1}	Reference
GAM42a	L-C-gProt-1027-a-A-17	γ-proteobacteria	5´-GCC TTC CCA CAT CGT TT-3´/ fluorescein	23S	1027-1043	35%-40%/1.5-o.n./3.9	(Manz et al., 1992)
Rcas470	S-Rob1s-0471-a-A-21	*Robbea* sp.1 symbiont	5´-TGC GTA ACG TCA AGA CCC TGG -3´/ Cy3	16S	471-491	35%/1.53./2.3	(Bayer et al., 2009)
Rss456	S-*-Rob2s-0457-a-A-21	*Robbea* sp.2 symbiont, I*nanidrilus leukodermatus* endosymbiont 1 (AJ890100)	5´-ACC CTG AGC TAT TAA CCC AAG -3´/ Cy3	16S	457-477	35%/o.n./2.3	(Bayer et al., 2009)
Rhs465	S-Rob3s-a-A-21	*Robbea* sp.3 symbiont	5´-AAC GTC AGG ATC CCG AGC TAT -3´/ Cy3	16S	466-486	35%/32./2.3	(Bayer et al., 2009)

[a]according to Alm et al., 1996

[b]16SrRNA position, *E. coli* numbering according to Brosius et al., 1978

[c]23SrRNA position, *E. coli* numbering according to Brosius et al., 1981

o.n.: overnight

Figures

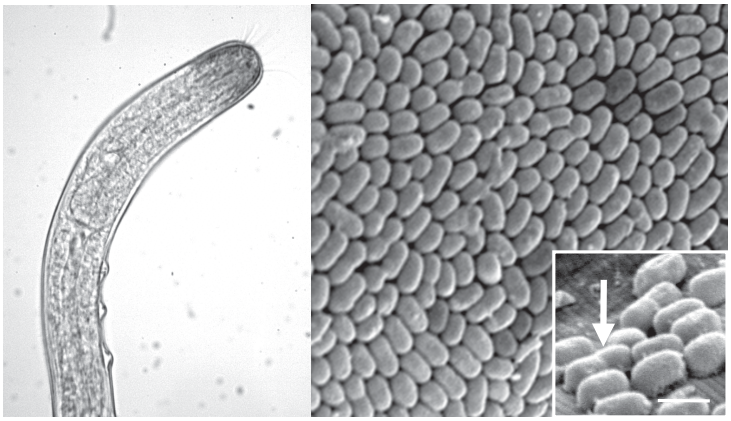

Figure 1: Light and scanning electron micrographs showing the *Robbea* sp.1 host on the left and its monospecific bacterial symbiont coat on the right. *Robbea* sp.1 symbionts are coccoid and clearly form a tightly packed monolayer on their host's cuticle. Scale bar is 2 µm, arrow points to a dividing symbiont.

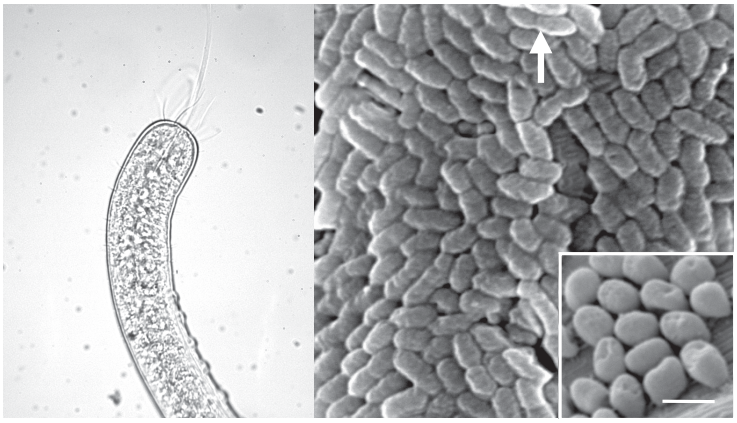

Figure 2: Light and scanning electron micrographs showing the *Robbea* sp.2 host on the left and its bacterial symbiont coat on the right. *Robbea* sp. 2 symbionts are coccoid and seem to form a multilayer, although in a less ordered arrangement than the symbionts on the two other *Robbea* species. Scale bar is 2 µm, arrow points to a dividing symbiont. Light microscopy picture by courtesy of Jörg A. Ott, SEM picture by courtesy of Christoph Bayer and Janek von Byern.

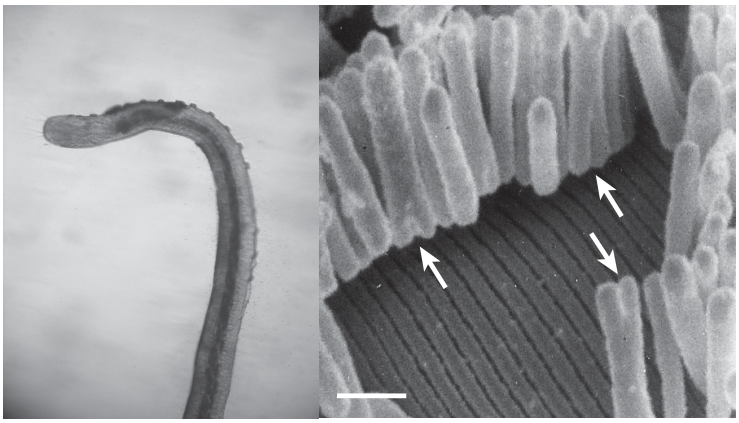

Figure 3: Light and scanning electron micrographs showing the *Robbea* sp.3 host and its monospecific bacterial symbiont coat. *Robbea* sp.3 symbionts are rod shaped and form a monolayer, standing perpendicular on their host's cuticle. Note the fission plane of dividing symbionts, which is always parallel to the longitudinal axis of the bacterial cells. Scale bar is 2 µm, arrows point to dividing symbiont. Light microscopy picture by courtesy of Jörg A. Ott, SEM picture by courtesy of Monika Bright.

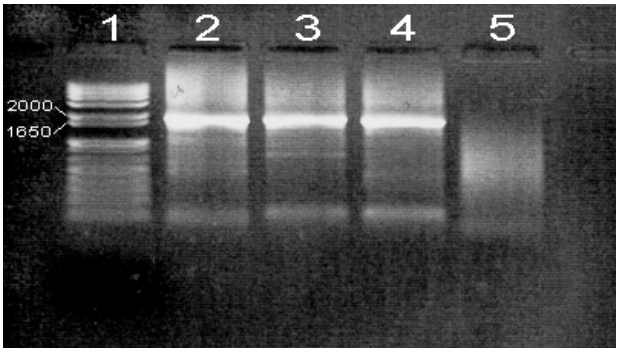

Figure 4: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 1.8 kb, corresponding to metazoan 18S rRNA were obtained from genomic DNA of three different individuals of *Robbea* sp.1 with 1F_short and 2023R primers. **lane 1:** 1 kb ladder **lane 2:** 1F_short and 2023R primers on genomic DNA of worm #1 **lane 3:** 1F_short and 2023R primers on genomic DNA of worm #2 **lane 4:** 1F_short and 2023R primers on genomic DNA of worm #3 **lane 5:** negative control with 1F_short and 2023R primers on ultrapure water as template.

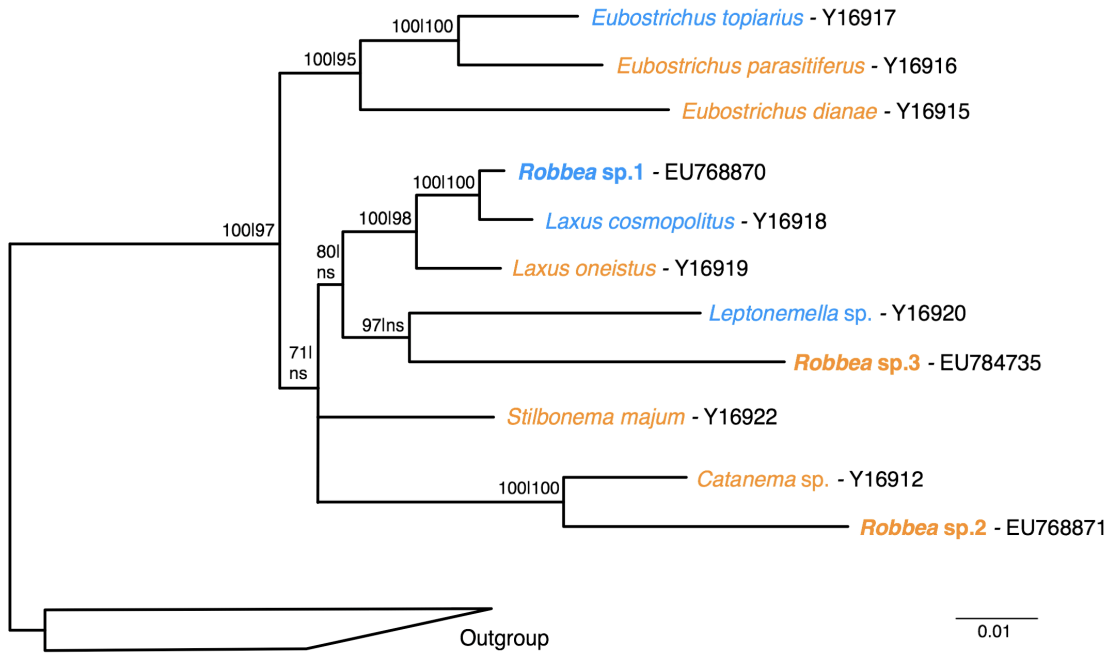

Figure 5: 18S rRNA-gene based phylogenetic consensus tree of Bayesian inference and Maximum Likelihood analysis showing the relationship of the *Robbea* species (in bold) within other Stilbonematinae. Bootstrap support values are depicted besides the respective nodes (MrBayes|Maximum Likelihood; **ns:** not supported, i.e. support value below 70 %). **Orange:** Caribbean species **Light Blue:** Mediterranean species. Scale bar represents 1 % estimated sequence divergence.

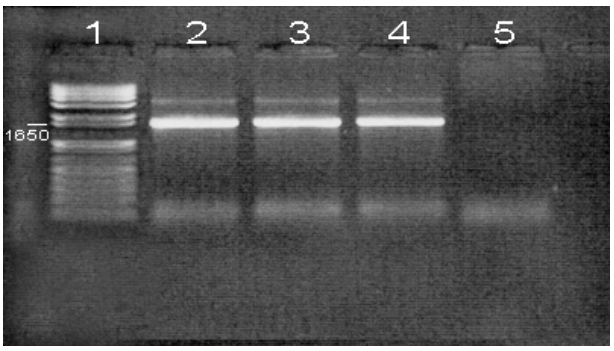

Figure 6: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 1.5 kb, corresponding to bacterial 16S rRNA were obtained from genomic DNA of three different individuals of Robbea sp.1 with 616V and 1492R primers. **lane 1:** 1 kb ladder **lane 2:** 616V and 1492R primers on genomic DNA of worm #1 **lane 3:** 616V and 1492R primers on genomic DNA of worm #2 **lane 4:** 616V and 1492R primers on genomic DNA of worm #3 **lane 5:** negative control with 616V and 1492R primers on ultrapure water as template.

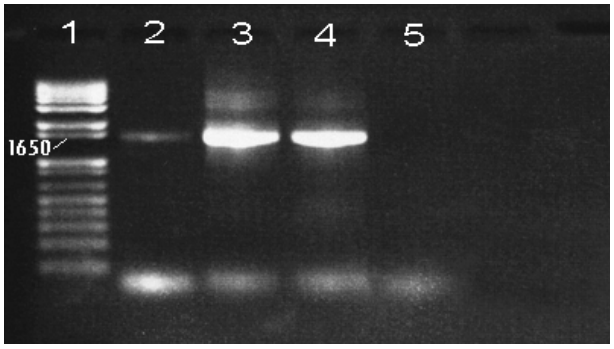

Figure 7: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 1.5 kb, corresponding to bacterial 16S rRNA were obtained from genomic DNA of three different individuals of Robbea sp.3 with 616V and 1492R primers. **lane 1:** 1 kb ladder **lane 2:** 616V and 1492R primers on genomic DNA of worm #1 **lane 3:** 616V and 1492R primers on genomic DNA of worm #2 **lane 4:** 616V and 1492R primers on genomic DNA of worm #3 **lane 5:** negative control with 616V and 1492R primers on ultrapure water as template.

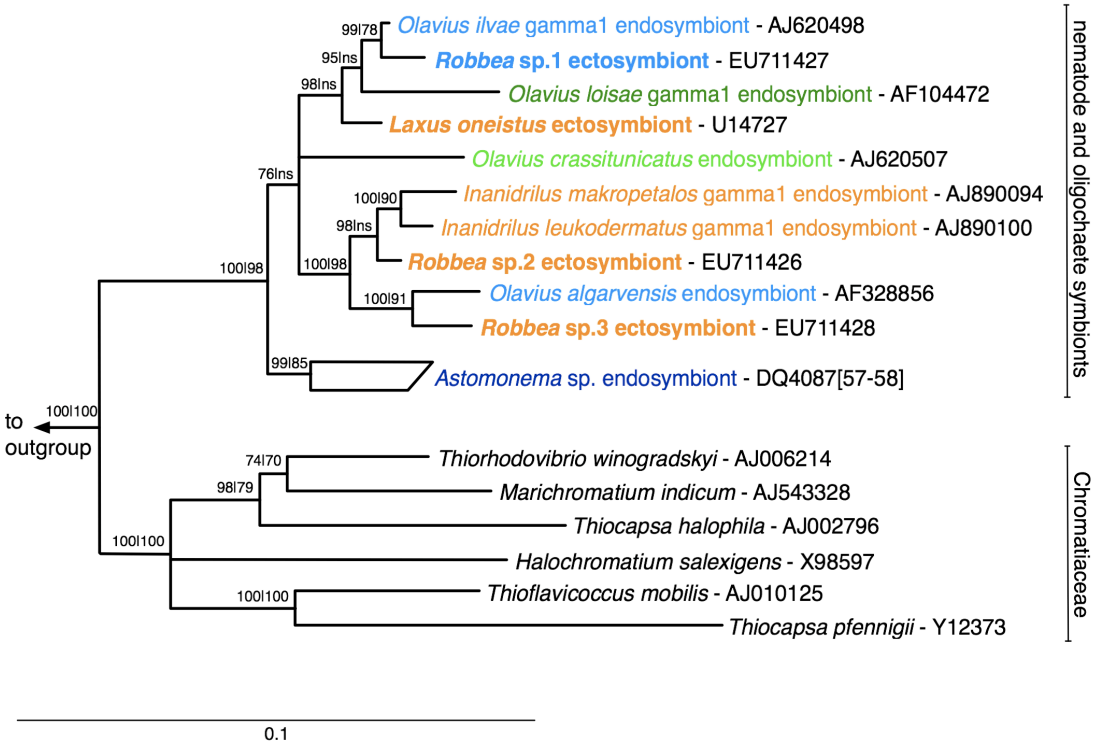

Figure 8: 16S rRNA-gene based phylogenetic consensus tree of Bayesian inference and Maximum Likelihood analysis showing the relationship of the *Robbea* symbionts with other stilbonematid and oligochaete symbionts, as well as other bacteria belonging to the Chromatiaceae and other vestimentiferan and mussel symbionts. Sequences of stilbonematid symbionts are in bold letters. Bootstrap support values are depicted besides the respective nodes (MrBayes|Maximum Likelihood; ns: not supported, i.e. support value below 70 %). Orange: Caribbean species Light Blue: Mediterranean species Dark Blue: North Atlantic species Light Green: North Pacific species Dark Green: South Atlantic species. Scale bar represents 10 % estimated sequence divergence.

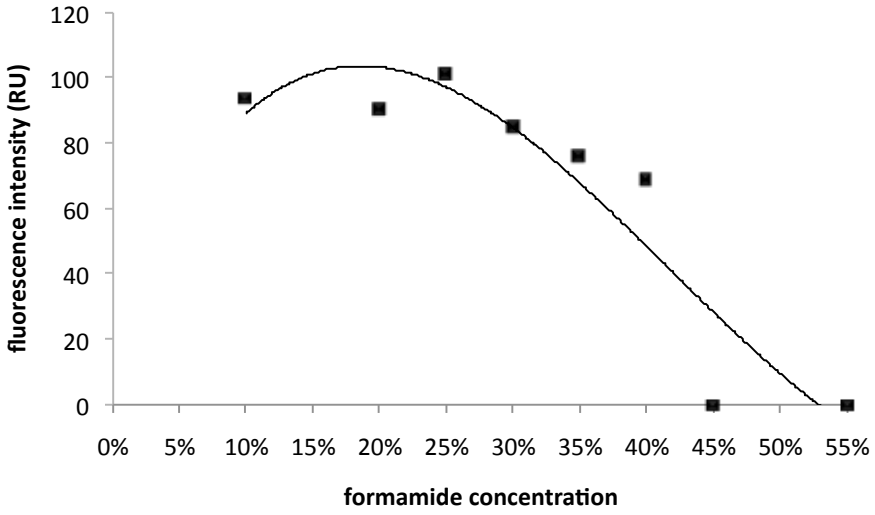

Figure 9: Graph displaying the polynomial regression of the mean fluorescence intensities (vertical axis) at each tested formamide concentration (horizontal axis) in the CloneFiSH hybridisation series. A peak in fluorescence intensity can be observed at approximately 20% formamide.

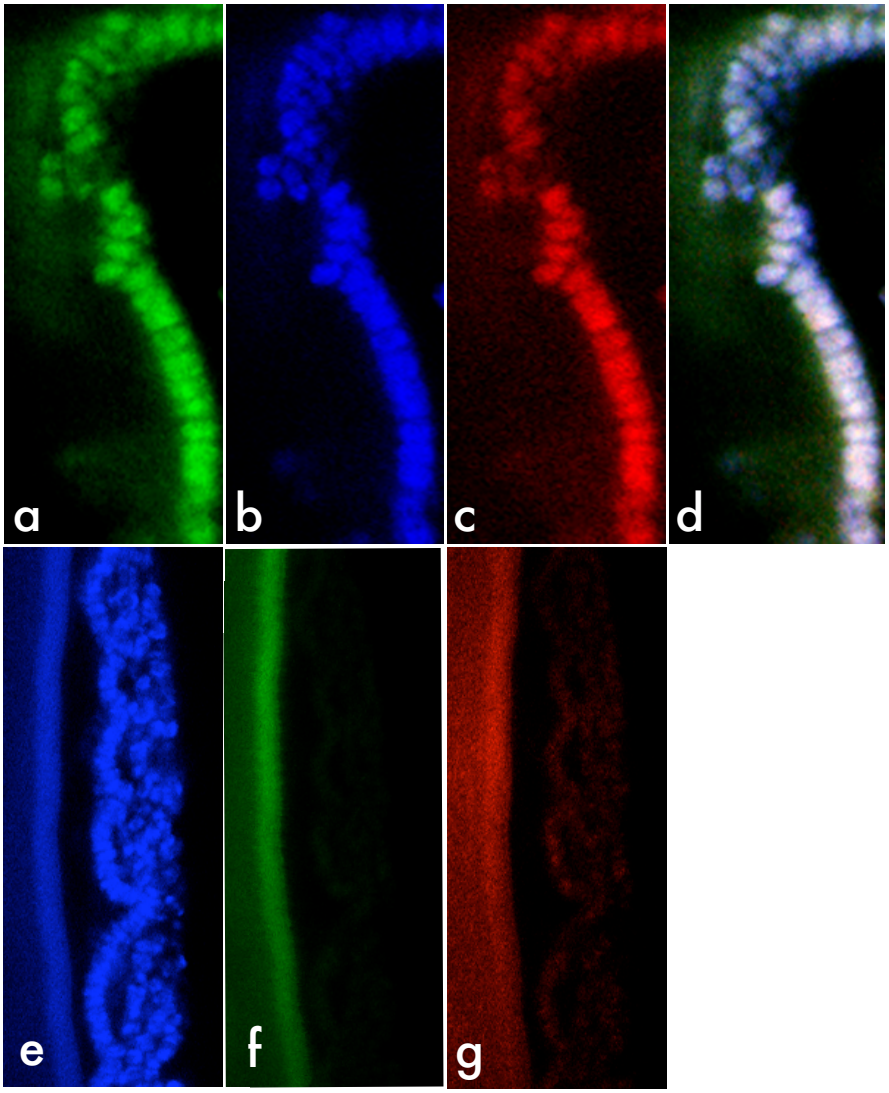

Figure 10: Fluorescence in situ hybridisation on *Robbea* sp.1 single worm targeting the symbiotic 16S rDNA. Images show symbionts attached to a worm's cuticle and stained as follows: **a** EUB338, **b** GAM42a and **c** Rca470. An overlay of the three stainings is shown in **d**. In parallel, a control experiment with another worm has been performed with **e** GAM42a, **f** BET42a and **g** NON338. Note the monolayer of the almost coccoid bacteria where the worm's cuticle is stretched and compare to Figure 1a.

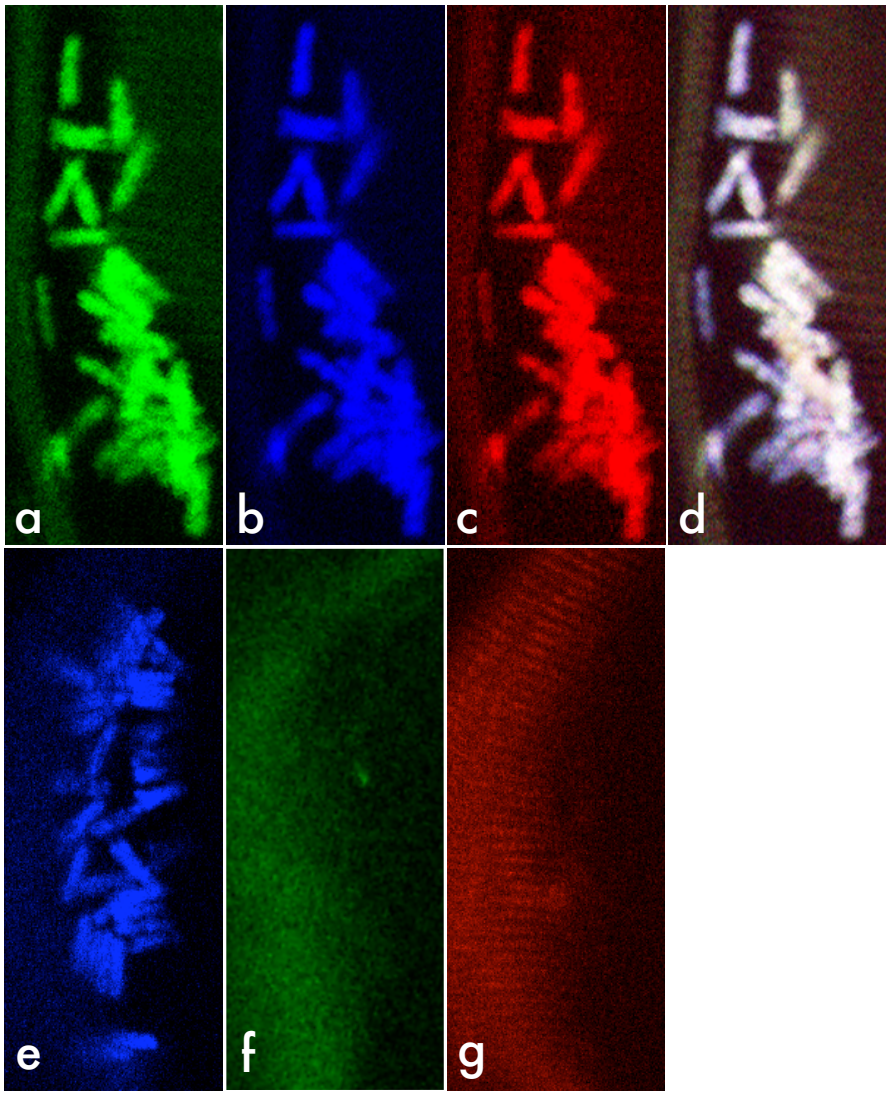

Figure 11: Fluorescence in situ hybridisation on *Robbea* sp.3 single worm targeting the symbiotic 16S rDNA. Images show symbionts detached from a worm's cuticle and stained as follows: **a** EUB338, **b** GAM42a and **c** Rhs465. An overlay of the three stainings is shown in **d**. In parallel, a control with another specimen has been performed with **e** GAM42a, **f** BET42a and **g** NON338.

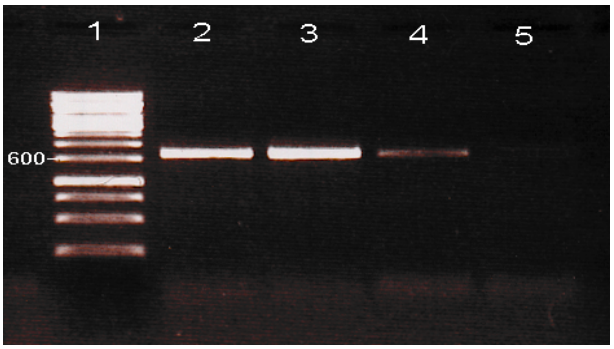

Figure 12: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 600 bp, corresponding to the Robbea sp.1 symbiont ITS were obtained from genomic DNA of three different individuals of Robbea sp.1 with Rcas626F and 1035R+ primers. **lane 1:** 100 bp ladder **lane 2:** Rcas626F and 1035R+ primers on genomic DNA of worm #1 **lane 3:** Rcas626F and 1035R+ primers on genomic DNA of worm #2 **lane 4:** Rcas626F and 1035R+ primers on genomic DNA of worm #3 **lane 5:** negative control with Rcas626F and 1035R+ primers on ultrapure water as template.

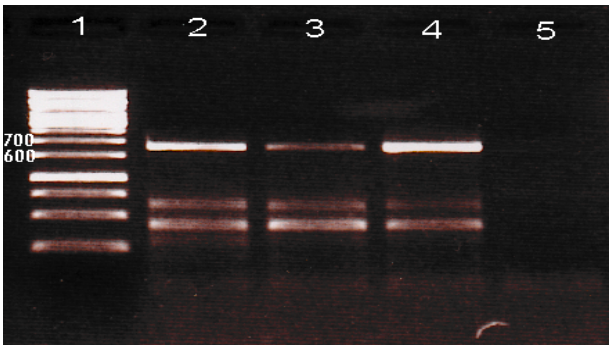

Figure 13: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 600 bp, corresponding to the Robbea sp.1 symbiont ITS were obtained from genomic DNA of three different individuals of Robbea sp.3 with Rhs438F and 1035R+ primers. **lane 1:** 100 bp ladder **lane 2:** Rhs438F and 1035R+ primers on genomic DNA of worm #1 **lane 3:** Rhs438F and 1035R+ primers on genomic DNA of worm #2 **lane 4:** Rhs438F and 1035R+ primers on genomic DNA of worm #3 **lane 5:** negative control with Rhs438F and 1035R+ primers on ultrapure water as template.

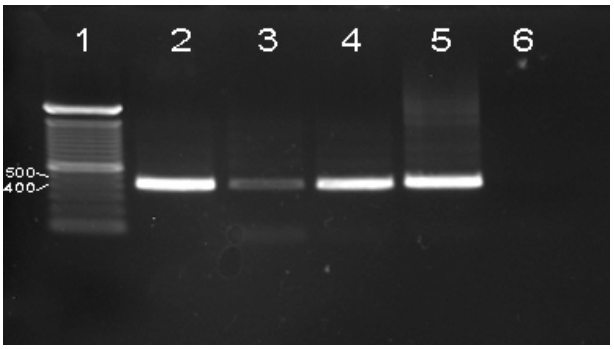

Figure 14: UV light image of ethidium bromide stained agarose gel. PCR fragments of approximately 400 bp were obtained from four different environmental 16S pools with Rcas635F and Rcas1012R primers, specifically targeting the 16S rRNA gene of the *Robbea* sp.1 symbiont. **lane 1:** 100 bp DNA ladder **lane 2:** Rcas635F and Rcas1012R primers on CAY W 16S pool as template **lane 3:** Rcas635F and Rcas1012R primers on CBC W 16S pool as template **lane 4:** Rcas635F and Rcas1012R primers on CAL W 16S pool as template **lane 5:** Rcas635F and Rcas1012R primers on LAS W 16S pool as template **lane 6:** negative control with Rcas635F and Rcas1012R primers on randomly amplified ultrapure water.

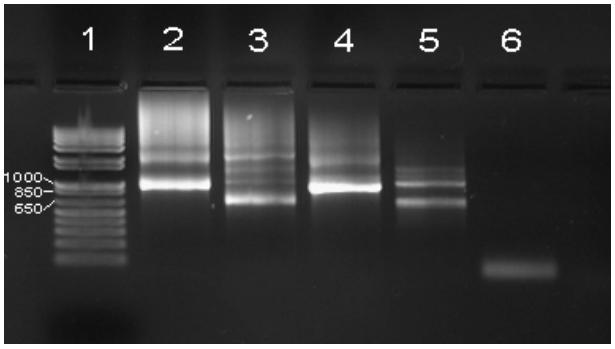

Figure 15: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 1 kb and 850 bp were obtained from two different environmental 16S pools with semispecific primer sets targeting the 16S rRNA gene of the *Robbea* sp.1 symbiont. **lane 1:** 1 kb DNA ladder **lane 2:** 616V and Rcas1012R primers on 16S pool obtained from CAY W **lane 3:** Rcas 626F and 1492R primers on 16S pool obtained from CAY W **lane 4:** 616V and Rcas1012R primers on 16S pool obtained from CAL W **lane 5:** Rcas 626F and 1492R primers on 16S pool obtained from CAL W **lane 6:** negative control with Rcas 616V and Rcas1012R primers on randomly amplified ultrapure water **lane 7:** negative control with Rcas626F and 1492R primers on randomly amplified ultrapure water.

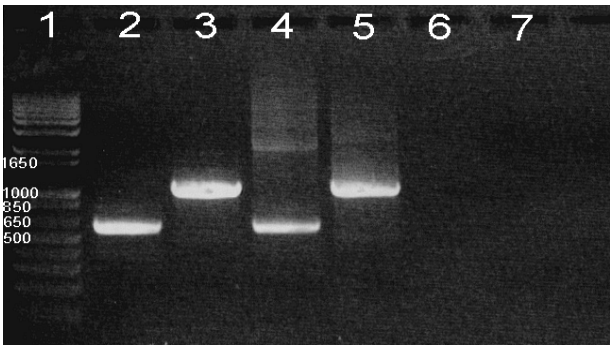

Figure 16: UV light image of ethidium bromide stained agarose gel. PCR fragments of approximately 1 kb obtained from two different environmental 16S pools with primers specifically targeting the 16S rRNA gene of the *Robbea* sp.2 symbiont. **lane 1:** 1 kb DNA ladder **lane 2:** Rcs458F and Rcs1017R primers on CBC W 16S pool as template **lane 3:** Rcs458F and Rcs1449R primers on CBC W 16S pool as template **lane 4:** Rcs458F and Rcs1017R primers on CAL W 16S pool as template **lane 5:** Rcs458F and Rcs1449R primers on CAL W 16S pool as template **lane 6:** negative control with Rcs458F and Rcs1017R primers on randomly amplified ultrapure water **lane 7:** negative control with Rcs458F and Rcs1449R primers on randomly amplified ultrapure water.

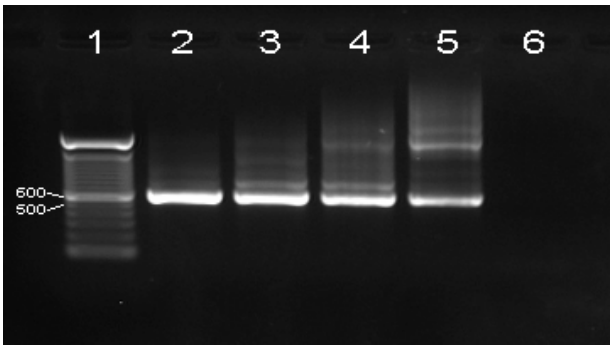

Figure 17: UV light image of ethidium bromide stained agarose gel. PCR fragments of approximately 600 bp were obtained from four different environmental 16S pools with Rhs438F and Rhs998R primers, specifically targeting the 16S rRNA gene of the *Robbea* sp.3 symbiont. **lane 1:** 100 bp ladder **lane 2:** 16S pool obtained from CAY W 16S pool as template **lane 3:** 16S pool obtained from CBC W 16S pool as template **lane 4:** 16S pool obtained from CAL W 16S pool as template **lane 5:** 16S pool obtained from LAS W 16S pool as template **lane 6:** negative control with Rhs438F and Rhs998R primers on randomly amplified ultrapure water.

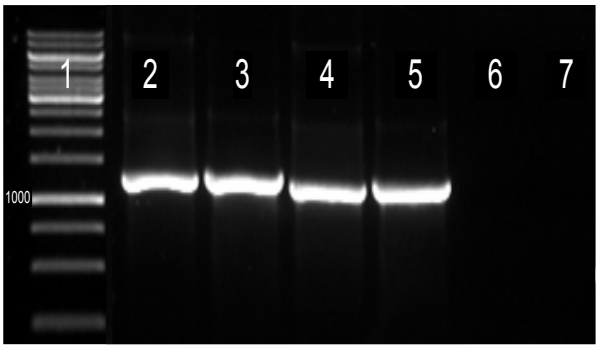

Figure 18: UV light image of ethidium bromide stained agarose gel. PCR fragments with sizes of approximately 1.1 kb and 1.0 kb were obtained from two different environmental 16S pools with semispecific primer sets targeting the 16S rRNA gene of the *Robbea* sp.3 symbiont. **lane 1:** 1 kb ladder **lane 2:** Rhs438F and 1492R primers on 16S pool obtained from CBC W **lane 3:** Rhs438F and 1492R primers on 16S pool obtained from CBC W **lane 4:** 616V and Rhs998R primers on 16S pool obtained from CBC W **lane 5:** 616V and Rhs998R primers on 16S pool obtained from CAL W **lane 6:** negative control with Rhs438F and 1492R primers on randomly amplified ultrapure water **lane 7:** negative control with 616V and Rhs998R primers on randomly amplified ultrapure water

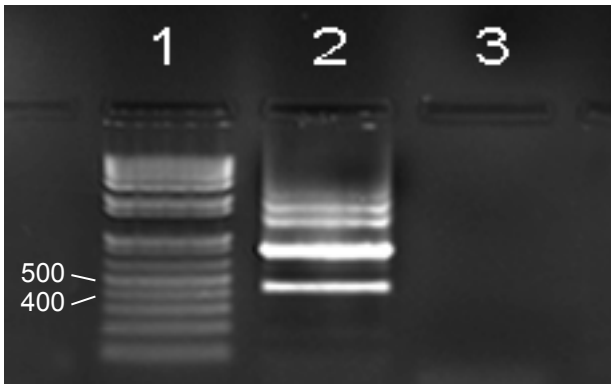

Figure 19: UV light image of ethidium bromide stained agarose gel. A PCR fragment of approximately 400 bp was obtained from a nested probe design with primers specifically targeting the 16S rRNA gene of the *Laxus oneistus* symbiont. **lane 1:** 1 kb ladder **lane 2:** fragment amplified with Los224F and Los646R primers from a template obtained with Los69F and Los1012R primers on the CBC W 16S pool **lane 3:** negative control with Los224F and Los646R primers on ultrapure water as template.

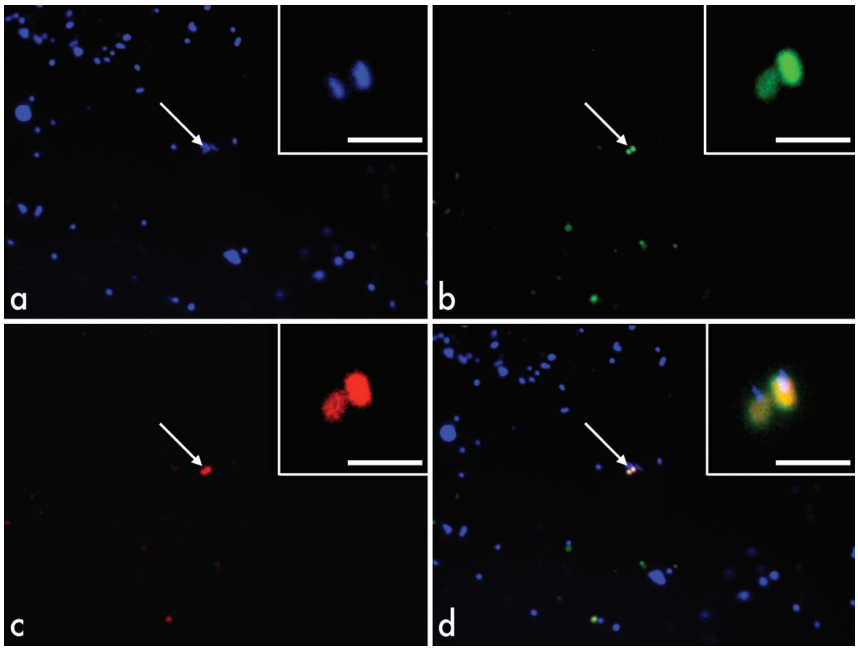

Figure 20: Fluorescence in situ hybridisation on environmental samples identifying free living forms of the *Robbea* sp.1 symbiont. The stainings result from **a** DAPI, **b** GAM42a, **c** Rca470 probe hybridisation and are combined in **d** to an overlay image of all three stainings. Arrows point to triple positives. Scale bars represent 3 µm.

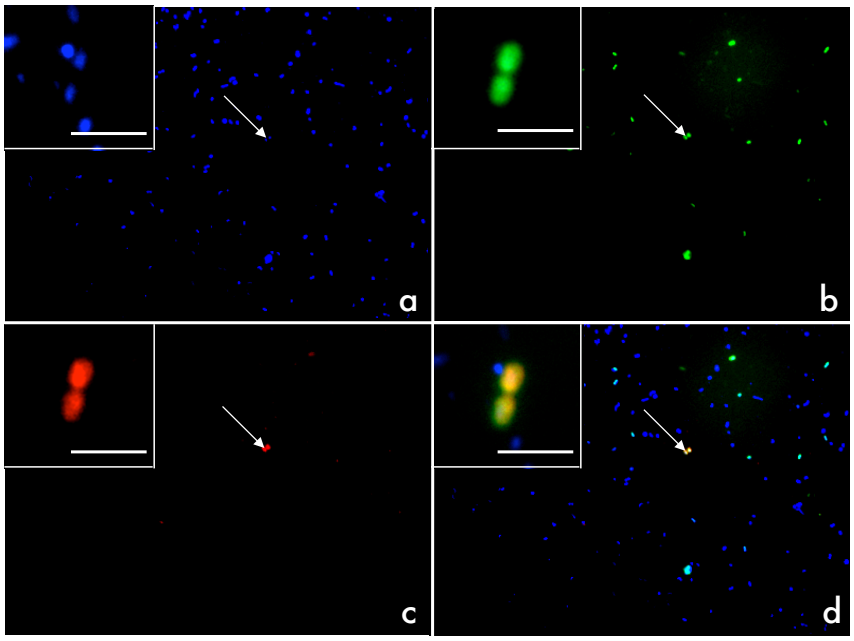

Figure 21: Fluorescence in situ hybridisation on environmental samples identifying free living forms of the *Robbea* sp.2 an *Inanidrilus leukodermatus* symbionts. The stainings result from **a** DAPI, **b** GAM42a, **c** Rca470 probe hybridisation and are combined in **d** to an overlay image of all three stainings. Arrows point to triple positives. Scale bars represent 4µm.

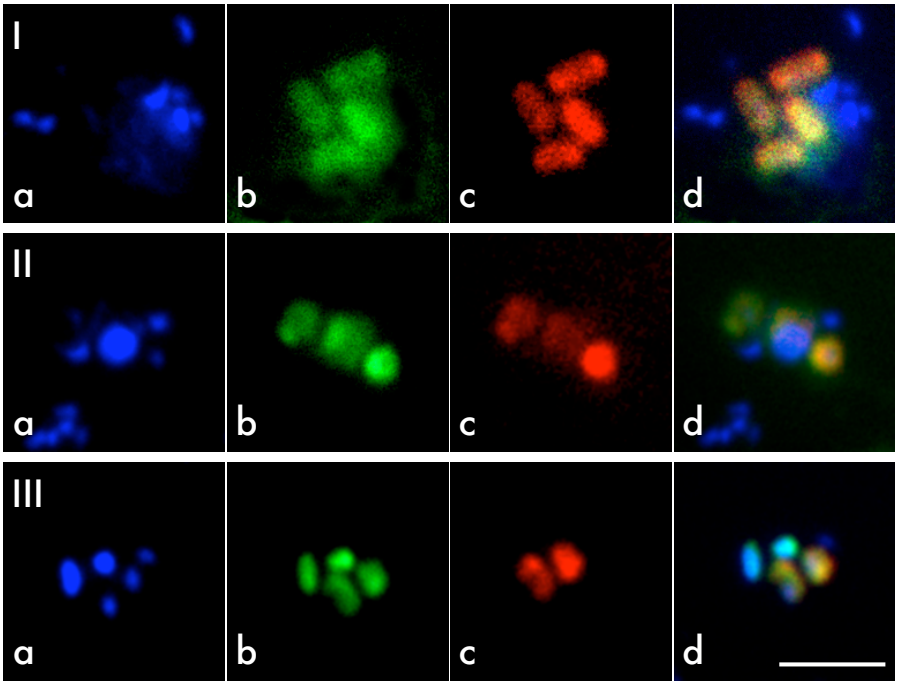

Figure 22: Fluorescence in situ hybridisation on environmental samples identifying free living forms of the *Robbea* sp.2 an *Inanidrilus leukodermatus* symbionts. The co-stainings I - III result from **a** DAPI and **b** GAM42a, **c** Rca470 probe hybridisation and are combined in **d** to an overlay image of all three stainings. As shown here, bacteria stained specifically are often found in consortia with other, γ- or non-γ-proteobacteria. This finding corresponds to the subcuticular situation in oligochaete hosts, where γ-proteobacterial sulphur-oxidisers are associated with δ-proteobacterial sulphate-reducers and other γ-proteobacterial sulphur-oxidisers (Dubilier et al., 2008). Scale bar represents 4μm.

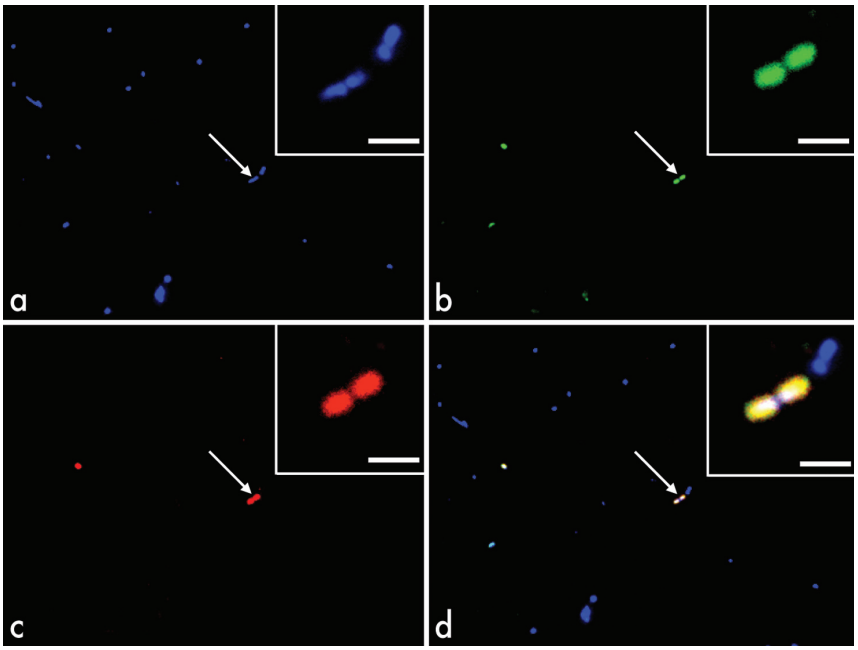

Figure 23: Fluorescence in situ hybridisation on environmental samples identifying free living forms of the *Robbea* sp.3 symbiont. The stainings result from **a** DAPI, **b** GAM42a, **c** Rca470 probe hybridisation and are combined in **d** to an overlay image of all three stainings. Arrows point to a triple positive, which is also shown in the inserts. Scale bars represent 4 µm.

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Acknowledgments

I thank Sebastian Lücker from the Department of Microbial Ecology at the University of Vienna for introducing me to CloneFISH and daime, Gerhard Spitzer from the Core Facility Cell Imaging and Ultrastructure Research (CIUS) at the University of Vienna for his support at the scanning electron microscope and the CIUS Cell Physiology & Scientific Film staff.

Zusammenfassung

Stilbonematinae (Chromadorea) sind freilebende Nematoden, die sulfidoxidierende Bakterien auf ihrer Kutikula tragen. In den Sandlückensystem aller Küsten weltweit wandern sie zwischen der oxidierten oberen und der darunterliegenden reduzierten Schicht, um ihre Bakterien mit dem als Elektronenakzeptor benötigten Sauerstoff und reduziertem Schwefel als Elektronendonator zu versorgen. Im Gegenzug dienen die bakteriellen Symbionten ihren Wirten als Hauptnahrungsquelle. Seit langem wird angenommen, daß jede neugeborene Wirtsgeneration die Symbionten von der Umwelt erwirbt. Da die Würmer während ihrer Entwicklung die Kutikula bis zu viermal abstreifen und ersetzen, muß sich auch hier der Bakterienmantel immer wieder von Neuem bilden. Die Vergesellschaftung der beiden Partner ist hochspezifisch und wird selbst gegen starke mechanische Beanspruchung, sowie gegen eine Vielzahl konkurrierender Bakterien aufrechterhalten. Anhand der Symbionten von *Laxus oneistus* konnte erstmals deren Zugehörigkeit zu einem distinkten Cluster symbiontischer Sulfidoxidierer innerhalb der γ-Proteobakterien nachgewiesen werden. In dieser Arbeit wurden die phylogenetischen Positionen dreier neu entdeckten stilbonematinen Symbiosen - *Robbea* sp.1, sp.2 und sp.3 - anhand der Nukleotidsequenzen der kleinen ribosomalen Untereinheiten bestimmt. Zur Vervollständigung des bereits vorhandenen Datensatzes wurden die 18S rRNA des *Robbea* sp.1 Wirtes, sowie die 16S rRNA der Symbionten von *Robbea* sp.1 und sp.3 sequenziert. Mithilfe der Fluoreszenz In Situ Hybridisierung (FISH) konnte die Monospezifität dieser Assoziationen nachgewiesen werden. Eine Analyse der *internal transcribed spacer region* (ITS) an *Robbea* sp.1 und sp.3 ergab, daß ein oder zwei Bakterien genügen, einen Symbiontenmantel gründen. Obwohl alle drei Arten in den wesentlichen Merkmalen der Gattung *Robbea* übereinstimmen, deutet die 18S rRNA basierte phylogenetische Rekonstruktion darauf hin, daß es sich um keine monophyletische Gruppe handelt. Ebenso verhält es sich im 16S rRNA basierten Stammbaum ihrer Symbionten, in dem sich die drei Bakterien innerhalb einer Gruppe sulfidoxidierender Symbionten von Nematoden und darmlosen Oligochaeten einordnen, gemeinsam aber kein distinkt abgesetztes Cluster bilden. In der fehlenden Kongruenz der beiden Stammbäume wird deutlich, daß stilbonematine Symbiosen im Laufe der Evolution mehrfach unabhängig voneinander entstanden sein müssen. Die fehlende Koevolution von Wirten und Symbionten gilt als typisch für horizontal übertragene Symbiosen und deckt sich

mit der bisherigen Vermutung, Stilbonematinae bezögen ihre bakteriellen Partner aus ihrer Umgebung. Weiterhin wird deutlich, daß bei Wirten wie Symbionten verwandtschaftliche Nähe nicht zwingend durch geographische Nähe bestimmt wird. Um eine daraus abgeleitete, zumindest atlantikweite Verbreitung nah verwandter, freilebender Phylotypen zu bestätigen, wurden mit Hilfe spezifischer Primer Fragmente der 16S rRNA Gene der Symbionten von *Robbea* sp.1, sp.2 und sp.3, sowie *Laxus oneistus* aus küstenfernem Oberflächenwasser amplifiziert. Am erfolgreichsten war die Suche nach der 16S rRNA des Symbionten des karibischen *Robbea* sp.3. Diese wurde nicht nur vollständig und mit vollkommener Sequenzübereinstimmung in karibischen Wasserproben gefunden, sondern auch in mediterranen. Mit FISH Experimenten konnte gezeigt werden, daß die freilebenden Phylotypen sogar metabolisch aktiv sind, da sich einige der spezifisch angefärbten Bakterien zum Zeitpunkt der Fixierung im Teilungszustand befunden haben. Ein solcher Nachweis von Bakterien eines sulfidoxidierenden Symbiontencluster in einem Habitat, das chemisch, physikalisch und geograpisch derart von dem ihrer Wirte abweicht, ist bislang beispiellos.

Danksagung

Mein größter Dank gilt meinen Eltern, Irene Elisabeth Katharina Heindl-Schack und Wolfgang Alexander Heindl, die mich im Laufe meiner recht umwegigen Ausbildung nicht nur finanziell, sondern auch stets moralisch unterstützt haben.

Besonders danken möchte ich mich meinen beiden Betreuern, sowie meinen Kollegen der Flachwassersymbiosengruppe:

Dr. Silvia Bulgheresi für ihre tägliche, ausgezeichnete und höchst engagierte Betreuung, ihre Geduld, Freundschaftlichkeit und Bereitschaft, mir nicht nur all ihr methodisches Wissen angedeihen zu lassen, sondern mich auch in die wissenschaftliche Gemeinschaft einzuführen.

Prof. Dr. Jörg A. Ott dafür, daß er mich ohne zu Zögern in seine Abteilung aufgenommen hat, für sein großes Engagement in der Leitung unserer Flachwassersymbiosengruppe, sowie seine Freundschaftlichkeit und allzeitige Unterstützung bei jedweder Art von Schwierigkeiten.

Harald Gruber, Ulrich Dirks und Niko Leisch für die freundschaftliche Zusammenarbeit und den anregenden Wissenstausch.

Großer Dank gilt auch allen Mitgliedern der Tiefseegruppe und der Gruppen für benthische Ökologie und Phykologie, sowie allen anderen Post-Docs, Dissertanten, Diplomanden und technischen Assistenten des Departments für Meeresbiologie, für jede Art der Unterstützung, die ausgesprochen angenehme Atmosphäre und eine wunderschöne Zeit im Allgemeinen.

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